**The role of soil biota in the invasion success of legumes in Australia**

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Thesis submitted for the degree of Doctor of Philosophy

July 2012

*Dedicated to the loving memory of my father Rein Birnbaum*

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**Statement of candidate**

I certify that the work in this thesis “The role of soil biota in the invasion success of legumes in Australia” is original and has not previously been submitted in any form for a higher degree at any other university or institution.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparations of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. The research presented in this thesis did not require approval from the Macquarie University Ethics Review Committee.

Christina Birnbaum (41518446)

July 2012

**Thesis abstract**

It has been widely acknowledged that soil microbial communities may play a significant role in the processes that regulate successful invasion by exotic plant species. Species within the genus *Acacia* have been extensively introduced for horticultural and ornamental purposes into novel regions within Australia, and many of these have become invasive. These invasive acacias may have a significant negative effect on the co-occurring native flora and induce changes to below-ground microbial composition. This thesis examines the role of soil biota in the invasion success of four *Acacia* species (*A. cyclops, A. longifolia, A. melanoxylon, A. saligna*) and their close relative *Paraserianthes lophantha* introduced into novel areas within Australia. *Acacia cyclops*, *A. saligna* and *P. lophantha* are native to Western Australia but have become invasive or naturalised in the eastern states, whereas *A. longifolia* and *A. melanoxylon* are, *vice versa*, native to the eastern states of Australia but have become invasive in Western Australia.

For the purpose of this thesis a biogeographic approach was applied and soil and seed material was sampled for each species in both ranges (native and non-native) and from multiple populations and individuals within a population. The field collected material was then used for experiments described in Chapters 2, 3, 4 and 5.

In Chapter 1 (Introduction) the published literature on the role of soil biota in the invasion success of plant species introduced to novel environments is discussed. In addition, the five study species and their invasion ecology, history and introduction pathways are described in the local as well as global context.

In Chapter 2 the net role of soil microbial communities on the invasion success of the five legume study species was investigated. This Chapter describes the results from a common garden experiment that employs the plant-soil feedback approach. Plant-soil feedback experiments have been widely applied in invasion ecology to test for plant-soil reciprocal interactions. The results from this experiment showed that there was no significant effect of the soil origin on plant growth, however there was a significant effect of the seed origin on plant performance. This suggests that invasion success of these legumes in Australia is not limited by mutualistic soil biota or facilitated by the absence of soil pathogens in novel environments, but rather genetic adaptation to novel environments and human-mediated artificial selection could have influenced the invasion success of these acacias in Australia.

In Chapter 3 multiple complementary approaches, including a glasshouse experiment to assess plant growth in non-native compared to native range soils, estimation of abundance of nitrogen fixing bacteria (i.e. rhizobia) and molecular analysis of the rhizobial community composition across both native and non-native ranges, were used to comprehensively assess the role of rhizobia in the invasion success of the five study species. Only one of the species ( *A. longifolia*) had greater biomass when grown in its non-native range soils, rhizobial abundance was similar for all five study species across both ranges, and rhizobial community composition was significantly different between the ranges for western natives *A. cyclops*, *A. saligna* and *P. lophantha* but similar for eastern natives *A. longifolia* and *A. melanoxylon*. This study has revealed that overall invasive success of these five species is unlikely to be constrained by the absence of compatible rhizobia in novel ranges, although there appears to be variation in rhizobial communities across the ranges for some of the host species.

In Chapters 4 and 5 the diversity of free-living and nodulating nitrogen fixing bacterial and fungal communities, respectively, associating with the five study species were investigated using next-generation sequencing. The results from Chapter 4 revealed that free-living nitrogen fixing bacterial communities in the soils were different across the continent, while similar across the ranges in the nodules of the host legumes. These results indicate that despite fundamental differences in the bacterial communities across the continent these legumes are unlikely to be constrained by the absence of compatible symbionts in the introduced range since they appear to associate with the same common subsets of bacteria in eastern and western populations.

In Chapter 5 the fungal communities in the rhizospheres of the study species in their non-native compared to native ranges were described and analysed. Similarly to the results from Chapter 4, I found that the fungal communities in the rhizosphere of these legumes were different across the continent. Overall, these results indicate that it is unlikely that these legumes are constrained by novel fungal communities or have been released from harmful pathogens in their non-native ranges in Australia.

Chapter 6 (Discussion) summarizes the original findings of this thesis and places them in the broader context of the plant invasion literature.

**Acknowledgements**

I owe my deepest gratitude to my principal supervisor Michelle Leishman who has been by far the best supervisor that one could ever wish for. I thank her for motivating me to push myself and work harder, supporting and giving the best objective advice when needed and creating such an enthusiastic and at the same time calm working environment.

I am very grateful to my adjunct supervisor Peter Thrall (Plant Industry, CSIRO) for his kind support and guidance throughout most of my PhD. Plant Industry in Canberra became my ‘second academic home’ when away from Macquarie and I am indebted to Peter for his enthusiasm and for giving me the opportunity to work in such an inspiring environment.

I also thank Luke Barrett, Andrew Bissett, John Brockwell and Shamsul Hoque from CSIRO Plant Industry for kindly sharing their time, knowledge and expertise as well as opening the door of microbiology and genetics to me.

I am grateful to John Klironomos, Miranda Hart, Jeri Parrent and Alexander Koch for having me in their lab and providing with much needed helpful insights and advice in the early stages of this PhD. I extend my acknowledgements to Peter McGee, Leonie Whiffen and Linda Broadhurst for providing helpful advice.

I would like to thank the PIREL lab members (past and present): Julia, Carla, Ifeanna, Rachael, Jessica, James, Ian and Anthony for being such inspirational and wonderful colleagues and friends. I extend my gratitude to many more wonderful people in the Department of Biological Sciences at Macquarie University of whom many have one way or the other helped me in the glasshouse or given great advice when needed.

Thanks to Kerstin, Natasha, Stephanie and many more friends in Sydney for giving their endless support and keeping me sane during preparation of this PhD.

To my dearest Mum for her love, endless belief in me and encouragement via our weekly Skype calls.

To Paweł goes my most heartfelt thanks: for providing me with invaluable assistance as a colleague, endless support and love as a partner, great patience during the last couple of months waiting for me in Perth while I was writing up and for many reasons to have a great laugh every single day.

Finally, I am grateful for the financial support provided by Macquarie University’s Research Excellence Scholarship which provided a stipend for my candidature and the Postgraduate Research Fund for funding the conference travel.

**Thesis scope and structure**

This thesis examines the role of soil biota, with an emphasis on rhizobia and fungi, on the invasion success of four *Acacia* spp. and a close relative *Paraserianthes lophantha* in Australia.

This thesis consists of six Chapters – the Introduction, four Chapters based on primary data that have been prepared as manuscripts for submission to peer-reviewed journals and a Discussion. The Introduction reviews the thesis topic and highlights the importance of studying below-ground microbial communities in conjunction with above-ground plant traits to better understand the complex mechanisms that drive invasion success of plants. The following four Chapters assess more comprehensively the net effects of soil microbial communities as well as the role of beneficial and detrimental soil bacteria and fungi in the invasion success of these five legume species in Australia. The final Chapter of this thesis (the Discussion) summarizes the original findings of this thesis and places them in a broader context. In addition, future research directions are suggested.

Each data Chapter was prepared as a stand-alone manuscript and therefore there is unavoidable repetition of introductory material and methods. The structure and formatting of each Chapter varies according to the requirements for the journal for which it has been prepared, though referencing is consistent throughout the thesis. Chapter 2 is in revision at *Biological Invasions*, Chapter 3 is in press as Birnbaum C., Barrett, L.G., Thrall, P.H. and Leishman M.R. (2012) Mutualisms are not constraining the cross-continental invasion success of Australian acacias. *Diversity and Distributions* DOI: 10.1111/j.1472-4642.2012.00920.x. Chapters 4 and 5 have been prepared for submission to journals. Appendix A contains a copy of the accepted paper.

**Chapter 1: Introduction**

**BACKGROUND**

When organisms are introduced beyond their native range, novel interactions between the invader and native organisms of the recipient community may occur, resulting in fundamental changes to existing processes in the invaded ecosystem. With increasing globalization and trade, plant species are currently being introduced to novel ecosystems at an unprecedented rate (Eschtruth & Battles, 2009). The impacts of these new plant introductions on the native communities have been a major focus of invasion biology research and have been generally well documented (Vitousek & Walker, 1989; D'Antonio & Vitousek, 1992; Gaertner *et al.*, 2009; Pyšek *et al.*, 2012). For instance, it is often reported that invasive species, including plants, are able to modify the structural composition and functional processes of the invaded system and thus pose a significant threat to biodiversity and ecosystem functioning (Pyšek & Richardson, 2010).

Despite the vast literature on human-mediated plant invasions (Bradley *et al.*, 2010; Blackburn *et al.*, 2011; Bradley *et al.*, 2012) there are still many gaps in our knowledge, particularly on the role of the soil microbial community (Yannarell *et al.*, 2011). This is not surprising, since soil microbes are more difficult to study than the aboveground community and more susceptible to any disturbance (Wolfe & Klironomos, 2005), including physical distrurbance in glasshouse and laboratory manipulations. Yet plant-soil interactions have been shown to strongly influence plant and soil community composition and diversity (van der Heijden *et al.*, 1998; Bever, 2003; Schnitzer *et al.*, 2011) and thus should be acknowledged and included in plant invasion studies. Indeed, in recent years the substantial advances in of molecular approaches have enabled ecologists to better describe the soil microbial communities and assess the importance of these soil communities for the growth and establishment success of invasive plant species in their introduced sites. This is of great importance especially for restoration of native plant communities that have been affected by invasive plant species. Comprehensive information on

different aspects of the invasion ecology of a plant invader could advance knowledge about above- and belowground interactions which, in turn, can help inform management strategies.

This thesis is part of a larger project that aimed to comprehensively understand the invasion success of five invasive legumes (*Acacia cyclops*, *A. longifolia*, *A. melanoxylon*, *A. saligna* and *Paraserianthes lophantha*) by examining both the above- and belowground aspects of their invasion ecology across multiple non-native and native range populations in Australia. The aboveground studies investigated the changes in reproductive allocation, plant size and genetic diversity of these legumes across several native and non-native populations (Harris *et al.*, 2012). In this thesis, I describe research on the belowground soil microbial communities of these five legumes, with an emphasis on rhizobia and soil fungi, and their role in the invasion success of these plant species. Combining this new information from above- and belowground studies on these legumes will help to develop a more complete understanding of the relative importance of above- and below-ground interactions in determining invasion success. This research will also provide an important contribution to the invasion ecology discipline that aims to understand the complex mechanisms that affect the invasion success of a plant species introduced to a novel environment.

**THE ROLE OF SOIL BIOTA IN PLANT INVASIONS**

There is an intrinsic link between aboveground plant communities and belowground soil microbial communities that influences ecosystem and community processes as well as the outcome of the introduction of novel plant species to a site (Callaway *et al.*, 2004; Wardle *et al.*, 2004; Wolfe & Klironomos, 2005; Reinhart & Callaway, 2006; van der Putten *et al.*, 2009; Inderjit & van der Putten, 2010). There are several hypotheses that test the plausible pathways of plant invasions (MacDougall *et al.*, 2009). However, studies of plant-soil interactions in invasions have predominantly focussed on the absence of soil enemies in the novel range (Mitchell & Power, 2003). More recently the significance of soil mutualists in plant invasions has been highlighted

by several authors (Parker *et al.*, 2006; Nuñez *et al.*, 2009; Rodríguez-Echeverria, 2010; Callaway *et al.*, 2011).

**The role of soil pathogens in plant invasions**

Soil pathogens may play a central role in regulating the processes that could affect the invasion success of a species in its new range. One of the most frequently tested hypotheses in invasion ecology is the enemy release hypothesis (Keane & Crawley, 2002) which postulates that plant species will, upon introduction into a novel region experience a decreased load of natural enemies (e.g. herbivores and pathogens) which could result in a rapid increase in invader establishment success, distribution and abundance.

Plant-soil feedbacks have been studied widely in invasion ecology to test for enemy-release effects but also to inform more generally on plant abundance, persistence and succession (Bever, 2003; Callaway *et al.*, 2004; Diez *et al.*, 2010). Generally, it is expected that the invader experiences stronger negative feedback in the native range and stronger positive feedback in the non-native range (Callaway *et al.*, 2004). The sign of feedback is strongly correlated with the host’s specificity and selectivity of certain soil microbial functional groups (Bever *et al.*, 1997; Grayston *et al.*, 1998; Bever, 2003).

Several studies of North American plant invaders such as *Centaurea maculosa* and *Alliaria petiolata* (Callaway *et al.*, 2004; Callaway *et al.*, 2008), have found evidence for positive plant-soil feedback. However, more recent reports indicate that invaders can also experience negative feedback in their non-native ranges (Andonian *et al.*, 2011a; Andonian *et al.*, 2011b). Thus plant-soil feedback effects are not always consistently positive in the non-native range and negative in the native range, suggesting that other biotic and abiotic factors and their interactions may influence the outcome of plant-soil feedbacks (e.g. climate, genetic adaptation and human imposed artificial selection).

**Importance of soil mutualisms in plant invasions**

Curiously, the role of soil mutualists (e.g. nitrogen-fixing bacteria and mycorrhizal fungi) in plant invasion success has been generally less studied than the role of pathogens (Callaway *et al.*, 2011). However these nutritional modes are widely distributed among many plant groups and the absence of compatible soil mutualists in the novel range could have a significant negative effect on plant establishment (Pacovsky *et al.*, 1986; Richardson *et al.*, 2000; Parker, 2001; Stanton-Geddes & Anderson, 2011).

Similarly to pathogens, soil symbionts may influence the outcome in plant community composition and diversity (van der Heijden *et al.*, 2008). For instance, van der Heijden *et al*. (2008) suggested that the presence of nitrogen fixing symbionts enhances host performance and competitive ability, which in turn could influence vegetation succession (Vitousek & Walker, 1989) and plant invasibility (Parker *et al.*, 2006). Mycorrhizal fungi on the other hand can provide the host with resistance to drought and disease in addition to supplying limiting nutrients such as nitrogen, phosphorus, copper, iron and zinc in exchange for carbon (van der Heijden *et al.*, 2008). Thus soil symbionts may play a crucial part in plant establishment and growth both in the native and non-native ranges and the absence or lack of suitable partners could significantly hinder invasion success.

**ACACIAS AS INVADERS**

*Acacia* (Fabaceae, subfam. Mimosoideae) is a large genus of shrubs and trees with a cosmopolitan distribution across all continents except Antarctica (Lewis, 2005). In Australia, *Acacia* is one of the most dominant plant clades with 1012 species in the subgenus Phyllodineae being native to Australia (Richardson & Rejmánek, 2011b). *Acacia cyclops*, *A. longifolia*, *A. melanoxylon*, *A. saligna* and *Paraserianthes lophantha* (a closely related species of *Acacia* subgenus Phyllodineae (Brown *et al.*, 2008)) were chosen as study species because they are some of the most problematic invaders both in Australia as well as globally (Richardson *et al.*,

2011a; Richardson & Rejmánek, 2011b), and are known to associate with both nitrogen-fixing rhizobia and mycorrhizae in their native ranges.

*Acacia* spp. and *P. lophantha* (henceforth acacias) have a long history of anthropogenic trans-continental and trans-regional introductions (Carruthers *et al.*, 2011). They have been intentionally introduced for multiple purposes such as for forestry, ornamental purposes and sand dune rehabilitation (Kull *et al.*, 2011). They are considered naturalized or invasive in many countries across the world (e.g. South Africa (Yelenik *et al.*, 2004), New Zealand (Weir *et al.*, 2004) and Portugal (Hellman *et al*., 2011; Rascher *et al*., 2011a; Rascher *et al*., 2011b) and pose a significant threat to many native plant communities worldwide (Richardson *et al.*, 2011a).

**The role of soil microbes in the invasion success of acacias**

Although there have recently been several published studies that have explored different aspects of acacias’ invasion ecology (e.g. reproductive biology (Gibson *et al.*, 2011) and functional traits (Gallagher *et al.*, 2011; Morris *et al.*, 2011)) there are relatively few studies that have comprehensively assessed the role of different soil microbial communities in mediating invasion success of acacias, especially in Australia.

Acacias in their introduced ranges have been shown to substantially modify soil properties (Hellmann *et al.*, 2011; González-Muñoza *et al.*, 2012) and impose long lasting impacts on the native plant and soil communities (Marchante *et al.*, 2008a; Rascher *et al.*, 2011b). Notably, acacias have been reported to change the soil chemical properties in soils in their non-native ranges (González-Muñoza *et al.*, 2012; Rascher *et al.*, 2012). This is generally explained by the fact that nitrogen fixing legumes have greater foliar N content than non-nitrogen fixing species (Rascher *et al.*, 2012).Thus alterations to soil chemical properties by these acacias inevitably leads to changes to the soil microbial community composition and functioning (Marchante *et al.*, 2009), although in some instances the effect on soil microorganisms depends more on the ecosystem type that is being invaded (Lorenzo *et al.*, 2010).

The role of nitrogen fixing bacteria in acacia invasion has received considerably more attention compared to other soil microbial communities (e.g. mycorrhizal fungi and soil pathogens). This is understandable since legumes, including acacias, are second only to Poaceae in agricultural and economic importance (Wojciechowski *et al.*, 2004) and have been widely adopted in agriculture and rehabilitation (Adams *et al.*, 2010) due to their symbiotic capacity for nitrogen fixation (Graham & Vance, 2003). Therefore a substantial amount of information exists describing the symbiotic nitrogen fixation in woody legumes, especially *Acacia* (Leary *et al.*, 2006). The ability to fix nitrogen has been also credited as one of the traits that plausibly has facilitated the invasion success of acacias beyond their original introduced populations (Sprent & Parsons, 2000). There are now several studies that have described the rhizobial communities associated with acacias in their non-native ranges, e.g. in Portugal (Rodrıguez-Echeverria *et al.*, 2009; Rodríguez-Echeverria, 2010), Spain (Rodríguez-Echeverría *et al.*, 2003), New Zealand (Weir *et al.*, 2004), South Africa (Marsudi *et al.*, 1999; Joubert, 2003) and in Australia (Lafay & Burdon, 2001; Hoque *et al.*, 2011). However differences in rhizobial composition and diversity associated with these acacias in Australia have been rarely evaluated as a plausible constraint or facilitation to the invasion success of these species.

There is considerably less information describing the soil fungal communities compared to the rhizobial communities associating with acacias, especially in Australia. It is generally accepted that acacias associate with both arbuscular (AM) and ectomycorrhizal (EM) fungi (Brundrett, 2009). However some authors have suggested that in northern and eastern Australia acacias associate with both EM and AM or only AM fungi (Warcup, 1980; Bellgard, 1991), while in south-west Australia they have been reported to associate only with AM fungi. Nevertheless, more data is needed to elucidate the fungal communities (e.g. mycorrhizae and soil pathogens) in both native and non-native ranges of these acacias and the possible effects of these on invasion success.

The main aim of this thesis was to examine the role of soil biota, with an emphasis on rhizobia and fungi, in the invasion success of four *Acacia* spp. and a close relative *Paraserianthes*

*lophantha* in Australia. Across four data Chapters following questions were addressed: (i) Do acacias experience positive plant-soil feedback in the non-native range and negative plant-soil feedback in the native range? (ii) Do soil mutualisms, such as rhizobia, constrain or facilitate the invasion success of acacias in their non-native range? (iii) Are these acacias specialists or generalists hosts? (iv) Is the overall composition of nitrogen fixing bacterial and fungal communities similar or different across the ranges and do these differences contribute to more successful invasion?

In this thesis, Chapter 2 describes an experiment using the “black box” approach and utilizing plant-soil feedback method to test for soil origin effects on the five legume study species by comparing plant biomass when grown in native versus non-native range population soils. In Chapter 3, I assessed the relative importance of rhizobia in the invasion success of these legumes by conducting a glasshouse experiment, estimating the rhizobial abundance and rhizobial community composition in the non-native and native range population soils. In Chapters 4 and 5, I comprehensively described the nitrogen fixing and fungal communities found in the rhizosphere of these legumes using 454 sequencing across multiple non-native and native range populations to assess whether these communities are different across the continent and whether these legumes are specialist or generalist hosts in Australia.

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**Chapter 2: The role of plant-soil feedbacks in the cross-continental invasion success of Australian legumes**

This manuscript has been submitted to *Biological Invasions* as ‘Birnbaum C. and Leishman M.R. ‘The role of plant-soil feedbacks in the cross-continental invasion success of Australian acacias’.

My contribution to the research and paper: Concept - 90%; Data collection - 100%; Analysis - 90%; Writing - 90%

**ABSTRACT**

Legumes, especially acacias, are considered amongst the most successful invaders globally. However there is still very little known about the role of soil microbial communities in their invasion success in novel ranges. We examined the role of the soil microbial community in the invasion success of four *Acacia* species (*A. cyclops*, *A. longifolia*, *A. melanoxylon* and *A. saligna*) and a close relative *Paraserianthes lophantha*, introduced into novel regions within Australia using a “black-box” approach. Seed and soil material were collected from multiple populations within each species’ native and non-native range within Australia and used in a plant-soil feedback experiment to assess the effect of the soil microbial community on plant growth and nodulation. We found no effect, either positive or negative, of soil origin on species’ performance, however there was a significant interaction between species and seed origin. Seed origin had a significant effect on the biomass of two species, *A. cyclops* and *A. saligna*. *Acacia cyclops* plants from the native range performed better across all soils than plants from the non-native range. The opposite trend was observed for *A. saligna*, with plants from the non-native range performing better overall than plants from the native range. Seed or soil origin did not have a significant effect on the presence and number of nodules suggesting that rhizobia do not constrain the invasion success of these legumes. Our results suggest that plant-soil feedbacks are unlikely to have played a significant role in the invasion success of these five species introduced into novel regions within Australia. This may be due to the widespread occurrence of acacias and their associated soil microbial communities throughout the Australian continent.

**Key-words:** Invasive species, Legumes, Novel ranges, Plant-soil interactions, Rhizobia

**INTRODUTION**

Increasingly it has been recognised that soil microbial communities may play a significant role in shaping the outcome of exotic plant invasions (Bever et al. 2010; Inderjit and van der Putten 2010; Klironomos 2002; Reinhart and Callaway 2006). Several direct and indirect pathways have been suggested to describe the below-ground biotic interactions between plants and their associated microbial communities (Inderjit and van der Putten 2010). For example, release from detrimental soil pathogens from the native range has been linked to the invasion success of some plant species in a range of environments (Mitchell and Power 2003; Wolfe and Klironomos 2005). Furthermore, presence of beneficial novel soil microbial community components, for example such as nitrogen fixing bacteria, has been proposed to be one of the key factors for successful establishment of an invader (Richardson et al. 2000; Simberloff and Von Holle 1999).

In addition, invaders may have detrimental effects on co-existing natives through indirect pathways (Inderjit and van der Putten 2010) via invader-induced changes to soil microbial communities. For example, allelopathic effects from plant invaders on native soil biota (Scharfy et al. 2011), accumulation of native soil pathogens in the presence of invasive plants (Eppinga et al. 2006), disruption of native mutualistic associations between native plants and their symbionts (Stinson et al. 2006) or nutrient release from exotic litter by decomposers (Marchante et al. 2008b) have all been suggested as indirect mechanisms that invaders may exploit to indirectly suppress the growth of native plants.

Direct or indirect pathways of below-ground trophic interactions between plants and their associated soil biota can generally have a net positive or net negative effect (termed ‘feedback’) on the plants. The plant-soil feedback approach has been used successfully in previous studies to test the effects of reciprocal interactions between plants and soil biota (Bever et al. 1997; Klironomos 2002; Levine et al. 2006; van Grunsven et al. 2009). This approach is based on two main assumptions (Kulmatiski et al. 2008). Firstly, plants will cause species-specific changes to soil biota, and secondly plants will show species-specific responses (i.e. plant-soil feedback) to

these changes (Dostál and Palečková 2010). Plant-soil feedbacks have been proposed to be either: (1) positive when the net effect on plant performance of the beneficial symbiotic community is stronger than the effect of parasites, pathogens or herbivores taken together (Bever et al. 1997); (2) neutral if the effect of these two groups is equal (van Grunsven et al. 2009); or (3) negative if the effect of the detrimental soil community is stronger than the effect of the beneficial soil biota (van Grunsven et al. 2009).

It is generally expected that a plant invader will experience more negative feedback in its native range and more positive feedback in its non-native range (Callaway et al. 2011; Callaway et al. 2004; Kulmatiski et al. 2008). For example *Centaurea maculosa* (spotted knotweed), an invasive perennial forb in North America, has been shown to be more inhibited by its native European soil microbial communities than by the soil biota in its non-native range in North America (Callaway et al. 2004). Another European invader in North America, *Alliaria petiolata* (garlic mustard), has been reported to inhibit the mycorrhizal fungal communities only in its invaded range, to the detriment of the co-existing native species that rely on those symbionts (Callaway et al. 2008). In another study, Engelkes and colleagues (2008) compared herbivore load, soil pathogens and plant performance of range-expanding plant species compared to native congeneric species. Range-expanding species were less affected by shoot herbivores compared to congeneric natives and also developed fewer pathogenic effects in their root-zone soil (Engelkes et al. 2008). Thus there is some evidence that invasive species in their non-native range and range expanding, potentially invasive species, experience overall less pressure from soil-borne pathogens and parasites compared to in their native range or to native species, respectively.

Although these studies have shown that some plant invaders are indeed able to escape their soil-borne enemies in their non-native range or that invaders are able to beneficially modify the soil communities to the detriment of co-existing natives, there are also several recent studies that have shown that invader success is not associated with positive plant-soil feedback in the non-native ranges (Andonian et al. 2011; Scharfy et al. 2010; te Beest et al. 2009). For example,

te Beest and colleagues (2009) showed that the highly invasive shrub *Chromolaena odorata* (Siam weed) was not released from its soil-borne enemies in non-native range soils. Instead, success in the non-native range of this species may be due to growth and allocation differences between seedlings from the non-native and native ranges (te Beest et al. 2009). Some authors have suggested that plant genotype may play an important role in shaping the belowground soil microbial composition. Schweitzer and colleagues (2008) showed in a common garden experiment using a model *Populus* system with replicated clones of known genotypes, that 78% of variation in soil microbial community composition could be attributed to plant genotype effects. Although genotype effects may be easily detected in some instances, other authors have found no effect of plant species within and across native and non-native range populations, suggesting the possibility of similar rhizosphere soil biota across populations and regions (Wagner et al. 2011).

In another study Scharfy et al. (2010) reported that the invasive perennial herb *Solidago gigantea* had a strong negative feedback on itself, with significantly less biomass produced when the inoculum came from a site invaded by *S. gigantea* (Scharfy et al. 2010). Similar results were reported by Andonian et al. (2011) for the invader *Centaurea solstitialis* (yellow starthistle). Thus there appears to be considerable variation between plant invaders and the role of the soil microbial community in their invasion success in novel ranges.

The main objective of this study was to assess whether there are consistent soil feedback effects among five closely-related legume species that are native to Australia and have been successfully introduced to novel sites beyond their native ranges within Australia (*A. cyclops, A. saligna,* *A. longifolia*, *A. melanoxylon*, and a close relative *Paraserianthes lophantha*, hereon collectively referred to as acacias). Most plant-soil feedback studies have focussed overwhelmingly on the impacts of the invader in the introduced range, however a biogeographical approach enables more comprehensive predictions on the invasion ecology and invasion outcome of a species in the non-native range (Hierro et al. 2005). We tested the performance of the five acacias using a plant-soil feedback experiment that assessed the net

effects of the soil microbial community by comparing each species’ performance using soil and seed material collected from multiple populations from both native and introduced ranges.

In addition, we recorded the number and distribution of effective root nodules of the five study species as it has been previously suggested that invasion success of legumes, including acacias, can be at least partly attributed to their ability to associate with nitrogen fixing bacteria (Rodriguez-Echeverria et al. 2009; Sprent and Parsons 2000). The role of soil mutualists on invasion outcomes is less well understood compared to the role of soil pathogens that have been more extensively studied in invasion ecology (Callaway et al. 2011). Therefore it is important to understand the role of soil mutualists such as rhizobia, especially for nitrogen fixing invasive species, and their plausible effects on the invasion outcome in the novel range (Birnbaum et al. 2012; Parker et al. 2007; Parker 2001). We predicted that acacias would perform better (positive soil feedback) when grown in their non-native range soils compared to native range soils, as they would be released from soil pathogens. We also hypothesized that acacia would not be constrained by rhizobia in the non-native range (Birnbaum et al. 2012). To our knowledge this study represents one of the few attempts to comprehensively describe the plant-soil feedback effects of legumes based on multiple species and population comparisons from both native and introduced ranges.

**MATERIALS AND METHODS**

**Study species**

We chose five species of the family Fabaceae subfamily Mimosoideae. Following Miller *et al*. (2011) *A. cyclops*, *A. longifolia* and *A. melanoxylon* are placed in the melanoxylon clade, whereas *A. saligna* belongs to the Pulchelloidea clade. *Paraserianthes lophantha* is a closely related species of *Acacia* subgenus Phyllodineae (Brown *et al.*, 2008). The five species are all shrubs to medium-sized trees that occupy wet or dry sclerophyll forests, rainforests and coastal communities in their native range within Australia (Maslin *et al.*, 2001b) (Table 1). *Acacia*

*cyclops* A.Cunn. ex G.Don, *A. saligna* (Labill.) H.L. Wendl and *P. lophantha* (Willd.) I.C. Nielsen are native to south-west Australia but have been introduced to the eastern states of Australia (New South Wales, Victoria and South Australia) where they have naturalised and become invasive. *Acacia cylops* has been widely used in eastern Australia for dune stabilisation and as an ornamental shrub (Virtue & Melland, 2003). *Acacia saligna* was introduced to eastern Australia for dune rehabilitation following sand mining and for ornamental purposes (Tame, 1992). *Paraserianthes lophantha* has been widely promoted as a garden plant and has become naturalised in eastern Australia, invading local bushland around towns and gardens in many areas (Cowan, 2001).

*Acacia longifolia* (Andrews) Willd. and *A. melanoxylon* R.Br. are native to south-east Australia (Queensland, New South Wales, Victoria and South Australia) but have been introduced to the south-west of Western Australia. Both species have been used as horticultural species and widely cultivated in the south-western region of Western Australia where they have become naturalised and are considered invasive (Maslin *et al.*, 2001a; Hussey *et al.*, 2007). *Acacia longifolia* comprises two subspecies, *longifolia* and *sophorae* (Entwisle *et al.*, 1996; Maslin *et al.*, 2001a)*,* which are capable of hybridization and are almost impossible to separate in the field. It is highly unlikely that any of these species have been transported naturally across the continent as the eastern and western ranges of each species are separated by the vast Nullarbor Desert, providing a major barrier to natural seed dispersal between native and introduced ranges (Jacobs & Wilson, 1996).

All species are also recognised as invasive or naturalised outside Australia. The five species have variously been introduced for forestry and rehabilitation purposes in South Africa (Roux, 1961), Portugal (Marchante *et al.*, 2003; Marchante *et al.*, 2008b; Rodríguez-Echeverria *et al.*, 2011), Hawaii (Wagner *et al.*, 1999) and New Zealand (Owen, 1997). Their success has been attributed to their ability to grow larger than native plants in non-native ranges (Morris *et al.*, 2011), their capacity to form extensive and persistent seed banks (Richardson & Kluge, 2008) and their ability to associate with a wide range of beneficial symbionts such as mycorrhizal fungi and

nitrogen fixing bacteria (Sprent & Parsons, 2000; Morris *et al.*, 2011; Rodríguez-Echeverria *et al.*, 2011).

**Soil and seed collection**

Soil and seed material was sampled across a wide area within the native and non-native ranges of each species (Table 1). Samples were collected in December 2009 across south-east and south-west Australia from Perth to Ravensthorpe in Western Australia and from Sydney to Yorke Peninsula in eastern Australia (Fig.1). For each species, we sampled five individuals from each of five populations within each range [5 species x 2 ranges (native and non-native) x 5 populations x 5 individuals]. From each individual plant we collected mature pods. Seeds were removed from pods in the laboratory for germination. We collected 1000 g of soil from underneath each individual plant as close to roots as possible at a depth of 10-15 cm. All seed and soil material were bulked within populations. Soils were kept in coolers in the field before being stored at 4oC in the laboratory prior to use in the experiment. All soil sampling and processing equipment was sterilized with 90% ethanol between sample collections. Soils were sieved through 2 mm sieve to separate out leaves and other coarse material and to homogenise the samples.

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**Plant-soil feedback experiment**

To test the effects of native and non-native range soils on plant growth and biomass allocation we performed a glasshouse experiment using field-collected soil as inoculum. In total, soil and seed material from 29 populations were used for the soil conditioning stage of the experiment and from 24 populations for the feedback stage (Table 1). The reduced number of seed by soil combinations compared with the conditioning stage was due to insufficient seed material for some populations. For example, the seeds from *P. lophantha* and *A. melanoxylon* native and non-native ranges, respectively, were heavily predated. It is important to note that we used a hybrid experimental approach to test for feedback responses between plants and their net soil microbial communities. This approach differs from other studies that have compared plant performances in home *vs* away treatments where “away” has been generally soil conditioned by heterospecific plants (Klironomos 2002; Mangan et al. 2010; Smith and Reynolds 2012; but see te Beest et al. 2009 and Callaway et al. 2011) ). In our study the “away” treatment was location, not another plant species.

***Stage 1: Soil conditioning***

We initially grew plants from each population for 11 weeks using its own soil as inoculum in order to amplify the soil microbial community associated with each population of each species (‘soil conditioning’). For *A. cyclops* we used soil/seed material from 4 native (N) and 3 non-native (NN) populations, for *A. longifolia* 3 N + 4 NN, for *A. melanoxylon* 3 N + 2 NN, for *A. saligna* 2 N + 3 NN and for *P. lophantha* 2N + 3 NN populations. The different number of seed and soil combinations was due to the variability of available seed material.

All seeds were treated as appropriate to maximise germination, rinsed with distilled water and placed in autoclaved sterilized sand (121oC 30 minutes wet cycle) in Petri dishes within growth cabinets for germination (25oC/18oC, light on/off 12 h). We controlled for potential differences

in seed mass between populations by only using the seeds that were within one standard deviation of the mean seed mass for a given species.

Each seed and soil combination was replicated 10 times, giving a total of 290 pots for the 29 populations. Seedlings at second leaf stage were transplanted to pots of 6.5 cm diameter and 21 cm height (total volume 0.64 L) that were filled with 1:2 sterilized (121oC 30 minutes wet cycle) coarse sand and sterilized (121oC 30 minutes wet cycle) fine river sand, respectively. Fifty mL of inoculum (native or non-native) of field soil was added to each pot as a separate layer. Then 50 mL of sterilized (121oC 30 minutes wet cycle) potting mix was added on top of the field soil layer. The location of pots within the glasshouse was randomised initially and fully re-randomised every two weeks. Plants were watered three times daily with tap water. During the growth period no nutrients were added as there was considered to be sufficient nutrients in the inoculum as well as in the potting mix layer to sustain the plants during the growing period. Addition of nutrients (e.g. nitrogen and phosphorus) was avoided as it could suppress the activity of nitrogen fixing bacteria and mycorrhizal fungi.

The glasshouse temperature ranged between 18oC night and 25oC day. At the end of the soil conditioning phase the sandy soil layer containing root fractions of each pot was collected, being careful to avoid mixing the soil layers. The sandy layer is likely to contain the highest concentration of soil microbes and nutrients, while the surface potting mix layer is likely to be contaminated with air-borne microbes from the glasshouse. The collected sandy soil layer from each of the 10 replicate pots from each seed/soil combination were bulked together. Root material was then cut into 1 cm pieces and homogenised with soil and placed in double zip-lock plastic bags and stored at 4oC to be used as inoculum in the next stage of the experiment. All soil sampling and processing equipment was sterilized with 90% ethanol between the samples to avoid contamination.

**Table 1.** Details of populations sampled for soil and seed material of five species in their native and non-native ranges and the examples of habitats they occupy in both their native and non-native ranges in Australia.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Species** | **Status** | **State** | **Location** | **Latitude** | **Longitude** | **Habitat** | |
| **Native range** | **Non-native range** |
| *1. A. cyclops* | Native  Native  Native  Native  Non-native  Non-native  Non-native | WA  WA  WA  WA  SA  SA  SA | Ravensthorpe  Bremer Bay  William Bay  Coogee  Yorke Peninsula  Yorke Peninsula  Victor Harbour | 33º 36´  34º 25´  35º 1´  32º 7´  34º 43´  35º 3´  35º 32´ | 120º 12´  119º 22´  117º 14´  115º 45´  137º 35´  137º 43´  138º 38´ | * Dry sclerophyllb * Coastal sand dunes and limestonea * Coastal heathb | * Dry sclerophyllb * Coastal sand dunes   and limestonea   * Coastal heathb |
| 2*. A. saligna* | Native  Native  Non-native  Non-native  Non-native | WA  WA  VIC  VIC  SA | Toodyay  Perth  Portland-Nelson Rd  Surf Coast Hwy  Mornington peninsula | 31º 33´  31º 51´  38º 7´  38º 16´  38º 13´ | 116º 27´  115º 45´  141º 14´  144º 19´  145º 5´ | * Dry sclerophyllc * Coastal dunesc * Near water courses and other wet areasc | * Dry sclerophylld * Coastal sand dunesd * Along major highwaysd * Open disturbed forestsd |
| **Table 1.** Details of populations sampled for soil and seed material of five species in their native and non-native ranges and the examples of habitats they occupy in both their native and non-native ranges in Australia (cont.). | | | | | | | |
| **Species** | **Status** | **State** | **Location** | **Latitude** | **Longitude** | **Habitat** | |
|  |  |  |  |  |  | **Native range** | **Non-native range** |
| 3*. P. lophantha* | Native  Native  Non-native  Non-native  Non-native | WA  WA  VIC  VIC  NSW | Gingilup Swamps NR  Mount Frankland NR  Port Fairy  Toora  Eden | 34º 18´  34º 31'  38º 23´  38º 38´  37º 04´ | 115º 24´  115º 42'  142º 12´  146º 17´  149º 54´ | * Winter-wet depressionsa * Near creeks or swampsa * Granite outcropsa | * Bushland around towns and gardense |
| 4. *A. longifolia* | Native  Native  Native  Non-native  Non-native  Non-native  Non-native | VIC  VIC  VIC  WA  WA  WA  WA | Portland-Nelson Rd  Cape Otway  Wilsons Promontory  Mt Barker  Gracetown  Watkins Road NR  Gidgegannup | 38º 11´  38º 51´  38º 56 ´  34º 39´  33º 51´  32º 18´  31º 47´ | 141º 20´  143º 30´  146º 16´  117º 33´  115º 01´  116º 00´  116º 11´ | * Dry sclerophylld * Coastal heath and scrubd * Sand on foredunesd | * Dry sclerophylld * Coastal heath and scrubd * Sand on foredunesd |
| **Table 1.** Details of populations sampled for soil and seed material of five species in their native and non-native ranges and the examples of habitats they occupy in both their native and non-native ranges in Australia (cont.). | | | | | | | |
| **Species** | **Status** | **State** | **Location** | **Latitude** | **Longitude** | **Habitat** | |
|  |  |  |  |  |  | **Native** | **Non-native** |
| 5. *A. melanoxylon* | Native  Native  Native  Non-native  Non-native | VIC  VIC  VIC  WA  WA | Port Fairy  Apollo Bay  Toora  Albany  Elleker | 38º 17´  38º 45´  38º 38´  35º 1´  35º 00´ | 142º 1´  143º 39´  146º 17´  117º 53´  117º 43´ | * Wet sclerophylld * Rainforestd | * Wet sclerophylld * Rainforestd |

a Descriptions by the Western Australian Herbarium, Department of Environment and Conservation. Text used with permission (http://florabase.dec.wa.gov.au/help/copyright). Accessed on Tuesday, 6 December 2011.

bR.S.Cowan, B.R.MaslinFlora of Australia. Volumes 11A (2001), 11B (2001) and 12 (1998).

c J.C. Doran, J.W. Turnbull (eds.) (1997) Australian Trees and Shrubs: species for land rehabilitation and farm planting in the tropics. Australian Centre for International Agricultural Research, Canberra [ACIAR books online: http://www.aciar.gov.au/publication/MN024 Accessed Nov. 2011]

d P.G. Kodela & G.J. Harden, (2002). Flora of NSW Vol. 2.

e R. S. Cowan, (2001). Flora of Australia Volume 11A. Edited by A.E. Orchard and A.J.G. Wilson.

***Stage 2: Feedback response***

We used the conditioned soil as inoculum to test for the effects of soil microbial communities from native and non-native soils on plant growth, measured as total biomass. Seeds from native and non-native range populations were grown with both native and non-native range soil inoculum in all possible combinations within each species. For *A. cyclops* we had 3 native (N) and 3 non-native (NN) populations, for *A. longifolia* 3 N and 3 NN, for *A. melanoxylon* 3 N and 1 NN, for *A. saligna* 2 N and 2 NN and for *P. lophantha* 1 N and 3 NN populations.

The total number of reciprocally grown seed/soil combinations for all species was 120 (for *A. cyclops* and *A. longifolia* 6x6 each; for *A.melanoxylon*, *A. saligna* and *P. lophantha* 4x4 each) with 8 replicates of each combination giving a total of 960 pots. For example, for *A. cyclops* we used seed and soil material from 3 (N) and 3 (NN) populations. Seedlings from each population were reciprocally grown with all *A. cyclops* soils from native and non-native populations. This gave a total of 36 soil x seed combinations for *A. cyclops* with four different treatments (native seed x native soil, native seed x non-native soil, non-native seed x non-native soil and non-native seed x native soil).

The experimental procedure was similar to the soil conditioning stage of the experiment, except plants were grown for 15 weeks. Nutrients were added once during the growth period to account for increasing plant requirements. We used Osmocote® plus low phosphorus formula that is designed specifically to match the nutritional needs of Australian plants. This formula contained 17% N, 1.6% P, 8.7% K, 3.7% S, 0.6% Mg, 0.01% B, 0.025% Cu, 0.2% Fe, 0.03% Mn, 0.01% Mo and 0.01% Zn. Four mL of slow-release fertilizer was added to each pot after 7 weeks. At harvest shoots and roots were separated. Roots were washed free from soil. Shoots and roots were dried at 75oC for 48 hours and weighed. The distribution and number of effective nodules (representation of presence of nitrogen fixing bacteria that are collectively termed rhizobia) was recorded for each replicate according to Corbin, Brockwell & Gault (1977) (Table 2). Nodule scores ranged from 0 – 5 (Corbin *et al.*, 1977), with low scores representing plants

with small number of effective nodules distributed more broadly along lateral roots, and high scores representing plants with higher number of effective nodules distributed mostly along the crown and few throughout the root system (Corbin et al. 1977). Small nodules occurring mostly along the lateral roots are likely to indicate a rather negligible impact on plant performance (Thrall et al. 2007), whereas bigger nodules located on the crown root suggest a more beneficial impact on plant performance. Nodule scores were averaged per population and treatment per host plant species and a binary table with presence/absence of nodules at a given score (0 – 5) per population was created (Table 6).

**Table 2**. Classification of nodulation used to score the nodules from the soil feedback experiment. Reproduced with permission from Corbin *et al.* (1977).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Nodule score | Distribution and number of effective nodules† | | | |
|
| Crown‡ | | Elsewhere | |
| 0 | 0 | | 0 | |
| 0.5 | 0 | | 1 - 4 | |
| 1 | 0 | | 5 - 9 | |
| 1.5 | 0 | | ≥10 | |
| 2 | Few | | 0 | |
| 2.5 | Few | | Few | |
| 3 | Many | | 0 | |
| 4 | Many | | Few | |
| 5 | Many | | Many | |
|  |  |  |  |  |
| † Effectiveness judged on basis of nodule size and internal | | | | |
| pigmentation; ineffective nodules not considered. | | | |  |
| ‡ Crown regarded as top 5 cm of root system. | | | |  |

**Soil chemical characteristics**

Soil chemistry was assessed to determine whether abiotic characteristics of soils varied between sites. A total of 27 sub-samples from 4 native (N) and 3 non-native (NN) soils for *A. cyclops*, 3 N and 4 NN for *A. longifolia,* 3 N and 1 NN for *A. melanoxylon*, 2 N and 3 NN soils of *A. saligna* and 1 N and 3 NN for *P. lophantha* was analysed*.* These were air dried and analysed at the Sydney Environmental & Soil Laboratory (SESL) for total nitrogen and phosphorus using LECO C:H:N and ICP-AAS (with HCl digest) respectively, total organic carbon using Dumas combustion (SESL in-house Method LECO 1), organic matter using calculation of TOC (based on LECO 1) and ammonium (NH4+) and nitrate (NO3-) using Mehlich 3 (SESL in-house CaCl2  extraction, followed by UV/Vis Spectro finish; APHA 4500-NO3-E (modified), respectively). Soil pH was measured in 1:5 soil to water preparations using a TPS digital pH meter (TPS Pty. Ltd., Brisbane).

**Statistical analyses**

Differences in total, above- and belowground biomass and nodules were analysed for all species using a mixed model ANOVA with soil and seed origin (native or non-native), species and their interactions as fixed factors and population nested within soil and seed origin as a random factor. Tukey’s post-hoc tests were performed to analyse for differences between species.

Mixed effects ANOVA was also performed on each species separately with soil and seed origin (native or non-native) as fixed factors and population nested within soil and seed origin as a random factor. One way ANOVA with Tukey’s post-hoc tests were performed to analyse differences between different soil and plant combination treatments within each species.

Data on soil chemical characteristics were analysed using one way ANOVA for each species separately with range (native or non-native) as the main factor. To meet the homogeneity and normality assumptions of ANOVA biomass data was log10 transformed and nodule data transformed to fit Poisson distribution. Total, above- and belowground biomass data, nodule data and soil chemistry data were analysed in SPSS version 21.0 (IBM SPSS Statistics) and plotted in R programming language (version R2.15.2) (R Development Core Team 2006).

**RESULTS**

**Plant biomass**

There was no significant effect of soil origin on plant biomass consistently across all species (Table 3). However there was a significant species by seed origin interaction for plant biomass, indicating that the effect of seed origin (native or non-native) on biomass varied among species (Table 3). Within-species analysis revealed a significant effect of seed origin on total biomass for two out of five species (e.g. *A. cyclops* and *A. saligna*) and for one species, *A. longifolia*, seed origin had near significant (*P* = 0.05) effect on total biomass (Table 4). Seedlings of *A. cyclops* grown from native range seed had *ca*. 24% greater total biomass than seedlings grown from non-native range seed when grown in both native and non-native range soils (Fig. 2). However Tukey’s post-hoc test did not confirm the differences in biomass between the treatments for *A. cyclops* (Table 5).

The opposite pattern was observed for *A. saligna* seedlings from the non-native range that performed better (19% greater total biomass) compared to the seedlings from the native range when grown in both native and non-native range soils (Fig. 2). Indeed, Tukey’s post-hoc test confirmed significant differences in aboveground biomass between the treatments (F3,4 = 11.63, *P* = 0.019) (Fig. 2, Table 5). Similarly to *A. saligna*, the seedlings of *A. longifolia* grown from the non-native range seed had ca. 31% greater biomass than seedlings grown from native range seed (Fig. 2), however post-hoc tests did not detect significant differences between the treatments (Table 5).

We observed no significant effect of seed origin on *A. melanoxylon* or *P. lophantha* biomass (Table 4). For the four *Acacia* species and *P. lophantha* there was overall no significant seed origin by soil origin interaction (Table 4). However for *P. lophantha* there was a significant effect of soil origin on total biomass (Table 4). Nevertheless Tukey’s post-hoc tests did not reveal significant differences in biomass for *A. melanoxylon* or *P. lophantha* (Fig. 2, Table 5), which may be due to low replication at the population level. For *P. lophantha* we could use seed material from only one native population and for *A. melanoxylon* from only one non-native population due to high seed predation, which limited our analyses. Population nested as a random factor within soil and seed origin was not significant in either the overall species model or any of the within-species analyses.

**Number and distribution of effective nodules**

We did not find a significant effect of soil or seed origin on the number and distribution of effective nodules across non-native and native range soils and hence nodule data was excluded from further analyses (Table 3). Nevertheless, some variation in the number and distribution across species and treatments could be observed based on the obtained scores (Table 6). For instance, both non-native and native seedlings of *A. cyclops* scored between zero to one across both non-native and native range soils suggesting that the majority of nodules occurred on lateral roots rather than on the crown roots. For *A. saligna*, *A. melanoxylon* and *P. lophantha* non-native seedlings had in many instances more nodules on the crown roots compared to native seedlings across both non-native and native range soils. Interestingly, *A. longifolia* seedlings from the non-native range had the highest number of effective nodules on the crown roots across both non-native and native range soils (Table 6).

**Soil chemical analysis**

We found no significant differences in organic matter, pH, total N, ammonium (NH4+), total P and total C between native and non-native range soils for any of the five species. However values were generally higher in soils of non-native populations of *P. lophantha* (Table 7). Ammonium (NH4+) was highest in soils of non-native populations of *A. longifolia* (Table 7). The only significant difference between species’ native and non-native soils was for *A. cyclops* in available nitrate (NO3-) which was higher in the non-native range. Statistical analyses could not be conducted for *P. lophantha* or *A. melanoxylon* due to low population numbers. Overall, soil properties were very similar across native and non-native ranges (Table 7).

**Table 3.** Overall mixed-model results from plant-soil feedback experiment for main effects describing the variation in total, above- and belowground biomass for four *Acacia* species and *Paraserianthes lophantha*.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Source of variation** | **Effect** | **Numerator d.f.** | **Denominator d.f.** | ***F*-Value** | ***P*** |
| Total biomass | Species | 4 | 33.22 | 49.24 | **< 0.001** |
|  | Soil origin | 1 | 7.97 | 0.01 | 0.94 |
|  | Seed origin | 1 | 24.61 | 2.19 | 0.15 |
|  | Species x Soil origin | 4 | 33.19 | 1.04 | 0.40 |
|  | Species x Seed origin | 4 | 24.66 | 4.74 | **0.006** |
| Aboveground biomass | Species | 4 | 33.31 | 32.97 | **< 0.001** |
|  | Soil origin | 1 | 8.29 | 0.00 | 0.99 |
|  | Seed origin | 1 | 25.00 | 2.65 | 0.11 |
|  | Species x Soil origin | 4 | 33.28 | 0.99 | 0.42 |
|  | Species x Seed origin | 4 | 25.05 | 4.77 | **0.005** |
| **Table 3.** Overall mixed-model results from plant-soil feedback experiment for main effects describing the variation in total, above- and belowground biomass for four *Acacia* species and *Paraserianthes lophantha* (cont.). | | | | | |
| Belowground biomass | Species | 4 | 28.17 | 99.57 | **< 0.001** |
|  | Soil origin | 1 | 6.95 | 0.99 | 0.35 |
|  | Seed origin | 1 | 8.15 | 0.39 | 0.54 |
|  | Species x Soil origin | 4 | 30.04 | 1.30 | 0.28 |
|  | Species x Seed origin | 4 | 20.42 | 2.94 | **0.046** |
| Nodules | Species | 4 | 28.75 | 0.65 | 0.62 |
|  | Soil origin | 1 | 4.25 | 0.01 | 0.93 |
|  | Seed origin | 1 | 24.02 | 0.45 | 0.50 |
|  | Species x Soil origin | 4 | 28.78 | 0.65 | 0.63 |
|  | Species x Seed origin | 4 | 24.10 | 0.76 | 0.55 |

**Table 4.** Mixed model results from plant-soil feedback experiment for fixed factors describing the variation in total, above-, belowground biomass and nodules for *Acacia cyclops*, *Acacia longifolia*, *Acacia melanoxylon*, *Acacia saligna* and *Paraserianthes lophantha*.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Species** | **Source** | **Effect** | **Numerator d.f.** | **Denominator d.f.** | | ***F*-Value** | ***P*** |
| *A. cyclops* | Total biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 5  5  5 | 0.04  9.54  0.04 | | 0.85  **0.03**  0.85 |
|  | Aboveground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 5  5  5 | 0.04  9.61  0.01 | | 0.85  **0.03**  0.93 |
|  | Belowground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 5  5  5 | 0.02  1.67  1.19 | | 0.88  0.25  0.32 |
| *A. longifolia* | Total biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 4.69  3.81  3.81 | 1.54  8.05  0.78 | | 0.27  0.05  0.42 |
|  | | | | | | | |
| **Table 4.**  Mixed model results from plant-soil feedback experiment for fixed factors describing the variation in total, above-, belowground biomass and nodules for *Acacia cyclops*, *Acacia longifolia*, *Acacia melanoxylon*, *Acacia saligna* and *Paraserianthes lophantha* (cont.). | | | | | | | |
| **Species** | **Source** | **Effect** | **Numerator d.f.** | **Denominator d.f.** | | ***F*-Value** | ***P*** |
| *A. longifolia* | Aboveground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 4.73  3.76  3.76 | | 1.73  6.83  0.47 | 0.24  0.06  0.53 |
|  | Belowground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 8  8  8 | | 0.00  9.35  1.78 | 0.99  **0.02**  0.21 |
| *A. melanoxylon* | Total biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 2  2  2 | | 0.03  0.06  0.61 | 0.86  0.82  0.51 |
|  | Aboveground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 2  2  2 | | 0.002  0.08  0.23 | 0.96  0.79  0.67 |
|  | | | | | | | |
| **Table 4.**  Mixed model results from plant-soil feedback experiment for fixed factors describing the variation in total, above-, belowground biomass and nodules for *Acacia cyclops*, *Acacia longifolia*, *Acacia melanoxylon*, *Acacia saligna* and *Paraserianthes lophantha* (cont.). | | | | | | | |
| **Species** | **Source** | **Effect** | **Numerator d.f.** | **Denominator d.f.** | | ***F*-Value** | ***P*** |
| *A. melanoxylon* | Belowground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 4  4  4 | | 1.45  0.50  0.50 | 0.29  0.51  0.51 |
| *A. saligna* | Total biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 4  4  4 | | 0.40  39.93  0.06 | 0.55  **0.003**  0.81 |
|  | Aboveground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 2  2  2 | | 0.01  78.91  0.15 | 0.94  **0.01**  0.73 |
|  | Belowground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 2  2  2 | | 0.66  0.01  0.19 | 0.50  0.93  0.90 |
|  | | | | | | | |
|  | | | | | | | |
| **Table 4.**  Mixed model results from plant-soil feedback experiment for fixed factors describing the variation in total, above-, belowground biomass and nodules for *Acacia cyclops*, *Acacia longifolia*, *Acacia melanoxylon*, *Acacia saligna* and *Paraserianthes lophantha* (cont.). | | | | | | | |
| **Species** | **Source** | **Effect** | **Numerator d.f.** | **Denominator d.f.** | | ***F*-Value** | ***P*** |
| *P. lophantha* | Total biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 4  4  4 | | 8.13  0.12  6.35 | 0.046  0.74  0.07 |
|  | Aboveground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 2  2  2 | | 5.00  0.04  11.12 | 0.15  0.86  0.08 |
|  | Belowground biomass | Soil origin  Seed origin  Soil origin x Seed origin | 1  1  1 | 4  4  4 | | 1.04  0.20  0.85 | 0.37  0.68  0.41 |
|  | | | | | | | |

**Table 5.** One way ANOVA results for mean total, above- and belowground biomass (±SE) and nodules (Poisson distribution, ±SE) following Tukey’s post-hoc tests after soil-feedback experiment harvest. Values are shown for seedlings from native range grown in native and non-native range soils and for seedlings from non-native range grown in native and non-native range soils.

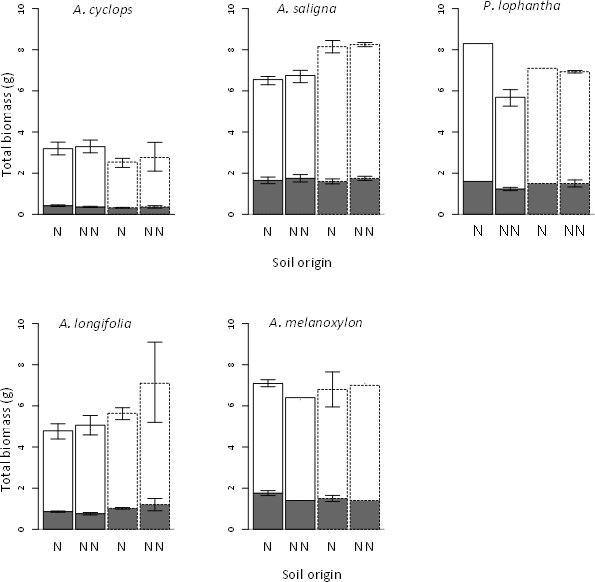
|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Total biomass** | | | |  |
| **Seedling origin** | Native | | Non-native | |  |
| **Soil origin** | Native | Non-native | Native | Non-native | ***P*** |
| **Species** |  |  |  |  |  |
| *A.cyclops* | 3.15 (±0.33) | 3.30 (±0.34) | 2.55 (±0.27) | 2.73 (±0.73) | 0.50 |
| *A.saligna* | 6.50 (±0.20) | 6.70 (±0.30) | 8.10 (±0.30) | 8.20 (±0.10) | **0.02** |
| *P.lophantha* | 8.30 | 5.66 (±0.40) | 7.10 | 6.93 (±0.06) | 0.06 |
| *A.longifolia* | 4.76 (±0.37) | 5.06 (±0.47) | 5.62 (±0.29) | 7.15 (±1.95) | 0.21 |
| *A.melanoxylon* | 7.10 (±0.17) | 6.30 | 6.80 (±0.85) | 7.10 | 0.92 |
|  | | | | | |
|  | **Aboveground biomass** | | | |  |
| **Seedling origin** | Native | | Non-native | |  |
| **Soil origin** | Native | Non-native | Native | Non-native | ***P*** |
| **Species** |  |  |  |  |  |
| *A.cyclops* | 2.77 (±0.31) | 2.93 (±0.31) | 2.22 (±0.22) | 2.40 (±0.70) | 0.49 |
| *A.saligna* | 4.90 (±0.00) | 5.00 (±0.40) | 6.55 (±0.25) | 6.50 (±0.20) | **0.02** |
| *P.lophantha* | 6.70 | 4.46 (±0.34) | 5.60 | 5.43 (±0.12) | 0.07 |
| *A.longifolia* | 3.93 (±0.43) | 4.30 (±0.40) | 4.62 (±0.26) | 5.90 (±1.60) | 0.27 |
| *A.melanoxylon* | 5.33 (±0.23) | 5.00 | 5.30 (±0.76) | 5.60 | 0.97 |
|  |  |  |  |  |  |
|  | **Belowground biomass** | | | |  |
| **Seedling origin** | Native | | Non-native | |  |
| **Soil origin** | Native | Non-native | Native | Non-native | ***P*** |
| **Species** |  |  |  |  |  |
| *A.cyclops* | 0.42 (±0.04) | 0.36 (±0.03) | 0.32 (±0.02) | 0.36 (±0.06) | 0.45 |
| *A.saligna* | 1.65 (±0.25) | 1.75 (±0.15) | 1.60 (±0.00) | 1.75 (±0.05) | 0.84 |
| *P.lophantha* | 1.60 | 1.23 (±0.08) | 1.50 | 1.50 (±0.17) | 0.48 |
| *A.longifolia* | 0.86 (±0.03) | 0.76 (±0.06) | 1.02 (±0.04) | 1.20 (±0.30) | 0.07 |
| *A.melanoxylon* | 1.76 (±0.12) | 1.40 | 1.50 (±0.15) | 1.40 | 0.42 |
|  |  |  |  |  |  |

**Table 6.** A summary table of nodule scores (indicating distribution and number of effective nodules as per Corbin *et al.* classification) for each species, treatment and population. Each number in the table represents the number of treatment replicates that scored a value between 0-5 in a treatment. For example, for *Acacia cyclops*, in the treatment where native seedlings were grown in four native range populations’ soils, one population scored a “0” whereas in three other populations on average native seedlings scored “1”. For the native range *A. cyclops* seedlings grown in non-native soils, all three non-native populations obtained a score of “1”.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | *A. cyclops* | | | | *A. saligna* | | | | | *P. lophantha* | | | | | *A. longifolia* | | | | | *A. melanoxylon* | | | | |
| **Seed origin** | Native | | Non-native | | Native | | Non-native | | | Native | | Non-native | | | Native | | Non-native | | | Native | | Non-native | | |
| **Soil origin** | N | NN | N | NN | N | NN | | N | NN | N | NN | | N | NN | N | NN | | N | NN | N | NN | | N | NN |
| **Score** |  |  |  |  |  |  | |  |  |  |  | |  |  |  |  | |  |  |  |  | |  |  |
| **0** | 1 | 0 | 2 | 1 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| **0.5** | 0 | 0 | 0 | 1 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| **1** | 3 | 3 | 2 | 1 | 2 | 1 | | 0 | 0 | 0 | 1 | | 0 | 0 | 1 | 1 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| **1.5** | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |
| **2** | 0 | 0 | 0 | 0 | 0 | 1 | | 2 | 2 | 0 | 2 | | 0 | 0 | 2 | 2 | | 3 | 0 | 3 | 0 | | 1 | 0 |
| **2.5** | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 1 | 0 | | 1 | 1 | 0 | 0 | | 0 | 0 | 0 | 1 | | 0 | 0 |
| **3** | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 2 | 0 | 0 | | 1 | 1 | 0 | 0 | | 2 | 1 |
| **4** | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 1 | 0 | 0 | | 0 | 0 |
| **5** | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 |

**Table 7**. Chemical properties of native and non-native soils for populations of four *Acacia* species and *Paraserianthes lophantha*. Values are means of sites within each range ± S.E. Statistical analyses could not be conducted for *Paraserianthes lophantha* or *Acacia melanoxylon* due to low site numbers. Significant differences (*P* < 0.05) are shown in bold.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Soil properties | *A.cyclops*  N NN  (n=4) (n=3) | *A. saligna*  N NN  (n=2) (n=3) | *P. lophantha*  N NN  (n=1) (n=3) | *A. longifolia*  N NN  (n=3) (n=4) | *A. melanoxylon*  N NN  (n=3) (n=1) |
| pH | 7.3 ± 0.27 6 ± 0.1 | 7.1 ± 0.7 6.3 ± 1.1 | 7.3 6.0 ± 0.8 | 5.8 ± 0.6 5.3 ± 0.3 | 5.6 ± 0.4 5.3 |
| Organic matter (%) | 7.3 ± 1.6 45 ± 2.4 | 4.3 ± 1.0 5.0 ± 2.2 | 5.9 7.6 ± 1.6 | 5.8 ± 1.3 6.8 ± 2.6 | 7.3 ± 1.0. 7.8 |
| Total N (%) | 0.1 ± 0.0 0.2 ± 0.1 | 0.2 ± 0.1 0.2 ± 0.1 | 0.2 0.3 ± 0.9 | 0.3 ± 0.1 0.2 ± 0.1 | 0.3 ± 0.0 0.3 |
| Total P (mg/kg) | 180 ± 75 270 ± 33 | 200 ± 0.0 270 ± 33 | 100 570 ± 218 | 270 ± 67 120 ± 46 | 370 ± 80 200 |
| Total C (%) | 4.3 ± 1.0 2.7 ± 1.4 | 2.5 ± 0.6 3.0 ± 1.3 | 3.5 4.5 ± 1.0 | 3.4 ± 0.8 4.0 ± 1.5 | 4.3 ± 0.6 4.6 |
| N-NH4+ (mg/kg) | 1.6 ± 0.5 1.8 ± 0.8 | 5.0 ± 1.8 2.6 ± 1.1 | 1.0 6.0 ± 1.9 | 6.0 ± 1.4 10.0 ± 4.4 | 6.9 ± 0.5 6.5 |
| N-NO3- (mg/kg) | **2.4 ± 1.5 15.7 ± 4.7** | 9.4 ± 4.3 20.1 ± 7.0 | 10.9 45.4 ± 24.1 | 25.8 ± 7.7 4.9 ± 4.6 | 30.8 ± 15.2 12.6 |



**Figure 2.** Mean shoot (± SE) (white bars) and root biomass (± SE) (grey bars) of four *Acacia* species and *Paraserianthes lophantha* from the plant-soil feedback experiment. Dashed bars indicate treatments when seedlings from non-native (NN) range populationsweregrown in native (N) and non-native (NN) soils and solidbars represent treatments when seedlings from native range populations were grown in native (N) and non-native (NN) soils respectively.

**DISCUSSION**

**The influence of plant-soil feedback effects on plant biomass**

Our original prediction was that all five study species would perform better (i.e. have larger biomass and positive soil feedback) when grown in their non-native range soils compared to native range soils due to the direct effect of release from harmful soil pathogens in the non-native range. We found that soil origin (native or non-native) consistently had no effect on any of our five closely related study species, indicating that there was no significant plant-soil feedback effect (i.e. positive or negative) on plant performance.

There are relatively few studies that have investigated the net effect of soil microbial communities on the invasion success of acacias using a plant-soil feedback approach. A study from Portugal found evidence for positive feedback for *A. longifolia* that was reported to modify soil properties (increased levels of C and N) and microbial biomass, especially in long term invaded sites (Marchante et al. 2008a) and also modify the microbial communities to its own benefit (Rodríguez-Echeverria 2010). Similar results have been reported for *A. saligna* in South Africa where it was found to increase total soil N and organic matter (Yelenik et al. 2007), thus modifying the original pre-invader soil conditions and its microbial assemblages.

Despite those positive soil feedback effects of *Acacias* in Portugal and South Africa, we did not find evidence for positive plant-soil feedback effects in the non-native range in Australia, suggesting that other biotic and abiotic factors could be more important contributors to these species’ invasion success. This is consistent with recent studies that have shown that invasion dynamics of a plant species in the new environment is more complicated than previously reported and is possibly dependant on multiple abiotic and biotic factors that act in concert and shape the invasion outcome of the plant species in the non-native range (Andonian et al. 2011a; Andonian et al. 2011b). However, we found that soil chemistry was highly similar across the ranges for all species and thus it is unlikely that this abiotic factor significantly affects invasive success of these species. Nevertheless, since only a fraction of field soil was used in the

glasshouse experiments, we cannot entirely exclude the possibility that soil chemistry and its seasonal fluctuations and perturbations do not play an important role in the field conditions.

It is plausible that we did not find an effect of soil origin on plant performance due to the high floristic similarities between the non-native and native ranges of these acacias. Acacias are an important component of many vegetation types within Australia, often being the dominant species (Richardson et al. 2011). Thus it is likely that the microbial communities that associate with this genus are also ubiquitous in the soil. It is widely acknowledged that micro-organisms are not randomly distributed in soil, but display spatially predictable patterns that are influenced strongly by vegetation (Elgersma and Ehrenfeld 2011). For instance, the spatial distribution of rhizobial communities has been shown to follow closely that of the host plant distribution patterns across short and long distances (Parker and Spoerke 1998; Spoerke et al. 1996). The vegetation communities in the native and introduced ranges of each of the five species in this study were highly similar, being all Eucalypt-dominated woodlands with a shrubby understorey containing many species in the family *Fabaceae*. Consequently it is highly likely that there is high homogeneity in soil microbial communities across the native and introduced ranges of the study species due to the floristic similarity of the aboveground plant communities.

**The effect of seed origin on plant biomass**

We found that seed origin (native or non-native) had a significant effect on seedling biomass in two of the five species (*A. cyclops* and *A. saligna*). However these effects of seed origin were not consistent: in *A. cyclops* seedlings from native range seed performed better, in *A. saligna* and *A. longifolia* seedlings from non-native range seed performed better, and in *A. melanoxylon* and *P. lophantha* there was no effect of seed origin, possibly due to low replication of populations within the range. Thus although there was no plant-soil feedback effect in any of the study species, our evidence suggests that it is likely that genetic adaptation to novel

conditions in the introduced range has contributed to invasion success in *A. saligna*. Alternatively, since these acacias have been used widely for ornamental and dune rehabilitation purposes, human imposed artificial selection could be responsible for the distribution of larger genotypes. Interestingly, recent work did not find evidence for genetic differences between native and non-native populations of *A. cyclops*, whereas *A. saligna* had lower genetic diversity in the non-native range populations (Harris et al. 2012). Lower genetic diversity in the introduced range suggests that genetic bottlenecks and founder events may have occurred during the introduction and subsequent invasion process (Le Roux et al. 2011). It is apparent that reduced genetic diversity in the non-native range for *A. saligna* had not resulted in a reduction in plant viability, and therefore the introduction of particular phenotypes in the non-native range is a more plausible explanation of greater seedling biomass for *A. saligna*.

Furthermore, bigger seedlings of *A. saligna* in the non-native range is unlikely to be due to differences in seed size as in our study we controlled for differences in seed mass by only using seeds that were within one standard deviation of the mean seed mass for a given species. In a previous study, *A. cyclops* and *A. saligna* showed a trend, although not significant, of larger seedlings from native range seeds grown in native range soils (Birnbaum et al. 2012), which is consistent with this study’s results for *A. cyclops*, but is the opposite to that found for *A. saligna*. Interestingly, Harris *et al.* (unpubl.) examined plant size in field populations and found that *A. longifolia* plants from the non-native range were larger, however no differences in biomass were observed for *A. cyclops* or *A. saligna*.

Overall our results from the present study and evidence from glasshouse and field based studies (Harris et al. 2012) of the same species suggests that the invasion success of these acacias (*A. cyclops*, *A. longifolia*, *A. melanoxylon*, *A. saligna* and *P. lophantha*) is associated with other biotic and abiotic conditions, such as herbivory and precipitation (Alba and Hufbauer 2012), in the non-native range as well as with phenotypic and genetic variation expressed, rather than being facilitated by novel soil microbial communities.

**The role of rhizobia**

The significance of soil mutualists, for example such as rhizobia, in legume invasion is fundamentally unresolved. There is some evidence to suggest that legumes may be constrained by the absence of compatible mutualists (Parker 2001) or, on the contrary, benefit from newly acquired symbionts in the novel range (Callaway et al. 2011; Parker et al. 2007). In some instances it has been shown that rhizobia may have been co-introduced with the invader which may be facilitating the invasion success of the legumes in the non-native range (Chen et al. 2005; Rodríguez-Echeverria 2010). Hence, evidence suggests that there is some variability of legume dependence on its soil mutualists in the novel range.

In this study, we hypothesized that acacias will not be constrained by rhizobia in the non-native range populations. Indeed, we did not find a significant effect of soil or seed origin on the number and distribution of effective nodules across non-native and native range soils. This suggests that rhizobia is likely to be equally ubiquitous in non-native and native range population soils and is unlikely to act as a constraint for the invasion success of these legumes in Australia. This result confirms our previous findings that suggested that despite some rhizobial community compositional differences between the ranges for *A. cyclops*, *A. saligna* and *P. lophantha* these legumes do not appear to be constrained by the absence of compatible soil mutualisms in their non-native range in Australia as they generally grow equally well in non-native and native range population soils (Birnbaum et al. 2012).

Although we did not find any significant effect of seed or soil origin on the number of effective nodules, some variation in the distribution of effective nodules when seedlings were grown in non-native compared to native range populations’ soils was evident. For instance, *A. saligna*, *A. longifolia*, *A. melanoxylon* and *P. lophantha* seedlings from the non-native range had overall more nodules on the crown roots across both range soils suggesting a greater benefit for plant performance compared with higher number of smaller nodules along the lateral roots as could be observed for seedlings from the native range across both range soils.

It is important to note, though, that the presence of compatible microorganisms in the non-native range is probably more significant for the invader in its early invasion stages rather than for the already established invaders that have had the opportunity to ‘condition’ the soil for their own benefit. Unfortunately, for acacias little is known about their movement across Australia as they are considered native species and thus little or no record has been kept about their introductions outside their native ranges within Australia (Harris et al. 2012). Thus the outcome of plant-soil feedback experiments and the role of mutualisms could be significantly different between recently introduced acacias and long-established ones. This is plausible as there is evidence that acacias, and legumes generally, rely on their mutualistic relationships (e.g. rhizobia and AM fungi) to establish and grow successfully (Pacovsky et al. 1986; Sprent and Parsons 2000).

**Conclusions**

In this study we did not observe any significant effect of soil origin on plant biomass of our five study species. This could be due to the extensive similarity of vegetation in the non-native and native ranges of these species in Australia, at least for the sites we sampled. Vegetation across the continent of Australia is dominated by shrubby leguminous species so it is not unlikely that highly similar microbial communities exist in the non-native and native ranges of these species. Alternatively, other abiotic or biotic factors such as environmental conditions, genetic adaptation to novel environments and human-mediated artificial selection could have influenced the invasion success of these acacias in Australia. Further analyses (e.g. microbial community sequencing) are needed to elucidate the differences in soil microbial diversity in the non-native compared to native range of these acacias. While we did not observe significant plant-soil feedback effects for these acacias within Australia, it is possible that the soil microbial community may be an important influence in locations outside Australia where acacias have been introduced into quite different vegetation communities with associated different soil microbial communities.

**Acknowledgements**

We would like to thank three anonymous referees for constructive comments that improved the manuscript. We would also like to thank John Klironomos, Alexander Koch and Jeri Parrent for helpful insights and discussions on the experimental design at the early stages of this study. We thank Carla Harris and Paweł Waryszak for extensive help in the field and Rachael Gallagher for providing the map. We also wish to thank Ian Davidson, Anthony Manea, Paweł Waryszak, Felix Servane and Lars Roth for their help in the glasshouse. This work was supported by Macquarie University Research Excellence Scholarship to CB and by an Australian Research Council Discovery grant (DP0879494) to ML.

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**Chapter 3: Mutualisms are not constraining cross-continental invasion success of legumes within Australia**

This manuscript is in press as ‘Birnbaum C., Barrett, L.G., Thrall, P.H. and Leishman M.R. (2012) Mutualisms are not constraining cross-continental invasion success of *Acacia* species within Australia.’ *Diversity and Distributions* DOI: 10.1111/j.1472-4642.2012.00920.x. A pdf of the publication is included in the Appendix A.

My contribution to the research and paper: Concept - 90%; Data collection - 80%; Analysis - 80%; Writing - 80%

**ABSTRACT**

**Aim** Studyingplant-soil interactions of introduced species in different parts of their global range could assist in managing biological invasions by elucidating the level of host specificity of key mutualisms. We assessed the role of the soil microbial community (with an emphasis on symbiotic nitrogen-fixing bacteria, collectively termed rhizobia) in determining cross-continental invasion success of five woody legume species.

**Location** Australia

**Methods** For each species, we compared growth of plants in soils from their native and non-native ranges using a glasshouse study, a soil dilution method (MPN) and T-RFLP to assess rhizobial abundance and community composition, respectively.

**Results** *Acacia longifolia* was the only species that had significantly larger aboveground biomass when grown in soils from its non-native range. Rhizobial abundance was equally high across species and ranges, indicating plants are unlikely to be limited by soil rhizobial abundance in non-native ranges. *Acacia cyclops,* *A. saligna* and *Paraserianthes lophantha* formed associations

with different rhizobial communities in non-native vs. native range soils. *Acacia longifolia* and *A. melanoxylon* associated with similar rhizobial communities in their native and non-native ranges, suggesting that rhizobia may have been accidentally introduced into their novel range with seeds or seedlings.

**Main conclusions** Invasive success of these five legume species is not constrained by the abundance of rhizobia in novel ranges for established legume populations, at least within Australia. Although differences in rhizobial community composition were evident between the native and non-native ranges for three of the five species, these were not associated with differences in plant growth. Increased aboveground biomass of *A. longifolia* when grown in soil from its non-native range suggests that invasive success of this species may be associated with differences in the non-rhizobial components of soil microbial communities in the novel range. This information could assist in management practises by facilitating a more instructive and effective screening for invasiveness.

**Keywords**

Biological invasions, *nifD*, *nodA*, plant-soil interactions, rhizobia, symbionts

**INTRODUCTION**

A growing body of evidence reveals that interactions between plants and soil microbes influence invasion dynamics (Klironomos, 2002; Callaway *et al.*, 2004; Mangla & Callaway, 2008). A key factor for survival and successful establishment of many invaders is the presence of compatible mutualists (Simberloff & Von Holle, 1999; Richardson *et al.*, 2000). This is likely to be particularly important for legumes which, under natural conditions, largely depend on symbioses with nitrogen-fixing soil bacteria (rhizobia) to establish and grow successfully (Parker, 2001; Rodrıguez-Echeverria *et al.*, 2009). Although it is generally accepted that most legumes can nodulate with a wide range of rhizobia, some legumes introduced into novel environments fail to establish unless suitable compatible rhizobia are also introduced (Richardson *et al.*, 2000). Additionally, great variability in symbiotic success between legumes and rhizobia exists across different environments and hosts (Thrall *et al.*, 2000; Thrall *et al.*, 2008).

Despite the general importance of soil symbionts for plant fitness, a clear understanding of the role played by mutualists in plant invasions is not well developed and is less studied compared to the role of pathogens in plant invasions (Callaway *et al.*, 2011). To predict the role of mutualisms in plant invasions several competing hypotheses have been proposed. The Enhanced Mutualism Hypothesis (EMH) postulates that invasive plants may experience positive effects from novel soil mutualists in the invaded range as many ecosystems contain a broad array of potential mutualistic partners (e.g. generalist mycorrhizal fungi with wide host ranges and rhizobial strains with infectivity across genera) (Richardson *et al.*, 2000). Alternatively, associations between locally specific mutualists and host plants in the native range may not be supported in novel environments due to the absence of those mutualists such as rhizobia (Parker & Kennedy, 2006) or mycorrhizae (Pringle *et al.*, 2009), resulting in poor host plant performance (Constrained Mutualism Hypothesis) (Parker, 2001; Stanton-Geddes & Anderson, 2011). Thirdly, host plants may be able to effectively associate with a wide range of soil mutualists (i.e. they are generalists) so that they can associate with endemic rhizobia and mycorrhizae equally effectively in both the native and non-native ranges (Generalist Host

Hypothesis). Fourthly, plants introduced into a novel range may be accompanied by invasive mutualists with which they can form effective associations (Accompanying Mutualist Hypothesis) (Rodríguez-Echeverria, 2010). Finally, other components of soil microbial communities may play an important role in plant invasions. For example, the Enemy Release Hypothesis suggests that invasive plants in the novel range may experience increased performance due to release from harmful soil-borne pathogens.

Legumes, especially woody perennial legumes such as *Acacia* species, are considered among the most damaging weeds of temperate ecosystems both in Australia (Emms *et al.*, 2005) and globally (Witkowski, 1991; Kutiel *et al.*, 2004; Paynter & Flanagan, 2004; Marchante *et al.*, 2008a; Le Maitre *et al.*, 2011; Richardson *et al.*, 2011a; Richardson & Rejmánek, 2011b). The genus *Acacia* is highly diverse and widespread in Australia, with acacias being a major component of many Australian ecosystems (Maslin *et al.*, 2001a; Maslin *et al.*, 2001b) due to their abundance, biomass and contribution to vegetation structure (Burdon *et al.*, 1999). A large number of *Acacia* species have been introduced outside their native distribution, with approximately 23 species recognised as invasive, both within and beyond Australia (Richardson *et al.*, 2011a; Richardson & Rejmánek, 2011b). Acacias are strong competitors due to their fast growth rates (Peperkorn *et al.*, 2005; Morris *et al.*, 2011), pre-adaptation to nutrient-poor soils and high seed output (Witkowski, 1991), often resulting in monospecific stands in invaded ranges (Werner *et al.*, 2010) and reduced native diversity (Marchante *et al.*, 2003; Gaertner *et al.*, 2009). In addition, invasive acacias can modify below-ground microbial communities (Rodríguez-Echeverria, 2010) as well as above-ground plant communities through altered nutrient dynamics (Marchante *et al.*, 2007).

While several studies have explored variation in rhizobial effectiveness and host specificity within and among different *Acacia* species in Australia (Burdon *et al.*, 1999; Thrall *et al.*, 2000), much less attention has been given to the biology of invasive acacias and the role of soil microbial communities, especially rhizobia, in invasion success within Australia. Understanding

the rhizobial-legume interaction as an ecologically significant below-ground trait (e.g. nitrogen-fixation) may be important in explaining the mechanisms behind the invasion success of these acacias and facilitate more effective management strategies.

The aim of this study was to examine the role of the soil microbial community, with an emphasis on rhizobia, in the success of acacias in novel ranges within Australia. We asked (i) Do soil microbial communities in non-native *vs*. native range soils differentially influence plant performance?; and (ii) is there a difference in rhizobial abundance and community composition between non-native and native range soils? We focused on four species of *Acacia* (*A. cyclops, A. longifolia, A. melanoxylon* and *A. saligna*) plus a close relative (*Paraserianthes lophantha*) that have been introduced and become naturalised or invasive in novel environments within Australia (Richardson & Rejmánek, 2011). According to Miller *et al.* (2011) *A. cyclops*, *A. longifolia* and *A. melanoxylon* are placed in the melanoxylon clade, whereas *A. saligna* belongs to the Pulchelloidea clade. *Paraserianthes lophantha* is a closely related species of *Acacia* subgenus *Phyllodineae* (Brown *et al.*, 2008). We used a glasshouse experiment to assess plant performance using soils from local populations of each species’ native and non-native ranges as inoculum sources. To investigate the potential for variation in rhizobial abundance to influence plant performance, we estimated rhizobial population sizes in soils from the native and non-native ranges of each species. Finally, we characterized rhizobial community composition of host populations within native and non-native ranges using terminal restriction fragment length polymorphism (T-RFLP). We then assessed the hypotheses described above in light of our experimental results. To our knowledge this study represents one of the few attempts to comprehensively describe the role of rhizobia in legume invasion based on multiple species and population comparisons from both native and introduced ranges.

**METHODS**

**Study species**

The five study species are members of the family Fabaceae (subfamily Mimosoideae). They are all shrubs to medium-sized trees that variously occupy wet or dry sclerophyll forests, rainforests and coastal communities in their native range within Australia (Maslin *et al.*, 2001b).

*Acacia longifolia* (Andrews) Willd. and *A. melanoxylon* R.Br. are native to south-east Australia (New South Wales, Victoria and South Australia). Both species have been widely cultivated as horticultural species in south-west Western Australia where they have become naturalised and are considered invasive (Maslin *et al.*, 2001b; Hussey *et al.*, 2007). *Acacia longifolia* comprises two subspecies, *longifolia* and *sophorae* (Entwisle *et al.*, 1996; Maslin *et al.*, 2001b) which are capable of hybridization and are almost impossible to separate in the field. *Acacia cyclops* A.Cunn. ex G.Don, *A. saligna* (Labill.) H.L. Wendl. and *P. lophantha* (Willd.) I.C. Nielsen are native to south-west Australia but have been introduced to eastern Australia. Both *A. cyclops* and *A. saligna* have been used for dune rehabilitation following sand mining and as ornamentals. *Paraserianthes lophantha* has been widely promoted as a garden plant and has become naturalised in eastern Australia, invading disturbed bushland margins before invading adjacent undisturbed areas (Muyt, 2001). It is highly unlikely that any of these five species have dispersed naturally across the continent as the eastern and western species ranges are separated by the vast Nullarbor Desert, providing a major geographic barrier to natural seed dispersal between eastern and western Australia (Jacobs & Wilson, 1996).

**Soil and seed collection**

Our aim was to sample populations widely dispersed within the native and non-native range of each species (Table 1, Chapter 2). Soil and seed samples were collected in December 2009 across south-east and south-west Australia from Perth to Ravensthorpe in Western Australia

and from Sydney to Yorke Peninsula in eastern Australia (Fig. 1, Chapter 2). For each species, we sampled five individuals from each of five populations within each range [5 species x 2 ranges (native and non-native) x 5 populations x 5 individuals]. A total of 1000 g of soil was collected beneath each individual as close to roots as possible at a depth of 10-15 cm and then bulked for each population. Soils were kept in a cooler in the field before being stored at 4o C. Soil sampling and processing equipment was sterilized with 90% ethanol between populations. Soils were sieved through 2 mm and 4 mm sieves to remove leaves and other coarse material and to homogenise samples. Mature seeds were collected from the same host individuals as soils. Seeds were bulked across individuals within populations.

**Soil chemical characteristics**

We assessed soil chemistry to determine whether abiotic characteristics of soils varied between sites. A total of 27 sub-samples from 4 native (N) and 3 non-native (NN) soils for *A. cyclops*, 3 N and 4 NN for *A. longifolia,* 3 N and 1 NN for *A. melanoxylon*, 2 N and 3 NN soils of *A. saligna* and 1 N and 3 NN for *P. lophantha* were analysed*.* These were air dried and analysed at the Sydney Environmental & Soil Laboratory (SESL) for total nitrogen and phosphorus using LECO C:H:N and ICP-AAS (with HCl digest) respectively, total organic carbon using Dumas combustion (SESL in-house Method LECO 1), organic matter using calculation of TOC (based on LECO 1) and ammonium (NH4+ ) and nitrate (NO3 -) using Mehlich 3 (SESL in-house CaCl2  extraction, followed by UV/Vis Spectro finish; APHA 4500-NO3-E (modified), respectively). Soil pH was measured in 1:5 soil to water preparations using a TPS digital pH meter (TPS Pty. Ltd., Brisbane).

**Plant growth response experiment**

We tested for plant growth differences between populations within native and non-native ranges for each host species. Seeds from each population of each species were grown in pots in the glasshouse using field collected soil from their own population as an inoculum source. In total, soil and seed material from 29 populations were used (Table 1, Chapter 2). For *A. cyclops* we used soil/seed material from 4 native (N) and 3 non-native (NN) populations, for *A. longifolia* 3 N + 4 NN populations, for *A. melanoxylon* 3 N + 2 NN, for *A. saligna* 2 N + 3 NN and for *P. lophantha* 2N + 3 NN populations. The different number of seed by soil combinations was determined by availability of seed material. Each soil/seed combination was replicated 10 times for a total of 290 pots.

Seeds were heat treated or manually scarified with sandpaper to promote germination. All seeds were surface-sterilized in 90% ethanol for 1 minute and 6% bleach for 5 minutes, rinsed with distilled water and sown on autoclaved sterilized sand (121oC 30 minutes wet cycle) in Petri dishes. These were placed in growth cabinets for germination (25oC/18oC, light on/off 12 h). Seedlings of uniform size were transplanted from Petri dishes to pots 6.5 cm diameter and 21 cm depth, filled with 1:2 ratio of sterilized (121oC 30 minutes wet cycle) coarse sand and fine river sand. A separate layer of 50 mL of inoculum (field-collected soil from a given population of each species) was added to each pot, and then covered by 50 mL of sterilized potting mix. The location of pots within the glasshouse was randomised initially and fully re-randomised every two weeks. Plants were watered three times daily with tap water. During the trial no nutrients were added as the small amount of nutrients in the inoculum and potting mix was considered sufficient to sustain seedlings for the duration of the experiment. Addition of nutrients was avoided given the potential (especially of nitrogen) to suppress the activity of nitrogen fixing bacteria. Glasshouse temperatures ranged between 18oC and 25oC (night/day). After 11 weeks aboveground biomass of each individual was harvested and dried at 75oC for 48 hours.

Aboveground dry weights were used as the primary response variable in analyses of plant performance.

**Estimates of rhizobial abundance**

We used the Most Probable Number (MPN) method to estimate number of rhizobia per gram of soil (Vincent, 1970). This method allowed us to specifically characterize only the abundance of active bacteria found in the nodules that is in the symbiotic association with the host. All MPNs of soils were counted using serial-dilution, nodulation-frequency plant infection tests (Brockwell, 1963). Host plants were grown in sterilized Thornton (1930) tubes (150 x 18 mm) containing washed vermiculite moistened with N-free McKnight’s (1949) seedling nutrient solution and closed with polyurethane foam plugs. Tubes were arranged in wooden racks and placed in a glasshouse with a temperature of 18-25oC. All plants were harvested after 7 weeks. At harvest, plants were scored for presence/absence of nodules and nodulation effectiveness. Red-brown coloured nodules contain leghaemoglobin which is a characteristic indicator of nitrogen fixation in plant root nodules (Atlas & Bartha, 1987). The MPN of rhizobia in the original sample was calculated from the proportion of test plants forming nodules at each dilution level (Brockwell, 1963).

Estimates of rhizobial abundance were calculated from two separate assays. First, we estimated rhizobial population size in soils for all five species including both native and non-native ranges. Here our aim was to estimate the number of rhizobia that plants from different localities were exposed to in their own soils, with local soil and seed material matched for each of 28 populations. Availability of seed material determined the number of seed by soil combinations used. For *A. cyclops* we used soil/seed material from 4 N and 3 NN populations, for *A. longifolia* 3 native (N) and 4 non-native (NN) populations, for *A. melanoxylon* 3 N and 2 NN populations, for *A. saligna* 2 N and 3 NN populations and for *P. lophantha* 1N and 3 NN populations. Most seeds of *P. lophantha* from its native range were heavily predated and thus we only had

sufficient material from one native population. For each soil by seed combination we made six 10-fold serial dilutions with three replicates for each level of dilution (28 x 6 x 3) giving a total of 504 tubes. After heat treatment seeds were germinated in Petri dishes filled with wet sterilized (121°C 30 minutes wet cycle) sand. Seedlings of uniform size were transplanted to tubes.

In the second assay reciprocal cross-inoculation assays were used to evaluate whether plants generally favour rhizobia from their own source population relative to rhizobia from other populations (including native vs. non-native comparisons). We focused on *A. longifolia* and *A. saligna* as they are the most problematic invaders both in Australia and overseas (Witkowski, 1991; Rascher *et al.*, 2010). For *A. longifolia*, we used seed and soil material from 3 native (N) and 4 non-native (NN) populations. Seedlings from each population were reciprocally inoculated with all *A. longifolia* soils from native and non-native populations. This gave a total of 39 soil x seed combinations with four different treatments (native seed x native soil, native seed x non-native soil, non-native seed x non-native soil and non-native seed x native soil). For each soil by seed combination we made six 10-fold serial dilutions with three replicates for each level of dilution (39 x 6 x 3) giving a total of 702 tubes. We undertook the same process for *A. saligna* with 2 N and 3 NN populations giving a total of 25 seed by soil combinations and 450 tubes (25 x 6 x 3).

**DNA isolation and T-RFLP**

T-RFLP was used to characterize the rhizobial diversity as it provides high resolution profile of the bacterial community and has been extensively used to study the soil microbial community structure and diversity (Anderson & Cairney, 2004; Singh *et al.*, 2006), including rhizobia (Singh *et al.*, 2006; Smalla *et al.*, 2007). To assess rhizobial diversity we extracted DNA from nodules collected from plant roots after harvesting the glasshouse experiment. During the harvest 2-5 nodules per plant were collected and surface sterilized with 90% ethanol and distilled water. Nodules were stored in the freezer at -20oC in a plastic jar filled with silica beads and cotton

wool. Nodules from 10 replicate plants from each of the 29 population/range soil combinations were pooled for DNA extraction. Nodules (0.05 g) were crushed in liquid nitrogen to create a homogenised sample for DNA extraction.

DNA from nodules was isolated using a PowerPlant DNA isolation kit following the manufacturer’s protocol (MO Bio Laboratories, Inc. Carlsbad, CA). *NifD* and *nodA* genes were amplified using nifD2F and nifD1R (Fedorov *et al.*, 2008) and nodA-2 and nodA-1 (Haukka *et al.*, 1998) primers, respectively. Both genes are required to form effective symbioses with host plants (Haukka *et al.*, 1998). *NifD* is the Fe protein subunit of nitrogenase and nif genes produce the nitrogen-fixing nitrogenase enzyme (Haukka *et al.*, 1998; Fedorov *et al.*, 2008). *NodA* is a host-specific determinant of fatty acid transfer in Nod factor biosynthesis (Ritsema *et al.*, 1996; Roche *et al.*, 1996). Nod genes encode the production of Nod factors responsible for production of symbiotic nodules and are unique to rhizobia (Haukka *et al.*, 1998). A touch-down PCR program was adopted from Korbie & Mattick (2008) with initial hot-start activation of Taq DNA polymerase at 95ºC for 10 min, 10 cycles of denaturation at 95ºC for 30 sec, annealing at 55ºC for 45 sec and elongation at 72ºC for 1 min and at each next step the annealing temperature was decreased by 1ºC reaching at final step 48ºC and final extension at 72ºC for 5 min., followed by 25 cycles of 95ºC for 30 sec, 55ºC for 45 sec and 72ºC for 1 min.

PCR products were cleaned using Microcon Centrifugal Filters (Millipore Corporation) according to manufacturer’s instructions. All PCR products were digested with *AluI*, *HinfI*, *MspI*, *RsaI*, *MboII* and *Sau969* (New England BioLabs) restriction enzymes in 30 µl reaction mixtures. A fragment size analysis was carried out with a 3130xl genetic analyser (Applied Biosystems, Warrington, United Kingdom) using a product size matched fluorescent lane standard LIZ600. T-RFLP profiles were produced using GeneMarker V1.95 (Softgenetics, State College, PA, USA). Terminal restriction fragments (TRFs) were quantified using local southern method (Southern, 1979). Only peaks over 50 bp were considered for the analysis to avoid TRFs caused by primer-dimers (Singh *et al.*, 2006).

**Statistical analyses**

Biomass (aboveground dry weight) data were analysed using a mixed model ANOVA with range (native and non-native) and species as fixed factors and population nested within range as a random factor. Populations were used as true replicates for each species within a range (native or non-native). Bonferroni post-hoc tests were applied to test for significant differences between species. When necessary, data were log10 transformed to meet the homogeneity and normality assumptions of ANOVA.

MPN data was transformed to fit a Poisson distribution. Data from the first assay was analysed using a mixed model ANOVA with range (native and non-native) and species as fixed factors and population nested within soil range (native or non-native) as a random factor. Bonferroni post-hoc tests were applied to test for significant differences between species. MPN data from the second assay using *A. longifolia* and *A. saligna* were analysed separately with a mixed model ANOVA with soil source population (native, non-native and own) as a fixed factor and population nested within soil range (native or non-native) as a random factor. Bonferroni post-hoc tests were applied to test for significant differences between soil source populations.

Principal coordinates analysis (PCA) based on a Jaccard similarity matrix was carried out on binary data generated from T-RFLPs. PERMANOVA was used to test for significant differences between the five species, between native and non-native ranges across all species, and between the eastern and western Australian natives. PCA analysis and ordinations were performed in the R programming language (version R2.13.0) using vegan package (Oksanen *et al.*, 2011).

Data on soil chemical characteristics were analysed using one way ANOVA for each species separately with range (native or non-native) as the main factor. Biomass (aboveground dry weight), MPN and soil chemical characteristics were analysed in SPSS version 18.0 (IBM SPSS Statistics).

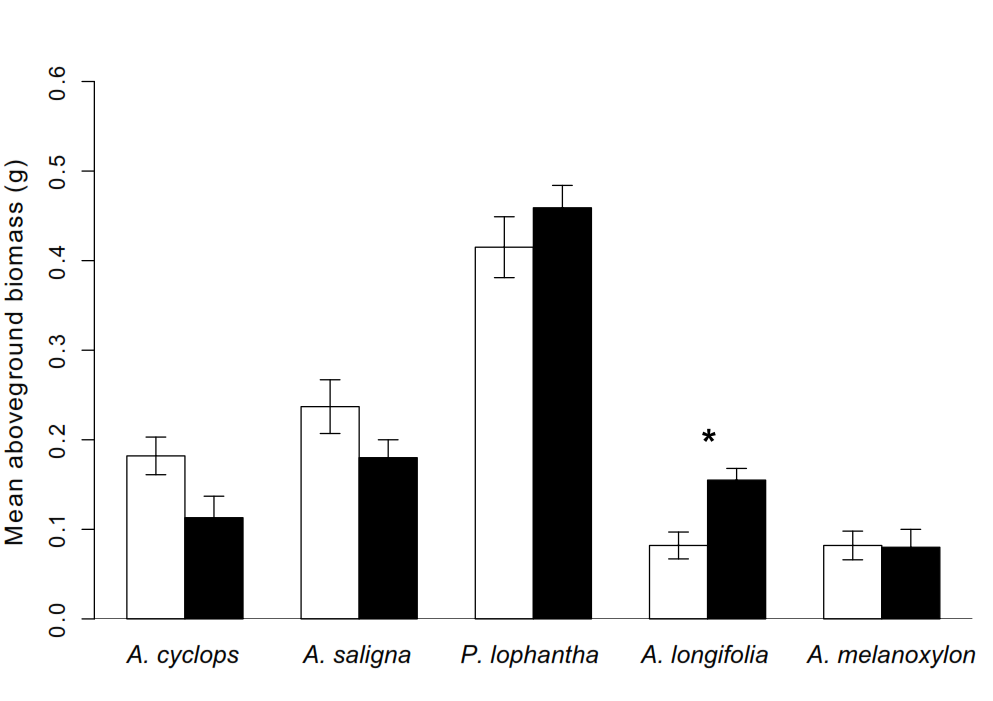
**RESULTS**

**Soil chemical analysis**

There were no significant differences between native and non-native range soils for any of the five species in organic matter, pH, total N, N-NH4+ , total P and total C, although values were generally higher in soils of non-native populations of *P. lophantha* and N-NH4+ was highest in soils of non-native populations of *A. longifolia* (Table 6, Chapter 2). The only significant difference between species’ native and non-native soils was for *A. cyclops* in available N-NO3- (*F*1,5 = 8.8, *P* = 0.03), which was higher in the non-native range. Statistical analyses could not be conducted for *P. lophantha* or *A. melanoxylon* due to low population numbers. Overall, soil properties were very similar across native and non-native ranges (Table 6, Chapter 2).

**Plant growth response in non-native compared to native range soils**

There was a significant interaction between species and range (*F*4,237 = 11.6, *P* < 0.001) indicating that differences in biomass between native and non-native range populations varied among species. However population (nested within range) was not significant (Wald Z = 0.13, *P* = 0.9). Within-species comparisons of seedling biomass from native versus non-native soils showed that eastern native *A. longifolia* seedlings grown in soil from the non-native range (i.e. Western Australia) were significantly larger than seedlings grown in native soils (*F*1,5 = 16.3, *P* = 0.01) (Fig. 2). Both of the western native *Acacia* species, *A. cyclops* and *A. saligna*, showed the opposite pattern of performing better in their native range soils, however these differences were not significant (*F*1,5 = 4.1, *P* = 0.08; *F*1,3 = 3.9, *P* = 0.14, respectively). *Acacia melanoxylon* and *P. lophantha* seedling biomass did not differ significantly between native and non-native range soils (*F*1,3 = 0.1, *P* = 0.78 and *F*1,3 = 2.5, *P* = 0.12, respectively). Thus there was no consistent pattern of increased performance in non-native soils across the five species.

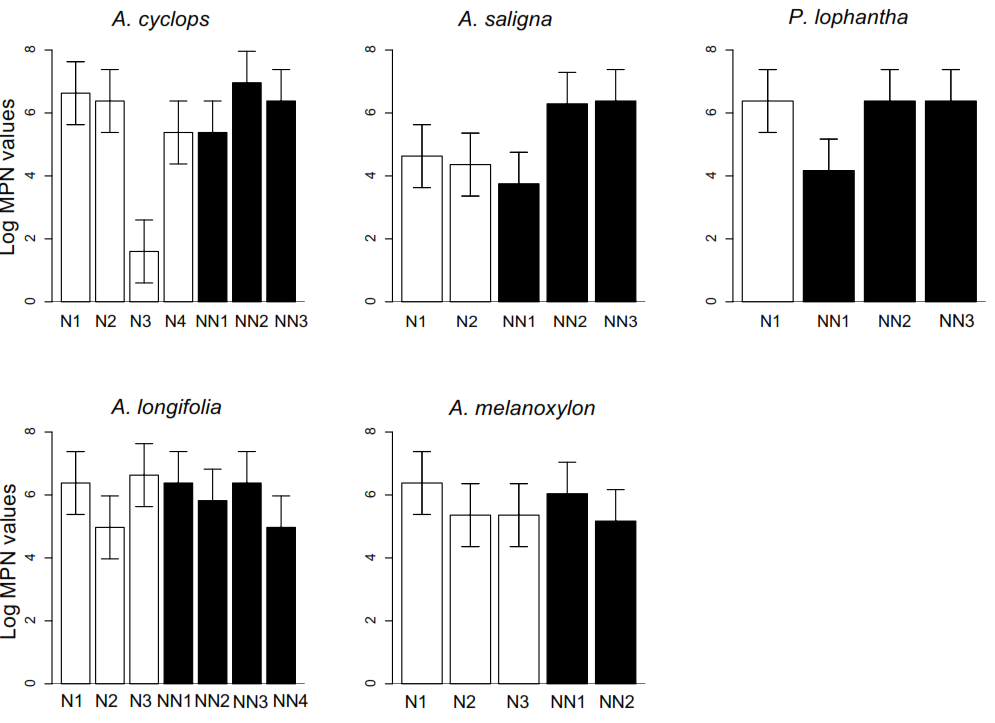


**Figure 2.** Mean aboveground biomass for four *Acacia* species and *Paraserianthes lophantha* grown in soils collected from native and non-native range populations. White bars represent soils from native and black bars soils from non-native populations, respectively. Asterisk indicates significant differences within-species in aboveground biomass between plants grown in soil from native and non-native populations.

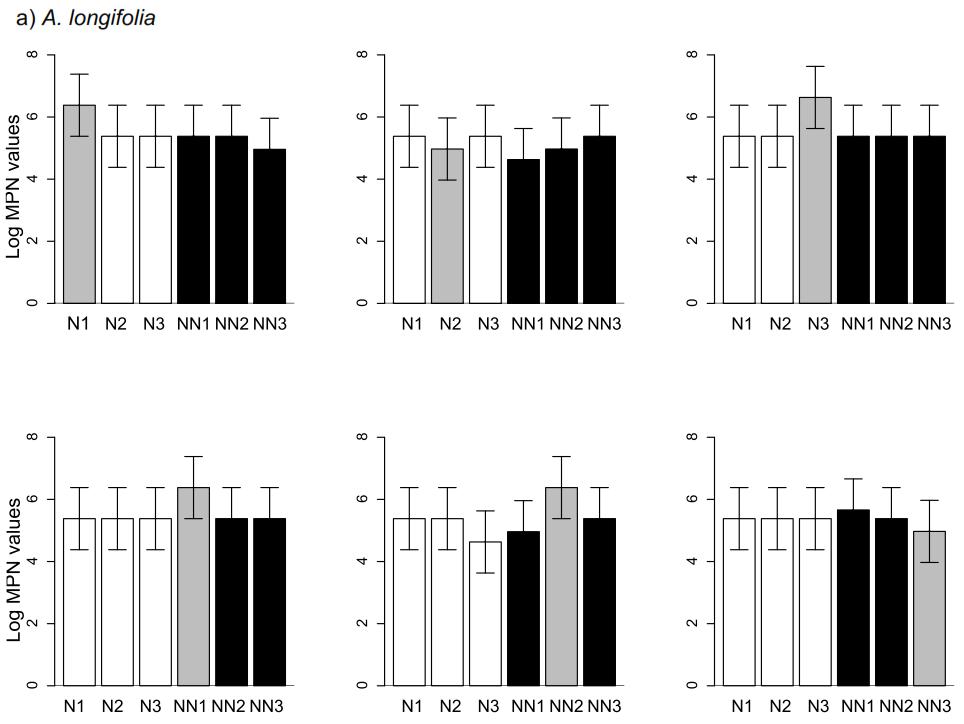
**Rhizobial abundance in non-native compared to native range populations**

We observed sufficiently high numbers of rhizobia per gram of soil in all species’ soils across both native and non-native ranges (Fig. 3) to indicate that rhizobial abundance was unlikely to be a limiting factor. MPN values for plants grown in soils from populations within both native and non-native ranges ranged between 0.4 x 102 to 933 x 104 rhizobia per gram of soil. There was no consistent difference in rhizobial abundance between soils from native and non-native populations across species (*F*1,22 = 0.2, *P* = 0.663), however there were significant differences between species (*F*4,22 = 3.1, *P* = 0.03).

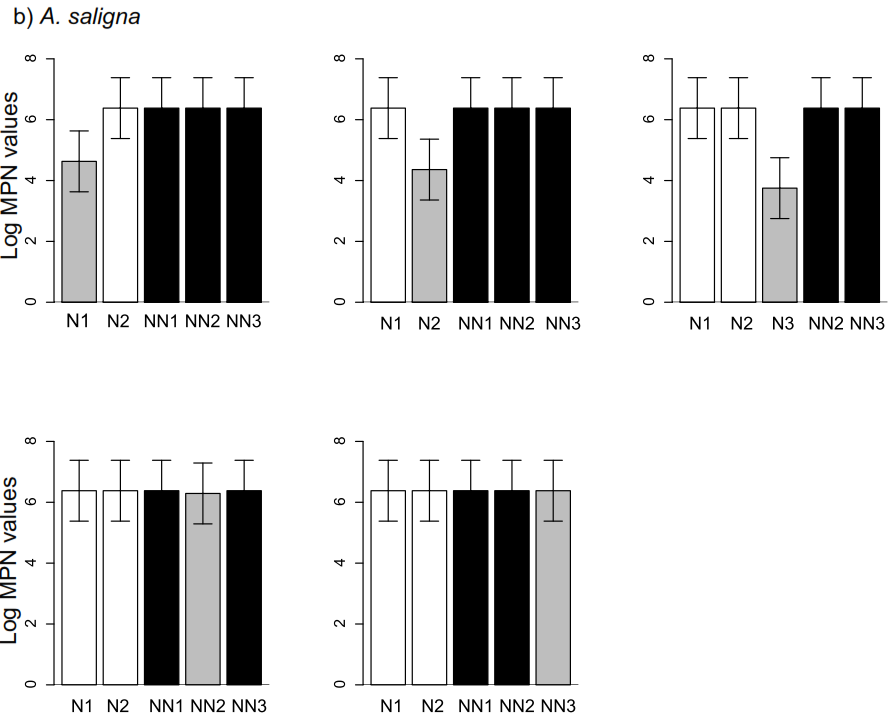
MPN values for *A. longifolia* were between 42.7 x 103 to 933 x 104 rhizobia per gram of soil (Fig. 4a). There were significant differences in MPN values when plants were grown in their native, non-native or reciprocally inoculated soils (*F*2,33 = 3.62, *P* = 0.03). In four instances out of six *A. longifolia* detected more rhizobia per gram of soil in its ‘own’ soils (Fig. 4a). However Bonferroni post-hoc pairwise comparisons did not detect any significant differences relating to soil origin. MPN values for *A. saligna* were between 5.6 x 103 and 933 x 104 rhizobia per gram of soil. No significant differences in MPN values between soil from native and non-native populations were detected (*F*2,22 = 1.9,*P* = 0.16, Fig. 4b).



**Figure 3.** Most probable number (MPN) of rhizobia g-1 soil (log values) for soils from native (N) or non-native (NN) ranges of four *Acacia* species and *Paraserianthes lophantha*. White bars represent native and black bars non-native soils, respectively. The numbers represent population numbers. The error bars represent standard error (±1 log unit).



**Figure 4a**. Most probable number (MPN) of rhizobia g-1 soil (log values) for native (N, white bars), non-native (NN, black bars) and ‘own’ (N or NN, grey bars) soils for populations of *Acacia longifolia*.The numbers represent population numbers. For example, N1 seedlings from N1 population were grown in their ‘own’ N1 soils and in the soils of other two native populations (i.e. N2 and N3, white bars) and in three non-native population soils (i.e. NN1, NN2 and NN3, black bars) separately. Then N2 seedlings were grown in their ‘own’ N2 soils as well as in the soils of two other native populations (i.e. N1 and N3, white bars) and in three non-native populations (i.e. NN1, NN2 and NN3, black bars). The same procedure was repeated for *Acacia longifolia* N3 population and three non-native populations. The error bars represent standard error (±1 log unit).



**Figure 4b**. Most probable number (MPN) of rhizobia g-1 soil (log values) for native (N, white bars), non-native (NN, black bars) and ‘own’ (N or NN, grey bars) soils for populations of *Acacia saligna*.The numbers represent population numbers. For example, N1 seedlings from N1 population were grown in their ‘own’ N1 soils and in the soil of other native population (i.e. N2, white bar) and in three non-native population soils (i.e. NN1, NN2 and NN3, black bars) separately. Then N2 seedlings were grown in their ‘own’ N2 soils as well as in the soil of other native populations (i.e. N1, white bar) and in three non-native populations (i.e. NN1, NN2 and NN3, black bars). The same procedure was repeated for *Acacia saligna* N3 population and three non-native populations. The error bars represent standard error (±1 log unit).

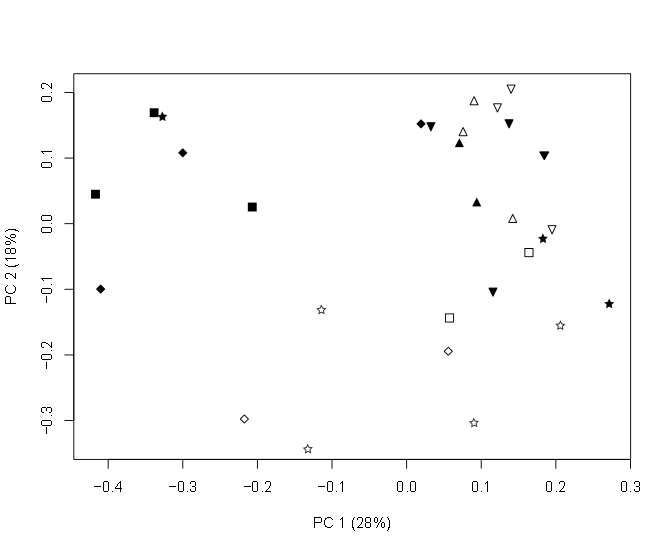
**Rhizobial community composition in non-native versus native range populations**

The first six dimensions of the PCA using the T-RFLP data accounted for c. 88% of the variance in rhizobial community composition. PC1 explained 28%, PC2 18% and PC3 16% of variation (Fig. 5). The ordination diagram showed a clear separation between bacterial communities in soils from non-native and native populations of the species native to Western Australia (*A. cyclops*, *A. saligna*, *P. lophantha*). In contrast, differences in rhizobial communities between the native and non-native populations of the eastern Australian species (*A. longifolia*, *A. melanoxylon*) were much less obvious (Fig. 5).

PERMANOVA analysis of T-RFLP variation in nod and nif genes from nodules collected from the glasshouse study indicated significant differences in perceived rhizobial community composition between native and non-native ranges (*P* = 0.03, Table 4) and between species (*P* = 0.002, Table 4). Western and eastern natives were analysed separately to separate the effect of origin, following inspection of the PCA ordination. Considering only the western Australian species, there was a significant difference in rhizobial community composition between native and non-native range populations (*P* = 0.02) across the three species, while species identity alone had no significant influence on rhizobial genetic structure (Table 4, Fig. 5). In contrast, for eastern Australian species, we found no significant differences in rhizobial community composition between native and non-native ranges, but a significant difference between species (*P* = 0.02, Table 4). We did not analyse within-species differences separately due to the low number of population replicates within native or non-native range of each species.

**Table 4.** Summary of PERMANOVA results for species and range effects following T-RFLP analysis on the DNA from nodules collected from the five study species following the glasshouse experiment.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Model** | **df** | **SS** | **MS** | ***F*** | ***P*** |
| **Overall model** |  |  |  |  |  |
| Range | 1 | 0.42 | 0.42 | 1.45 | 0.03 |
| Species | 1 | 0.60 | 0.60 | 2.07 | 0.002 |
| Residuals  Total | 21  28 | 6.12  8.79 | 0.29 |  |  |
| **Western natives** |  |  |  |  |  |
| Range | 1 | 0.53 | 0.53 | 1.61 | 0.02 |
| Species | 1 | 0.30 | 0.30 | 0.9 | 0.60 |
| Residuals  Total | 13  16 | 4.28  5.45 | 0.32 |  |  |
| **Eastern natives** |  |  |  |  |  |
| Range | 1 | 0.26 | 0.26 | 1.16 | 0.22 |
| Species | 1 | 0.33 | 0.33 | 1.45 | 0.02 |
| Residuals  Total | 8  11 | 1.83  2.67 | 0.22 |  |  |



**Figure 5.** PCA for soil rhizobial communities based on extracted DNA from nodules associated with four *Acacia* species and *Paraserianthes lophantha.* Open and closed symbols represent native and non-native populations, respectively. – *Acacia cyclops*, ∇ ▼ –*Acacia longifolia*, ∆ ▲ – *Acacia melanoxylon*, □ ■ – *Acacia saligna*, ◊ ♦ – *Paraserianthes lophantha*.



**DISCUSSION**

**A conceptual framework for the role of mutualisms in plant invasions**

It has been well documented that invading plants must overcome major biotic and abiotic constraints before successful establishment in novel environments (Williamson & Harrison, 2002; Levine *et al.*, 2004; Mitchell *et al.*, 2006). Recently, much attention has been focused on interactions of exotic plants with soil biota as these have been shown to be important in shaping invasion success (van der Heijden *et al.*, 1998; Klironomos, 2002; Bever, 2003). In this regard, the role of parasites and pathogens and the potential for escape from these in novel environments has been extensively studied in invasion ecology (e.g. Enemy Release Hypothesis). Comparatively, we still know very little about the role of soil mutualists (e.g. rhizobia) in mediating plant invasions (Callaway *et al.*, 2011). In this paper we have outlined and then tested several non-exclusive hypotheses regarding the effects of the belowground microbial community, particularly the role of rhizobia on invasion success of legumes in Australia (Table 3).

**Are mutualisms important in invasions?**

We asked firstly whether species show differences in performance when grown in their non-native compared to native range soils. Given that all five species in our study associate with rhizobia, our *a priori* expectation was that differences in plant performance could be at least partially explained by differences in rhizobial abundance and community composition in native *vs*. non-native soils. For example, we expected soils with more rhizobia to be associated with increased plant growth (Thrall *et al.*, 2007; Rodrıguez-Echeverria *et al.*, 2009). We note that even if increased performance in novel environments is positively correlated with nodulation, it is still possible that release from enemies is playing a role in invasion success.

For four of the five plant species we found no evidence that variation in the soil microbial community between native and non-native range soils influenced plant growth (i.e. plant performance did not depend on soil origin). The single exception was *A. longifolia* which performed significantly better in its non-native range soils (Fig. 2). Furthermore, as indicated by MPN values, rhizobial population sizes were similar between the non-native and native ranges of all five species, suggesting that these N-fixing species are unlikely to be limited by low rhizobial abundance when introduced to novel areas outside their native range, at least within Australia. However for three (*A. cyclops,* *A. saligna,* and *P. lophantha*) out of five species we found significant differences in rhizobial community composition in non-native compared to native range soils.

Overall, we found supporting evidence for the Generalist Host Hypothesis for four of five study species (*A. cyclops, A. saligna, A. melanoxylon* and *P. lophantha,* Table 3), suggesting that these species do not require specific rhizobial communities to establish successfully outside of their native ranges, at least within Australia. This is supported more generally by experimental evidence that geographically widespread species of *Acacia* tend to be more promiscuous with regard to forming symbiotic associations than narrowly distributed host species (Thrall *et al*. 2000). More recently, a study of two Australian acacia species (*A. stenophylla* and *A. salicina*) found no evidence of local adaptation (i.e. absence of mutualism constraints) within their own ranges (Barrett *et al.*, 2011). However, earlier work found that one of these species, *A. stenophylla*, strongly preferred its own soil communities to those from *A. salicina* populations (Thrall *et al*. 2007) while *A salicina* can be characterised as more of a generalist. Interestingly, *A. salicina* is also considered as an invasive species outside its native range in Australia (Richardson *et al.*, 2011a).

We found supporting evidence for the Accompanying Mutualist Hypothesis (Table 3) for one of the five study species (*A. melanoxylon*). For this species we found no differences in biomass,

MPN values or rhizobial composition across both ranges (Table 3), suggesting that its rhizobial communities may have been introduced with its seeds or seedlings. Introduction of rhizobia

with the host into the non-native range has been reported previously for *A. longifolia* in Portugal (Rodríguez-Echeverria, 2010) and *Mimosa pigra* in Taiwan (Chen *et al.*, 2005). While our results indicate that *A. melanoxylon* communities are very similar in terms of the rhizobial identity, abundance and effectiveness, we cannot make conclusive statements regarding rhizobial community composition since T-RFLP analysis is limited in its ability to discriminate among similar genotypes. We found no supporting evidence for the Enhanced or Constrained Mutualism hypotheses for any of our five study species (Table 3).

Several authors have reported that acacias and other legumes in Australia can vary considerably in their rhizobial associations (Burdon *et al.*, 1999; Thrall *et al.*, 2000) and thus it is unclear how dependent they are on those symbioses. For example, Thrall *et al*. (2000) reported that some legumes (*Acacia mearnsii* and *Indigofera australis*) were variable in their responses to different rhizobial isolates whereas others (*A. melanoxylon* and *Hardenbergia violacea*) performed well with most isolates. Similar results were shown by Burdon *et al*. (1999) who concluded that there was significant host-based variability in the ability to form effective symbiotic associations.

Overall, though, based on the results of our inoculation and genetic studies, we suggest that rhizobial mutualists are generally not limiting acacia success in new ranges within Australia, at least not in natural ecosystems where native legumes are present, as is commonly the case in Australia. It is generally accepted that legumes are dependent on mutualists (e.g. rhizobia and arbuscular mycorrhizal fungi) to establish and grow successfully (Pacovsky *et al.*, 1986; Sprent & Parsons, 2000; Stanton-Geddes & Anderson, 2011) and thus mutualism constraints could be more significant at the early stages of legume colonization in novel areas (Thrall *et al.*, 2005; Stanton-Geddes & Anderson, 2011).

**Evidence for other biotic or abiotic effects**

The increased performance of *A. longifolia* in non-native soils may be due to either differences in soil chemistry or in soil microbial communities between the ranges. However, we found no significant difference in nutrient or organic matter content between native and non-native soils for this species (Table 2), suggesting that soil chemistry does not account for plant performance differences. Furthermore, no differences between the non-native and native range were detected in rhizobial abundance or rhizobial community composition for this species. Thus one possible explanation for greater biomass of *A. longifolia* grown in non-native range soils is release from soil-borne pathogens (Enemy Release Hypothesis (Table 3, Fig. 2)). However, it is likely that the combination of mutualistic as well as antagonistic organisms in our inoculum and their combined effects (positive and/or negative) have contributed to the observed results for *A. longifolia*, rather than just escape from soil-borne pathogens. Additional analyses of the non-rhizobial microbial community of *A. longifolia* are needed to definitively identify the underlying causes of the differences in plant growth we observed.

**Predicting the success of plant invasions**

Overall, our results show that acacias are not constrained by lack of compatible rhizobia in their non-native range, at least in Australia (Table 3). Similar results were reported in a recent comparative study by Callaway and colleagues (2011). These authors reported that the invasive legume *Robinia pseudoacacia* had successfully acquired rhizobial partners across its native and non-native ranges, showing no differences in nodule production across the ranges. Furthermore, no biogeographic pattern in the community composition of rhizobia associating with *R. pseudoacacia* was found. This suggests that N-fixing bacteria are not limiting this species’ distribution in its new range (Callaway *et al.*, 2011), which is consistent with our results.

Other studies that have investigated rhizobial communities of acacias over broader geographic scales (i.e. outside of Australia) have found contrasting results. For example, it has been found that *A. cyclops* non-specifically nodulates with both fast- and slow growing rhizobia in Libya (Mohamed *et al.*, 2000). Thus, in novel ranges it could experience either enhanced mutualisms or it may be a generalist host outside its native range in Australia (Table 3). *Acacia saligna* has been reported to associate with more diverse rhizobia in its native Australia compared to Portugal (Rodríguez-Echeverria *et al.*, 2011), suggesting the possibility that evolutionary history may influence the complexity of legume-rhizobial interactions. However, other strains of N-fixing bacteria (e.g. *Ensifer* (formerly *Sinorhizobium spp*.)) that have not previously been found in Australia have been reported from North Africa for *A. saligna* (Amrani *et al.*, 2010) providing further support for the generalist host hypothesis for this species in its novel range in North Africa.

Similarly to *A. saligna*, eastern native *A. melanoxylon* associates in its non-native range in North Africa with rhizobial communities that have not been previously reported from Australia (Swelim *et al.*, 1997; Marsudi *et al.*, 1999; Lafay & Burdon, 2001; Yates *et al.*, 2004; Amrani *et al.*, 2010). This suggests that *A. melanoxylon* could also be a generalist host outside Australia (Table 3). In contrast, a recent study of *A. longifolia* in its invasive range in Portugal showed that it had established rhizobial communities similar to those from its native Australian soils (Rodríguez-Echeverria, 2010). An earlier study reported that *A. longifolia* nodulated profusely compared to the native legumes (*Ulex europaeus* and *Cytisus grandiflorus*) and this was correlated with increased aboveground growth (Rodrıguez-Echeverria *et al.*, 2009). Overall, this suggests that interactions between invasive legumes and rhizobia may vary considerably outside their native ranges, making it difficult to predict the invasion success of a species based solely on its symbiotic associations.

Our results suggest that the widespread distribution of acacias in their native ranges across the Australian continent paves the way for other acacia taxa to increase their ranges when moved

by humans to areas far removed from their native ranges. Compatible rhizobia that associate with acacias may be ubiquitous, although clearly there is considerable variation among rhizobial communities (Lafay & Burdon, 1998; Barrett *et al.*, 2011; Hoque *et al.*, 2011). Nevertheless, in Australia it has been reported that both fast- (i.e. *Rhizobia*) and slow-growing (i.e. *Bradyrhizobium*) rhizobia occur naturally (Lafay & Burdon, 2001), however *Bradyrhizobium* is the predominant rhizobia throughout the continent (Lange, 1961; Lawrie, 1983; Barnet *et al.*, 1985; Barnet & Catt, 1991). Further analyses (e.g. sequencing) on both rhizobial and non-rhizobial communities are needed to elucidate the different functional groups of the soil biota (e.g. mutualists and pathogens) and their role in the invasion success of these acacias.

Our attempt to elucidate and evaluate the importance of key soil mutualisms (e.g. rhizobia) in mediating invasive success of these acacias along with recent comprehensive research on other major aspects of acacias ecology, e.g. reproductive biology (Gibson *et al.*, 2011) and ecophysiological traits (Morris *et al.*, 2011) highlight the complexity of understanding all the dimensions of invasive potential of a species.

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| **Table 3.** A summary table showing the results, the hypotheses supported by the evidence and the interpretation for each species. Additional information is given for other similar findings from outside Australia (list not exhaustive). | | | | | | |
| **Species** | **Results** | **Hypotheses supported** | | **Description** | | **Other studies** | |
| *Acacia cyclops* | No biomass difference between native and non-native soils  No difference in rhizobial abundance between native and non-native soils  Differences in rhizobial community composition between native and non-native range | Generalist Host Hypothesis | Plants are not limited in the introduced range as they are able to effectively associate with a wide range of soil mutualists. | | Non-specific nodulation with both fast- and slow growing rhizobia was reported from Libya (Mohamed *et al.*, 2000). | |
| *Acacia saligna* | No biomass difference between native and non-native soils  No difference in rhizobial abundance between native and non-native soils  Differences in rhizobial community composition between native and non-native range | Generalist Host Hypothesis | Plants are not limited in the introduced range as they are able to effectively associate with a wide range of soil mutualists. | | Higher diversity of rhizobia was found in Australia than in Portugal (Rodríguez-Echeverria *et al.*, 2011). Other strains of N-fixing bacteria (e.g. *Ensifer spp*.) not found in Australia reported from North Africa (Amrani *et al.*, 2010). | |
| **Table 3.** A summary table showing the results, the hypotheses supported by the evidence and the interpretation for each species. Additional information is given for other similar findings from outside Australia (list not exhaustive) (cont.). | | | | | | |
| **Species** | **Results** | **Hypotheses supported** | **Description** | | **Other studies** | |
| *Paraserianthes lophantha* | No biomass difference between native and non-native soils  No difference in rhizobial abundance between native and non-native soils  Differences in rhizobial community composition between native and non-native range | Generalist Host Hypothesis | Plants are not limited in the introduced range as they are able to effectively associate with a wide range of soil mutualists. | | No known reports on rhizobial communities in native or non-native ranges. | |
| *Acacia longifolia* | Greater biomass in non-native soils  No difference in rhizobial abundance between native and non-native soils  No difference in rhizobial community composition between native and non-native soils. | Enemy Release Hypothesis | Plants are released in the novel range from soil pathogens that have a detrimental effect in the native range. | | Rhizobial communities in Portugal similar to those in Australia (Rodriguez-Echeverria *et al.*, 2007; Rodríguez-Echeverria, 2010). | |
| **Table 3.** A summary table showing the results, the hypotheses supported by the evidence and the interpretation for each species. Additional information is given for other similar findings from outside Australia (list not exhaustive) (cont.). | | | | | | |
| **Species** | **Results** | **Hypotheses supported** | **Description** | | **Other studies** | |
| *Acacia melanoxylon* | No biomass difference between native and non-native soils  No difference in rhizobial abundance between native and non-native soils  No difference in rhizobial community composition between native and non-native soils. | Accompanying Mutualist Hypothesis  Generalist Host Hypothesis | Host plants are not limited in the introduced range as they have brought their specialist mutualists with them (e.g. with seeds or seedlings).  Plants are not limited in the introduced range as they are able to effectively associate with a wide range of soil mutualists. | | May be different rhizobial communities in native range in Australia compared to non-native range in North Africa (Swelim *et al.*, 1997; Lafay & Burdon, 2001; Amrani *et al.*, 2010). | |

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**Chapter 4: Nitrogen fixing bacterial communities in the rhizosphere of invasive legumes in Australia are different across the continent**

This manuscript has been prepared for submission to *FEMS Microbiology Ecology* as ‘Birnbaum, C., Bissett, A., Thrall, P.H. and Leishman M.R. ‘Nitrogen fixing bacterial communities in the rhizosphere of invasive legumes in Australia are different across the continent.’

My contribution to the research and paper: Concept - 90%; Data collection - 70%; Statistical analysis - 90%; Bioinformatic analysis - 50%; Writing - 80%

**Abstract**

The objective of this study was to describe the nitrogen fixing bacterial communities associated with *Acacia cyclops*, *A. longifolia, A. melanoxylon* and *A. saligna* and a close relative *Paraserianthes lophantha* in their non-native and native ranges in Australia. Free-living nitrogen fixing bacterial communities were amplified from soils that were collected from the rhizosphere of multiple populations of each species in both non-native and native ranges. Additionally, nitrogen fixing bacterial communities were amplified directly from nodules collected from plants that were previously grown in the glasshouse using field-collected soil as inoculum. Nitrogenfixing bacteria were sequenced using 454 pyrosequencing of the *nifH* gene and sequences assigned to taxonomic groups based on *nifH* consensus taxonomy. Our results suggest that the free-living nitrogen fixing bacterial communities associating with invasive legumes throughout Australia are generally different across the ranges at the genus level which is likely driven by fundamental differences in soil microbial composition across the continent. However the bacterial communities that these hosts associate with via the symbiotic pathway in the nodules are fundamentally similar across the continent. The abundance of common nitrogen fixing bacteria in the rhizosphere of all five species across their native and non-native

ranges suggests it is unlikely that these plant species are constrained by the absence of compatible nitrogen fixing bacteria in their novel range.

**Keywords** rhizobia, legumes**,** free-living nitrogen fixers, novel ranges, invasion, mutualism

**Introduction**

Soil microbes are increasingly acknowledged to play a significant part in invasion outcomes for many plant species introduced to novel environments (Wolfe & Klironomos, 2005; Reinhart & Callaway, 2006; van der Putten *et al.*, 2007). The role of soil mutualists in plant invasions, however, is still relatively unknown compared to that of soil pathogens, which have been more extensively studied (Callaway *et al.*, 2011). It has been suggested, however, that the spread of invaders may depend on the successful establishment of key mutualisms in the new range (Simberloff & Von Holle, 1999; Richardson *et al.*, 2000). This may be particularly true for plants which have specific inter-dependencies with symbiotic mutualists (e.g., legumes and nitrogen-fixing rhizobia).

Generally, it has been proposed that invaders can be either constrained by lack of mutualisms in the absence of suitable partners (Parker, 2001) or benefit from newly acquired symbionts in the novel range (Marler *et al.*, 1999; Parker *et al.*, 2007). There is certainly evidence that lack of compatible soil mutualists may serve as a constraint for successful establishment in the novel range for some species (Díez, 2005; Nuñez *et al.*, 2009; Dickie *et al.*, 2010). Legumes have been reported to rely extensively on mutualisms (e.g., rhizobia, mycorrhizal fungi) to successfully colonize and establish in novel areas (Pacovsky *et al.*, 1986; Sprent & Parsons, 2000; Parker, 2001). For example, Stanton-Geddes & Anderson (2011) showed that seedlings of the legume *Chamaecrista fasciculata* (Partridge Pea) were less likely to be nodulated than experimentally inoculated plants when transplanted beyond their native range. Additionally, they found that there was always an inoculation benefit for *C. fasciculata* plants, suggesting that absence or low densities of compatible rhizobia may limit range expansion of this legume species (Stanton-Geddes & Anderson, 2011).

Broad symbiotic promiscuity and ability to nodulate at low rhizobial abundance have been described as significant advantages for invading legumes (Parker, 2001; Perez-Fernanndez & Lamont, 2003; Rodríguez-Echeverria *et al.*, 2011). For example, promiscuous legumes (i.e.,

generalists) are more likely to find compatible symbionts in novel ranges compared to legumes with narrow symbiotic specificity (i.e., specialists) (Rodríguez-Echeverria *et al.*, 2011). Recent studies have shown that some invasive woody legumes were able to profusely nodulate (Lafay & Burdon, 2006) and associate with novel bacterial communities in their exotic ranges (Marsudi *et al.*, 1999; Amrani *et al.*, 2010).

Despite the evidence for promiscuity of some host species, there are also reports that despite being global invaders, some invasive legume species associate preferentially with rhizobia from their native range. For example, Chen *et al*. (2005) found that *Mimosa pigra* (mimosa) in Taiwan associated predominantly with *Burkholderia* strains that appeared to have originated from *M. pigra*’s native range in South America, rather than with nitrogen-fixing bacteria from the Taiwanese native range. Similar results were reported by Rodriguez-Echeverria (2010), who showed that *Acacia longifolia* in Portugal associated with rhizobial communities that were very similar to the rhizobial communities from *A. longifolia*’s native range in Australia. This suggests that rhizobial communities may have been accidentally introduced with seeds or seedlings of this species into Portugal (Rodríguez-Echeverria, 2010). Thus, there appears to be considerable variation in host-soil symbiont associations across non-native and native ranges making it difficult to generalise regarding the importance of soil mutualists in invasion success.

Woody legumes, especially *Acacia* species, are considered some of the worst invaders globally (Richardson *et al.*, 2011a; Richardson & Rejmánek, 2011b). The invasion success of acacias has been largely credited to their global use in agro-forestry and horticulture (Griffin *et al.*, 2011). This, in turn, opened a path to the colonization of novel communities beyond their natural range and has, in some cases, resulted in significant impacts on invaded ecosystems via induced changes to soil chemistry and microbial assemblages (Marchante *et al.*, 2003; Marchante *et al.*, 2008a; Le Maitre *et al.*, 2011). There is some evidence to suggest that more widely distributed acacias within their native range could be more promiscuous, i.e., they are able to associate with more diverse rhizobia, whereas narrowly distributed species strongly prefer their own

symbionts (Thrall *et al.*, 2000). However, there is also evidence to suggest that some acacias (e.g., *A. cyclops*) are able to non-specifically nodulate with both fast- and slow-growing rhizobia in their novel range (Marsudi *et al.*, 1999; Mohamed *et al.*, 2000; Lafay & Burdon, 2006). Thus, quantification of the role of mutualists such as rhizobia in determining invasion success could enhance our understanding of species’ invasion potential more generally.

The primary aim of this study was to describe the nitrogen fixing bacterial communities of four *Acacia spp*. (*A. cyclops, A. longifolia, A. melanoxylon* and *A. saligna*) and a close relative *Paraserianthes lophantha* (hereafter collectively termed legumes) in their non-native and native ranges in Australia. We analysed nitrogen fixing bacterial communities from (i) soils collected in the rhizosphere of each species in both non-native and native range populations across Australia; and (ii) nodules that were collected after harvesting plants grown in the glasshouse where field-collected soil was used as inoculum. We first asked: (i) do nitrogen fixing bacterial communities in soils differ across non-native and native range populations of these legumes in Australia and (ii) do host plants associate with similar or different nitrogen fixing bacterial communities in nodules depending on host range (non-native vs native)? We predicted that the extent to which nitrogen fixing bacterial communities in nodules differ across ranges would at least partly depend on whether these legumes are generalists or specialists. For example, specialists would be more likely to select similar subsets of symbionts in both native and non-native regions. To our knowledge this study represents one of the first attempts to comprehensively describe the nitrogen fixing bacterial communities across multiple invasive host taxa and biogeographic ranges by directly sequencing the *nifH* gene from both soil communities as well as from nodules.

**Materials and methods**

**Study species**

Four *Acacia* species (*A. cyclops* A.Cunn. ex G.Don*, A. saligna* (Labill.) H.L. Wendl*, A. longifolia* (Andrews) Willd. and *A. melanoxylon* R.Br.) and a close relative *P. lophantha* (Willd.) I.C. Nielsen

were used as host species. *Acacia cyclops*, *A. saligna* and *P. lophantha* are native to Western Australia but have been introduced to the eastern states of Australia (New South Wales, Victoria and South Australia) where they have naturalised and become invasive. *Acacia longifolia* and *A. melanoxylon* are native to south-east Australia but have been introduced to and become invasive in Western Australia. The eastern and western ranges of each species are separated by the vast Nullarbor Desert, providing a major barrier to natural seed dispersal between native and introduced ranges (Jacobs & Wilson, 1996). Thus it is unlikely that any of these species have been transported naturally across the continent. All of these species are also recognised as invasive or naturalised outside Australia, for example in South Africa (Roux, 1961), Portugal (Marchante *et al.* 2003; Marchante *et al.* 2008b; Rodriguez-Echeverria *et al.* 2011), Hawaii (Wagner *et al.*, 1999) and New Zealand (Owen, 1997).

**Study sites and soil sampling**

To characterise the diversity of nitrogen fixing bacteria associated with legumes we collected soil samples in December 2009 across south-east and south-west Australia from Perth to Esperance (from 31º 51´ S, 115º 45´ N to 33º 48´ S, 121º 49´ N, respectively) in Western Australia and from Sydney to Yorke Peninsula (from 33º 42´ S, 151º 16´ N to 34º 43´ S, 137º 35´ N, respectively) in eastern Australia (Fig. 1, Chapter 2). For each species, with the exception of *A. melanoxylon* for which we only had four populations in its non-native range, we sampled five individuals from each of five populations within each range [5 species x 2 ranges (native and non-native) x 5 populations x 5 individuals] (Table 1). A total of 1000 g of soil was collected beneath each individual as close to roots as possible at a depth of 10-15 cm and then bulked for each population. Soils were kept in a cooler in the field before being stored at 4oC. Soils were sieved to 2 mm to remove leaves and other coarse material and to homogenise samples. Soil

subsamples were placed in sterilized falcon tubes and stored in the freezer at -20oC until further analysis. All sampling and processing equipment, including sieves, were sterilized with 90% ethanol between populations.

**Nodule collection**

To assess nitrogen fixing bacterial diversity in nodules we extracted DNA from nodules that were collected from plant roots after harvesting the glasshouse experiment that is described in Chapter 3. The plants in that experiment were grown in field collected soils and assessed for growth in the non-native compared to native range soils. From each plant 2-5 nodules were collected and surface sterilized with 90% ethanol and distilled water before being stored in the freezer at -20oC in a plastic jar filled with silica beads and cotton wool (Somasegaran, 1994). Nodules from 10 replicate plants from each of 29 population/range soil combinations were pooled for DNA extraction. Nodules (0.05 g) were crushed in liquid nitrogen to create a homogenised sample for DNA extraction.

**DNA extraction and PCR**

DNA from soils and nodules was isolated using a PowerSoil and PowerPlant DNA isolation kit, respectively, following the manufacturer’s protocol (MO Bio Laboratories, Inc. Carlsbad, CA). *NifH* was amplified from soils and nodule DNA using a nested PCR with the internal primer pair *nifH* 1 (5´-TGY GAYCCN AAR GCN GA-3´) and *nifH* 2 (5´-ADN GCC ATC ATY TCN C-3´) (Zehr & McReynolds, 1989) and the external primers *nifH* 3 (5´-ATR TTR TTN GCN GCR TA-3´) and *nifH* 4 (5´-TTY TAY GGN AAR GGN GG-3´) (Zani *et al.*, 2000). *NifH* was chosen as it is considered the signature functional gene for studying the diversity of nitrogen-fixing organisms (Gaby & Buckley, 2011) and it remains the most thoroughly studied gene with an extensive collection of genes obtained from both cultured and uncultivated organisms from multiple environments (Izquierdo & Nüsslein, 2006). PCR amplifications were performed by the Research and Testing Laboratory (Lubbock, Texas, USA) following the protocol of Smith *et al*. (2010) and Dowd *et al*. (2008a). HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used for PCR under the

conditions described in detail in Dowd *et al*. (2008b). Additionally, a step 2 PCR was performed following the protocol of Dowd *et al.* (2008b) as it prevents amplification biases caused by inclusion of tag and linkers during initial template amplification reactions. After secondary PCR,

amplicon products were mixed in equal volumes and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) (Dowd *et al.*, 2008a).

**Tag-encoded FLX amplicon pyrosequencing**

Sequencing was performed by the Research and Testing Laboratory (Lubbock, Texas, USA) following the protocol of Dowd *et al*. (2008a). In summary the DNA fragments’ size and concentration were accurately measured by using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380 Fluorometer (Turner Biosystems, CA, USA). A 9.6 E+06 sample of double-stranded DNA molecules/μL with an average size of 625 bp were combined with 9.6 million DNA capture beads, and then amplified by emulsion PCR. After bead recovery and bead enrichment, the bead-attached DNAs were denatured with NaOH, and sequencing primers were annealed. A two-region 454 sequencing run was performed on a 70×75 GS PicoTiterPlate (PTP) by using a Genome Sequencer FLX System (Roche, Nutley, New Jersey). All FLX related procedures were performed following Genome Sequencer FLX System manufacturer’s instructions (Roche, Nutley, New Jersey).

**Bioinformatic analyses**

Downstream sequence analyses were performed on *nifH* sequences from nodules and soils in a combined file using mothur version 1.22.0 (Schloss *et al.*, 2009) following the adapted sequence quality-control pipeline analysis described in detail in Schloss *et al.* (2011), until chimeric sequences were removed. Sequences were included in subsequent analyses only if they carried the correct primer sequence, did not contain any nucleotide ambiguities, contained homopolymer runs >8 and were ≥150 bp and ≤400 bp long.

Following the above quality control, sequences were blasted with a BLAST e-value > e 0.001 against an existing *nifH* database that has 16 989 *nifH* sequences (Gaby & Buckley, 2011). Only the sequences that returned a blast hit of sequence identity ≥ 90% were kept and used for further downstream analysis. Approximately 13% of sequences did not match any existing

sequence in the database and were removed from further analysis. Remaining sequences were checked for chimeras against “self” using USEARCH (Edgar, 2010) and a further 2% were removed from the dataset. After checking for chimeric sequences, nodule and soil *nifH* sequences were split into separate groups (nodules and soils) and pre-clustered at 1% within each group. Following pre-clustering, nodule and soil sequences were classified using *nifH* consensus taxonomy (Gaby & Buckley, 2011) with the consensus confidence threshold of 51%. Furthermore, OTUs (Operational Taxonomic Unit) represented by only a single sequence (i.e., singletons) were omitted from the analysis as they are likely to result from pyrosequencing errors (Tedersoo *et al.*, 2010). Additionally, the relative abundance of nitrogen fixing bacterial OTUs in soil and nodule data was calculated for each host species and population.

**Statistical analyses**

Principal coordinates analysis (PCA) based on a Bray-Curtis dissimilarity matrix was carried out on presence/absence transformed data generated from the OTU species matrix for soil and nodule bacterial communities separately. This allowed visual inspection to identify differences between non-native and native range populations for each species separately. Upon inspection of PCA results, PERMANOVA (Anderson, 2001) was used to test for overall differences across all species between their ranges (i.e., native and non-native) and locations (i.e., eastern and western populations). A similarity percentage analysis (SIMPER) based on a Bray-Curtis index of dissimilarity was used to determine the dissimilarity in nitrogen fixing bacterial communities for nodule and soil nitrogen fixing bacterial communities separately within ranges and locations and to identify bacterial species that contributed strongly to that dissimilarity.

Mantel tests (Mantel, 1967; Mantel & Valand, 1970) based on Pearson’s product-moment correlation were performed to compare the soil and nodule dissimilarity matrices in order to determine if there was a significant correlation between the two matrices (i.e. nodule and soil matrices). One-way ANOVA was used to test for differences in the number of OTUs (i.e., richness) for nodule and soil bacterial communities separately across all host species.

Additionally, richness of bacterial communities between the non-native and native range was assessed for each species separately. All analyses and ordinations were performed in the R programming language (version R2.14.2) using vegan 2.0-3 (Oksanen *et al.*, 2011) package.

**Results**

A total of 777573 sequencing reads from the 454 sequencing analysis (Table 1) for amplified *nifH* gene fragments from soil and nodule DNA (49 and 29 samples, respectively) were obtained with a mean fragment length of 338 bp (max 769 bp, median 359 bp). Following quality control (trimming, blast against *nifH* database, chimera check) the total number of sequences was reduced to 619791 (i.e. 79% of all sequences). The final total of known *nifH* gene sequence fragments for soil and nodule samples was 358349 and 261442, respectively.

**Nitrogen fixing bacterial communities in soils**

A total of 119 OTUs were classified at the genus level as nitrogen fixing bacteria based on the amplified *nifH* gene from soil DNA collected from the rhizosphere of each of the study species across both native and non-native ranges within Australia. Overall we observed a significant range by location interaction for nitrogen fixing bacterial community structure for the five host species, suggesting that differences between the native and non-native ranges are strongly linked to the location (east vs west) of the populations (Fig. 1a, Table 2). Additionally, soil nitrogen fixing bacterial communities were significantly different across the five study species (Table 2). Notably, *A. cyclops*’ bacterial communities in the soil were significantly different from the soil nitrogen fixing bacterial communities of the other four legume taxa (Table 3). Across all

hosts, soil bacterial species richness (i.e., number of OTUs) was overall similar across eastern and western populations, 21 (±2) and 26 (±2) respectively (F1,47 = 2.52, *P* = 0.119). However, bacterial species richness of native compared to non-native range populations was significantly higher for two species, *A. cyclops* (F1,8 = 10.36, *P* = 0.0123, Table 1) and *A. saligna* (F1,8 = 5.86, *P* = 0.041) (Table 1). For the other three legumes there were no significant differences in species richness between their non-native and native range populations (Table 1).

The five most abundant bacterial genera out of the 51 taxa presented in Figure 2a included sequences that were unclassified (33% of all soil sequences), *Xanthomonas* (17%), *Leptothrix* 12%), *Rhodoblastus* (5%) and *Aminobacter* (3%) (Fig. 2a). *Rhodoblastus* was the dominant genus in *A. cyclops* soils across both ranges whereas the unclassified group dominated *A. saligna*, *A. melanoxylon* and *P. lophantha* soils across both ranges (Fig. 2a). Furthermore, *Xanthomonas* was also relatively abundant in *A. melanoxylon* soils across both ranges. *Xanthomonas*, a group which includes many plant disease-causing pathogens (Hayward *et al.*, 1993), was also highly abundant (up to 100%) in one native *A. longifolia* range population soil and was the dominant taxa in the other four native range population soils, but was relatively scarce in four non-native population soils (Fig. 2a). Interestingly, in one non-native population of *A. longifolia*, the soil had very high abundance (up to 80%) of the mesophilic *Methanocaldococcus* which was only found in very low abundance in the soils of two other species (*A. cyclops* and *A. saligna*) (Fig. 2a).

The 51 most common taxa presented in the rhizosphere heatmap shown in Fig. 2a across all hosts and populations included four OTUs (i.e., *Bradyrhizobium*, *Ensifer*, *Azorhizobium* and *Phyllobacterium*) that are classified as rhizobia (Weir, 2011). Although *Bradyrhizobium* has been previously reported as one of the predominant rhizobial genera found in the nodules of many invasive acacias in both native and non-native ranges (Rodríguez-Echeverria *et al.*, 2011), in our study this genus, although present in all soils (Fig. 2a), was found to be less common (i.e. relative abundance of 1-10%,) than other putative non-nodulating bacterial taxa. Similarly,

*Ensifer* was also surprisingly scarce and was not detected in soils of *A. longifolia* and *A. melanoxylon* and only in very low abundance for the other three study species. *Phyllobacterium* was only detected in one non-native population of *A. cyclops*. SIMPER analysis revealed that 26 bacterial OTUS contributed up to 50% of the average Bray-Curtis dissimilarity for location (east vs west) which included three rhizobial taxa *Bradyrhizobium*, *Ensifer* and *Azorhizobium* (Table 4).

**Nitrogen fixing bacterial communities in nodules**

A total of 30 OTUs were classified at the genus level as nitrogen fixing bacteria originating from legume nodules that were harvested after a glasshouse experiment (Chapter 3). All taxa found in nodules were also found in the rhizosphere and the Mantel test showed a significant correlation between the two matrices (Mantel statistic r = 0.2347, *P* = 0.026). Overall we found no significant differences between native and non-native ranges or eastern and western populations in nitrogen fixing bacterial communities detected from the nodules of these legumes (Table 2, Fig. 1b). However there were significant differences between the five host study species (Table 2). Notably, the nitrogen fixing bacterial communities in nodules of *A. cyclops* were significantly different from the bacterial communities of the two eastern native species *A. longifolia* and *A. melanoxylon* (Table 3).

With few exceptions, nodule nitrogen fixing bacterial community richness was similar (F1,27 = 2.86, *P* = 0.102) between eastern and western populations, 11 (±1) and 9 (±1), respectively (Table 1). However, species richness was significantly higher in non-native range vs. native range nodules of *A. cyclops* (F1,5 = 17.76, *P* = 0.008) and marginally higher in native range nodules of *A. longifolia* (F1,5 = 6.42, *P* = 0.052) (Table 1).

Out of 13 most common taxa presented in the heatmap shown in Fig. 2b the most abundant genera across all hosts and populations were *Aminobacter* (31% of all nodule sequences), unclassified bacteria (20% of all nodule sequences), sequences from the genus *Leptothrix* (17%),

*Xanthomonas* (20%), *Bradyrhizobium* (2%) and *Pseudocidovorax* (1%) (Fig. 2b). Bacterial communities in the nodules of *A. cyclops* were overall similar across the ranges with

*Aminobacter* being the dominant taxa found (Fig. 2b). The novel unclassified bacterial OTUs were the dominant group in non-native range nodules of *A. saligna* and *P. lophantha*,but overall the nodule bacterial communities were similar across the ranges for these two species (Fig. 2b). Notably, *A. cyclops* and *A. saligna* also had considerably higher numbers of *Rhodoblastus* (up to 80% and 70% respectively) in their native range nodules (Fig. 2b).

*Xanthomonas* was the most abundant taxa found in the non-native range nodules of *A. longifolia*, whereas the novel sequences at genus level were more dominant in nodules from the native range. Unclassified bacteria from the genus *Lepothrix* dominated *A. melanoxylon* nodules in both native and non-native range populations. Additionally, *Xanthomonas* comprised a third of all bacterial taxa in the native populations of *A. melanoxylon* (Fig. 2b).

*Bradyrhizobium* and *Ensifer* were the only two detected abundant OTUs classified as “rhizobia” at the genus level in nodules. *Bradyrhizobium* was present in the nodules of all hosts across both ranges but in relatively small numbers (10-20%) compared to other taxa (Fig. 2b). *Ensifer* was only detected in *A. cyclops* and *A. saligna* nodules from the non-native range (Fig. 2b). SIMPER analysis revealed that 6 bacterial OTUs contributed up to 50% of the average Bray-Curtis dissimilarity across all species for location (east vs. west) which included one rhiozbial taxa *Ensifer* (Table 4).

**Table 1**. Details of hosts and obtained sequence reads for amplified *nifH* gene from native and non-native soils (n=49) collected across Australia and from nodules (n=29) harvested after a glasshouse experiment using field collected soil inoculum for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) at the genus level is also presented for each host plant species for native and non-native ranges (mean (±SE)). Asterisks indicate significant differences between the ranges in OTU numbers (*P* < 0.05).

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
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| **No.** | **Host species, populations** | | **Range** | | | **Obtained sequence reads from soils** | | | | **Obtained sequence reads from nodules** | | | | | **State** | | | | | **Location** | | | | | | **Latitude, longitude** | | | | **Soils** | | **Nodules** | |
| 1. | *A. cyclops* | | Native | | | 23744 | | | 5993 | | | | | | WA | | | | | Ravensthorpe | | | | | | 33º 36´ 120º 12´ | | | | 23 (±3)\* | | | 9 (±1)\* | |
| 2. | *A. cyclops* | | Native | | | 2815 | | | 3014 | | | | | | WA | | | | | Bremer Bay | | | | | | 34º 25´ 119º 22´ | | | |  | |  | |
| 3. | *A. cyclops* | | Native | | | 13151 | | | NA | | | | | | WA | | | | | D`Entrecasteaux NP | | | | | | 34º 51´ 116º 1´ | | | |  | |  | |
| 4. | *A. cyclops* | | Native | | | 9614 | | | 5415 | | | | | | WA | | | | | William Bay | | | | | | 35º 1´ 117º 14´ | | | |  | |  | |
| 5. | *A. cyclops* | | Native | | | 8181 | | | 3427 | | | | | | WA | | | | | Coogee | | | | | | 32º 7´ 115º 45´ | | | |  | |  | |
| 6. | *A. cyclops* | | Non-native | | | 1847 | | | NA | | | | | | SA | | | | | Yorke peninsula | | | | | | 34º 3´ 137º 45´ | | | | 14 (±1)\* | | 14 (±1)\* | |
| 7. | *A. cyclops* | | Non-native | | | 3545 | | | 60108 | | | | | | SA | | | | | Yorke peninsula | | | | | | 34º 43´ 137º 35´ | | | |  | |  | |
| 8. | *A. cyclops* | | Non-native | | | 2585 | | | 3940 | | | | | | SA | | | | | Yorke peninsula | | | | | | 35º 3´ 137º 43´ | | | |  | |  | |
|  | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|  | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Table 1**. Details of hosts and obtained sequence reads for amplified *nifH* gene from native and non-native soils (n=49) collected across Australia and from nodules (n=29) harvested after a glasshouse experiment using field collected soil inoculum for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) at the genus level is also presented for each host plant species for native and non-native ranges (mean (±SE)). Asterisks indicate significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **No.** | **Host species, populations** | **Range** | | | **Obtained sequence reads from soils** | | | | **Obtained sequence reads from nodules** | | | | | **State** | | | | **Location** | | | | **Latitude, longitude** | | | | | **Soils** | | | | | **Nodules** | |
| 9. | *A. cyclops* | Non-native | | | 1220 | | | | NA | | | | | SA | | | | McLaren Vale | | | | 35º 14’ 138º 27´ | | | | |  | | | | |  | |
| 10. | *A. cyclops* | Non-native | | | 1714 | | | | 35470 | | | | | SA | | | | Victor harbour | | | | 35º 32´ 138º 38´ | | | | |  | | | | |  | |
| 11. | *A. saligna* | Native | | | 2102 | | | | NA | | | | | WA | | | | Wickepin | | | | 32º 37´ 117º 23´ | | | | | 33 (±5)\* | | | | | 13 (±3) | |
| 12. | *A. saligna* | Native | | | 20858 | | | | NA | | | | | WA | | | | Mordalup | | | | 34º 18´ 116º 44´ | | | | |  | | | | |  | |
| 13. | *A. saligna* | Native | | | 13685 | | | | NA | | | | | WA | | | | Yonderup | | | | 32º 34´ 115º 47´ | | | | |  | | | | |  | |
| 14. | *A. saligna* | Native | | | 14975 | | | | 23304 | | | | | WA | | | | Toodyay | | | | 31º 33´ 116º 27´ | | | | |  | | | | |  | |
| 15. | *A. saligna* | Native | | | 12950 | | | | 7590 | | | | | WA | | | | Perth | | | | 31º 51´ 115º 45´ | | | | |  | | | | |  | |
| 16. | *A. saligna* | Non-native | | | 5620 | | | | NA | | | | | NSW | | | | Tailem Bend | | | | 35º 16´ 139º 27´ | | | | | 19 (±4)\* | | | | | 10 (±2) | |
|  | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Table 1**. Details of hosts and obtained sequence reads for amplified *nifH* gene from native and non-native soils (n=49) collected across Australia and from nodules (n=29) harvested after a glasshouse experiment using field collected soil inoculum for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) at the genus level is also presented for each host plant species for native and non-native ranges (mean (±SE)). Asterisks indicate significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **No.** | **Host species, populations** | **Range** | | | **Obtained sequence reads from soils** | | | | | | | **Obtained sequence reads from nodules** | | | | | | | **State** | | **Location** | | | | **Latitude, longitude** | | | **Soils** | | | **Nodules** | | |
| 17. | *A. saligna* | Non-native | | | 1751 | | | | | | | 1162 | | | | | | | VIC | | Portland-Nelson Rd | | | | 38º 7´ 141º 14´ | | |  | | |  | | |
| 18. | *A. saligna* | Non-native | | | 7643 | | | | | | | 24660 | | | | | | | VIC | | Surf coast hwy | | | | 38º 16´ 144º 19´ | | |  | | |  | | |
| 19. | *A. saligna* | Non-native | | | 5284 | | | | | | | 23890 | | | | | | | SA | | Mornington peninsula | | | | 38º 13´ 145º 5´ | | |  | | |  | | |
| 20. | *A. saligna* | Non-native | | | 3120 | | | | | | | NA | | | | | | | SA | | Bega | | | | 36º 39´ 149º 49´ | | |  | | |  | | |
| 21. | *P. lophantha* | Native | | | 6664 | | | | | | | 3918 | | | | | | | WA | | Mt. Frankland NP | | | | 34º 4´ 116º 3´ | | | 32 (±6) | | | 10 (±1) | | |
| 22. | *P. lophantha* | Native | | | 6417 | | | | | | | NA | | | | | | | WA | | Pemberton | | | | 34º 24´ 116º 5´ | | |  | | |  | | |
| 23. | *P. lophantha* | Native | | | 5654 | | | | | | | 4194 | | | | | | | WA | | Gingilup Swamps NR | | | | 34º 18´ 115º 24´ | | |  | | |  | | |
| 24. | *P. lophantha* | Native | | | 17530 | | | | | | | NA | | | | | | | WA | | Serpentine NP | | | | 32º 22´ 116º 0´ | | |  | | |  | | |
|  | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Table 1**. Details of hosts and obtained sequence reads for amplified *nifH* gene from native and non-native soils (n=49) collected across Australia and from nodules (n=29) harvested after a glasshouse experiment using field collected soil inoculum for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) at the genus level is also presented for each host plant species for native and non-native ranges (mean (±SE)). Asterisks indicate significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|  |  | | |  | | | |  | | | | |  | | | |  | | | |  | |  | | | | |  | | | |  | |
| **No.** | **Host species, populations** | | | **Range** | | | | **Obtained sequence reads from soils** | | | | | **Obtained sequence reads from nodules** | | | | **State** | | | | **Location** | | **Latitude, longitude** | | | | | **Soils** | | | | **Nodules** | |
| 25. | *P. lophantha* | | | Native | | | | 12694 | | | | | NA | | | | WA | | | | Armadale | | 32º 9´ 116º 7´ | | | | |  | | | |  | |
| 26. | *P. lophantha* | | | Non-native | | | | 3196 | | | | | 6361 | | | | VIC | | | | Port Fairy | | 38º 23´ 142º 12´ | | | | | 23 (±4) | | | | 11 (±2) | |
| 27. | *P. lophantha* | | | Non-native | | | | 2494 | | | | | NA | | | | VIC | | | | Surf coast hwy | | 38º 18´ 144º 19´ | | | | |  | | | |  | |
| 28. | *P. lophantha* | | | Non-native | | | | 1477 | | | | | 2639 | | | | VIC | | | | Toora | | 38º 38´ 146º 17´ | | | | |  | | | |  | |
| 29. | *P. lophantha* | | | Non-native | | | | 4311 | | | | | 2884 | | | | NSW | | | | Eden | | 37º 04´ 149º 54´ | | | | |  | | | |  | |
| 30. | *P. lophantha* | | | Non-native | | | | 7825 | | | | | NA | | | | NSW | | | | Scarborough | | 34º 15´ 150º 57´ | | | | |  | | | |  | |
| 31. | *A. longifolia* | | | Native | | | | 2250 | | | | | 4211 | | | | VIC | | | | Portland-Nelson Rd | | 38º 11´ 141º 20´ | | | | | 18 (±5) | | | | 11 (±1) | |
| 32. | *A. longifolia* | | | Native | | | | 42735 | | | | | 3078 | | | | VIC | | | | Cape Otway | | 38º 51´ 143º 30´ | | | | |  | | | |  | |
| **Table 1**. Details of hosts and obtained sequence reads for amplified *nifH* gene from native and non-native soils (n=49) collected across Australia and from nodules (n=29) harvested after a glasshouse experiment using field collected soil inoculum for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) at the genus level is also presented for each host plant species for native and non-native ranges (mean (±SE)). Asterisks indicate significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|  | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **No.** | **Host species, populations** | **Range** | | | | | **Obtained sequence reads from soils** | | | | **Obtained sequence reads from nodules** | | | | | **State** | | | | | **Location** | | | **Latitude, longitude** | | | | | **Soils** | | | **Nodules** | |
| 33. | *A. longifolia* | Native | | | | | 15169 | | | | 2626 | | | | | VIC | | | | | Wilsons Promontory | | | 38º 56´ 146º 16´ | | | | |  | | |  | |
| 34. | *A. longifolia* | Native | | | | | 3258 | | | | NA | | | | | NSW | | | | | Croajingalong NP | | | 37º 29´ 149º 35´ | | | | |  | | |  | |
| 35. | *A. longifolia* | Native | | | | | 5991 | | | | NA | | | | | NSW | | | | | Lake Tabourie | | | 38º 51´ 150º 9´ | | | | |  | | |  | |
| 36. | *A. longifolia* | Non-native | | | | | 5821 | | | | NA | | | | | WA | | | | | Lake Powell NR | | | 35º 1´ 117º 45´ | | | | | 19 (±4) | | | 8 (±1) | |
| 37. | *A. longifolia* | Non-native | | | | | 12113 | | | | 4231 | | | | | WA | | | | | Mt Barker | | | 34º 39´ 117º 33´ | | | | |  | | |  | |
| 38. | *A. longifolia* | Non-native | | | | | 25388 | | | | 6319 | | | | | WA | | | | | Gracetown | | | 33º 51´ 115º 01´ | | | | |  | | |  | |
| 39. | *A. longifolia* | Non-native | | | | | 16953 | | | | 6161 | | | | | WA | | | | | Watkins Road NR | | | 32º 18´ 116º 0´ | | | | |  | | |  | |
| 40. | *A. longifolia* | Non-native | | | | | 18867 | | | | 5466 | | | | | WA | | | | | Gidgegannup | | | 31º 47´ 116º 11´ | | | | |  | | |  | |
|  |  |  | | | | |  | | | |  | | | | |  | | | | |  | | |  | | | | |  | | |  | |
| **Table 1**. Details of hosts and obtained sequence reads for amplified *nifH* gene from native and non-native soils (n=49) collected across Australia and from nodules (n=29) harvested after a glasshouse experiment using field collected soil inoculum for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) at the genus level is also presented for each host plant species for native and non-native ranges (mean (±SE)). Asterisks indicate significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|  | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **No.** | **Host species, populations** | | **Range** | | | **Obtained sequence reads from soils** | | | | **Obtained sequence reads from nodules** | | | | | **State** | | | | | **Location** | | **Latitude, longitude** | | | | | **Soils** | | | | | **Nodules** | |
| 41. | *A. melanoxylon* | | Native | | | 7644 | | | | NA | | | | | SA | | | | | Mt Lofty Summit | | 34º 58´ 138º 42´ | | | | | 29 (±8) | | | | | 11 (±0.3) | |
| 42. | *A. melanoxylon* | | Native | | | 73689 | | | | 9153 | | | | | VIC | | | | | Port Fairy | | 38º 17´ 142º 1´ | | | | |  | | | | |  | |
| 43. | *A. melanoxylon* | | Native | | | 5330 | | | | 8753 | | | | | VIC | | | | | Apollo Bay | | 38º 45´ 143º 39´ | | | | |  | | | | |  | |
| 44. | *A. melanoxylon* | | Native | | | 6351 | | | | 6850 | | | | | VIC | | | | | Toora | | 38º 4´ 146º 23´ | | | | |  | | | | |  | |
| 45. | *A. melanoxylon* | | Native | | | 1754 | | | | NA | | | | | NSW | | | | | South East Forest NP | | 37º 8´ 149º 28´ | | | | |  | | | | |  | |
| 46. | *A. melanoxylon* | | Non-native | | | 6797 | | | | 11780 | | | | | WA | | | | | Albany | | 35º 1´ 117º 53´ | | | | | 20 (±2) | | | | | 12 (±2) | |
| 47. | *A. melanoxylon* | | Non-native | | | 2339 | | | | 2305 | | | | | WA | | | | | Elleker | | 35º 00´ 117º 43´ | | | | |  | | | | |  | |
| 48. | *A.melanoxylon* | | Non-native | | | 3576 | | | | NA | | | | | WA | | | | | Quinninup | | 34º 25´ 116º 15´ | | | | |  | | | | |  | |
| 49. | *A. melanoxylon* | | Non-native | | | 2755 | | | | NA | | | | | WA | | | | | Perth | | 31º 51´ 115º 45´ | | | | |  | | | | |  | |

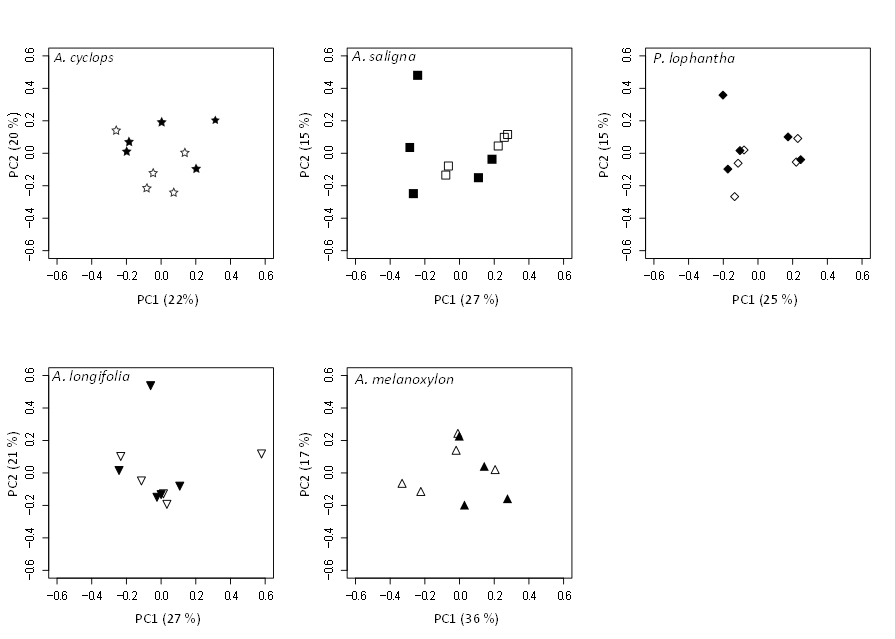
**Table 2**. Summary of PERMANOVA results for variation in nitrogen fixing bacterial communities in soils and nodules for four *Acacia* species and *Paraserianthes lophantha* across their non-native and native ranges within Australia.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Model | df | SS | MS | *F* | *P* |
| **Nitrogen fixing bacterial**  **communities found in soils** | | | | | |
| Species | 1 | 0.3855 | 0.3855 | 3.1212 | **0.0022** |
| Range | 1 | 0.2749 | 0.2749 | 2.2258 | **0.0188** |
| Location | 1 | 0.1509 | 0.1509 | 1.2222 | 0.2587 |
| Species x range | 1 | 0.0882 | 0.0882 | 0.7144 | 0.7257 |
| Species x location | 1 | 0.0581 | 0.0581 | 0.4706 | 0.9200 |
| Range x location | 1 | 0.3232 | 0.3232 | 2.6174 | **0.0065** |
| Species x range x location | 1 | 0.0973 | 0.0973 | 0.7876 | 0.6426 |
| Residuals | 41 | 5.0633 | 0.1235 |  |  |
| Total | 48 | 6.4415 |  |  |  |
|  |  |  |  |  |  |
| **Nitrogen fixing bacterial**  **communities found in nodules** | | | | | |
| Species | 1 | 0.2431 | 0.2431 | 6.0355 | **0.0012** |
| Range | 1 | 0.0854 | 0.0854 | 2.1219 | 0.0971 |
| Location | 1 | 0.0745 | 0.0745 | 1.8498 | 0.1348 |
| Species x range | 1 | 0.0369 | 0.0369 | 0.9174 | 0.4568 |
| Species x location | 1 | 0.0497 | 0.0497 | 1.2346 | 0.3052 |
| Range x location | 1 | 0.0203 | 0.0203 | 0.5063 | 0.7218 |
| Species x range x location | 1 | 0.0398 | 0.0398 | 0.9890 |  |
| Residuals | 21 | 0.8458 | 0.0402 |  |  |
| Total | 29 | 1.3958 |  |  |  |

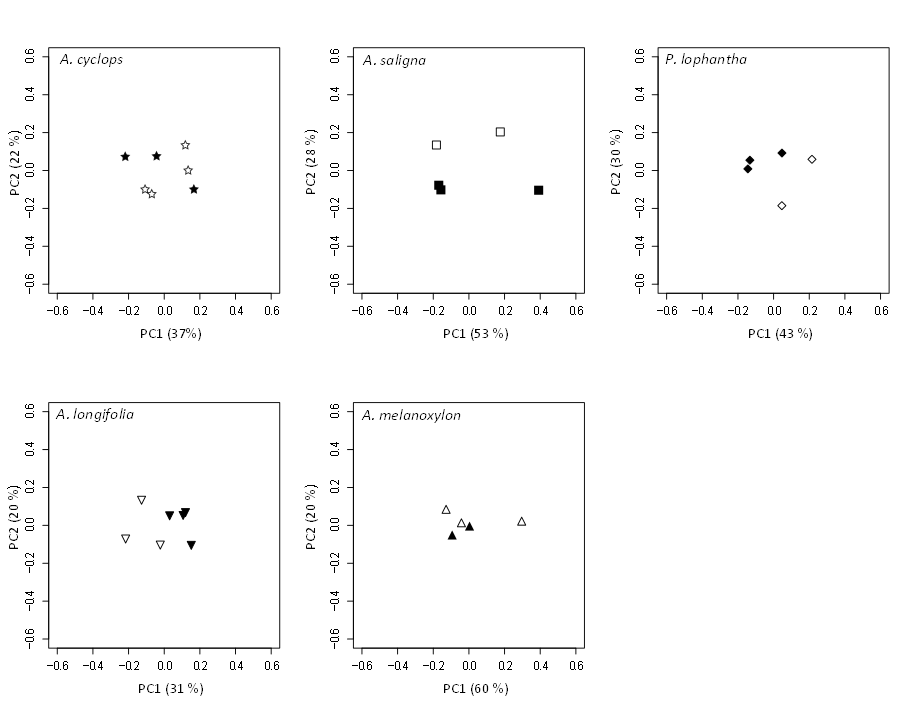
**Table 3.** Summary of PERMANOVA results for species’ comparisons in nitrogen fixing bacterial communities in soils and nodules for four *Acacia* species and *Paraserianthes lophantha* across their non-native and native ranges within Australia.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Model | df | SS | MS | *F* | *P* |
|  |  |  |  |  |  |
| **Differences in soil nitrogen fixing bacterial communities between five legumes** |  |  |  |  |  |
|  |  |  |  |  |  |
| *A. cyclops* x *P. lophantha* | 1,19 | 0.4324 | 0.4324 | 3.949 | **<0.0001** |
| *A. cyclops* x *A. saligna* | 1,19 | 0.3184 | 0.3184 | 2.6982 | **0.0075** |
| *A. cyclops* x *A. longifolia* | 1,19 | 0.3205 | 0.3205 | 2.5874 | **0.0041** |
| *A. cyclops* x *A. melanoxylon* | 1,18 | 0.4081 | 0.4081 | 3.7754 | **<0.0001** |
| *A. saligna* x *P. lophantha* | 1,19 | 0.1583 | 0.1583 | 1.2405 | 0.254 |
| *A. saligna* x *A. longifolia* | 1,19 | 0.1859 | 0.1859 | 1.3039 | 0.211 |
| *A. saligna* x *A. melanoxylon* | 1,18 | 0.1682 | 0.1682 | 1.3221 | 0.209 |
| *P.* *lophantha* x *A. melanoxylon* | 1,18 | 0.0700 | 0.0700 | 0.5921 | 0.856 |
| *P. lophantha* x *A. longifolia* | 1,19 | 0.2292 | 0.2292 | 1.7176 | 0.073 |
| *A. longifolia* x *A. melanoxylon* | 1,18 | 0.1391 | 0.1391 | 1.0424 | 0.408 |
|  |  |  |  |  |  |
| **Differences in nitrogen fixing bacterial communities in nodules between five legumes** |  |  |  |  |  |
|  |  |  |  |  |  |
| *A. cyclops* x *P. lophantha* | 1,10 | 0.0560 | 0.0560 | 1.374 | 0.261 |
| *A. cyclops* x *A. saligna* | 1,10 | 0.0455 | 0.0455 | 0.7114 | 0.569 |
| *A. cyclops* x *A. longifolia* | 1,13 | 0.1189 | 0.1189 | 3.2411 | **0.0138** |
| *A. cyclops* x *A. melanoxylon* | 1,10 | 0.2366 | 0.2366 | 6.196 | **0.0121** |
| *A. saligna* x *P. lophantha* | 1,9 | 0.0045 | 0.0045 | 0.0714 | 0.975 |
| *A. saligna* x *A. longifolia* | 1,11 | 0.0152 | 0.0152 | 0.2771 | 0.911 |
| *A. saligna* x *A. melanoxylon* | 1,9 | 0.0529 | 0.0529 | 0.8741 | 0.493 |
| *P.* *lophantha* x *A. melanoxylon* | 1,9 | 0.0694 | 0.0694 | 2.0178 | 0.156 |
| *P. lophantha* x *A. longifolia* | 1,11 | 0.0115 | 0.0115 | 0.3377 | 0.804 |
| *A. longifolia* x *A. melanoxylon* | 1,11 | 0.0805 | 0.0805 | 2.5321 | 0.087 |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Table 4**. Bacterial species contributing up to 50% of the average Bray-Curtis dissimilarity (using SIMPER analysis) in soils and nodules for four *Acacia* species and *Paraserianthes lophantha* between eastern and western populations. Bold indicates bacteria that are classified as rhizobia and are most commonly reported in legume nodules based on the existing literature. | | | | |
| In soils | | | | |
| No. | Genus | Class | Average abundance | Cumulative percentage |
| 1. | *Sulfitobacter* | Alphaproteobacteria | 0.2 | 2.8 |
| 2. | *Methanocaldococcus* | Methanococci | 0.3 | 5.1 |
| 3. | unclassified | Alphaproteobacteria | 0.6 | 7.5 |
| 4. | unclassified | Opitutae | 0.4 | 9.8 |
| 5. | *Allochromatium* | Gammaproteobacteria | 0.6 | 12.2 |
| 6. | *Alcaligenes* | Betaproteobacteria | 0.6 | 14.5 |
| 7. | *Azospirillum* | Alphaproteobacteria | 0.6 | 16.7 |
| 8. | *Methylocystis* | Alphaproteobacteria | 0.4 | 18.9 |
| 9. | ***Bradyrhizobium*** | Alphaproteobacteria | 0.6 | 21.0 |
| 10. | *Myxococcaceae* | Deltaproteobacteria | 0.4 | 23.2 |
| 11. | *Polaromonas* | Betaproteobacteria | 0.3 | 25.2 |
| 12. | *Novosphingobium* | Alphaproteobacteria | 0.2 | 27.3 |
| 13. | *Geobacter* | Deltaproteobacteria | 0.4 | 29.3 |
| 14. | *Pelobacter* | Deltaproteobacteria | 0.4 | 31.2 |
| 15. | *Pseudacidovorax* | Betaproteobacteria | 0.7 | 33.0 |
| 16. | *Halomonas* | Gammaproteobacteria | 0.7 | 34.8 |
| 17. | *Telmatospirillum* | Alphaproteobacteria | 0.2 | 36.5 |
| 18. | *Desulfatibacillum* | Deltaproteobacteria | 0.2 | 38.2 |
| 19. | *Magnetospirillum* | Alphaproteobacteria | 0.2 | 39.9 |
| 20. | ***Ensifer*** | Alphaproteobacteria | 0.2 | 41.5 |
| 21. | *Desulfuromonas* | Deltaproteobacteria | 0.2 | 43.1 |
| 22. | *Azohydromonas* | Betaproteobacteria | 0.2 | 44.6 |
| 23. | ***Azorhizobium*** | Alphaproteobacteria | 0.2 | 46.0 |
| 24. | *Zymomonas* | Alphaproteobacteria | 0.3 | 47.5 |
| 25. | environmental samples | Bacillales | 0.2 | 48.9 |
| 26. | *Rhodopseudomonas* | Alphaproteobacteria | 0.2 | 50.3 |
|  | | | | |
| In nodules | | | | |
| 1. | *Amorphomonas* | Alphaproteobacteria | 0.8 | 10.8 |
| 2. | *Alcaligenes* | Betaproteobacteria | 0.7 | 20.5 |
| 3. | *Rhodoblastus* | Alphaproteobacteria | 0.5 | 28.8 |
| 4. | *Novosphingobium* | Alphaproteobacteria | 0.6 | 36.9 |
| 5. | *Rubrivivax* | Betaproteobacteria | 0.5 | 44.9 |
| 6. | ***Ensifer*** | Alphaproteobacteria | 0.3 | 52.1 |



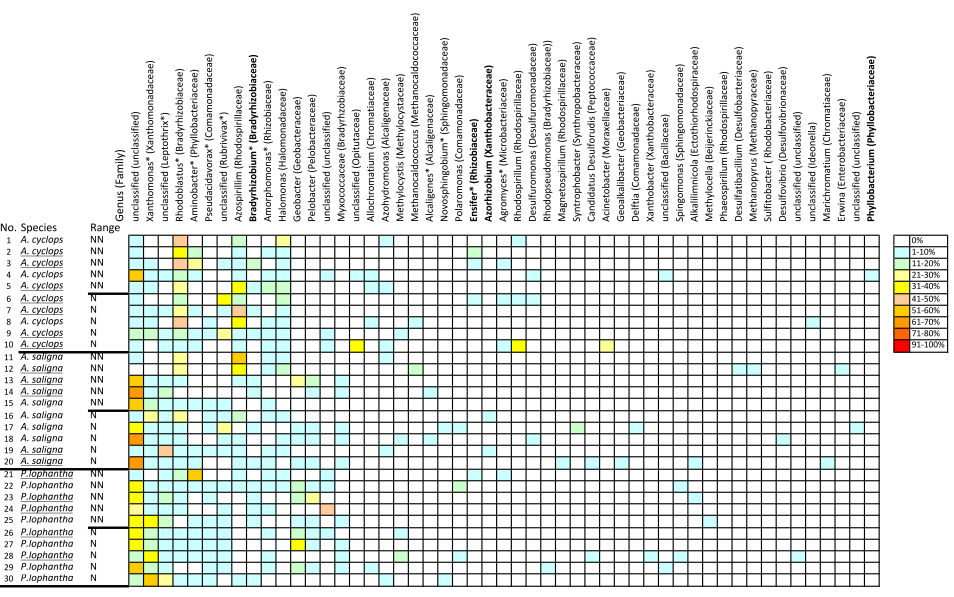
**Figure 1a.** PCA plots for soil nitrogen fixing bacterial communities based on extracted DNA from soils associated with four *Acacia* species and *Paraserianthes lophantha*. Open and closed symbols represent native and non-native populations, respectively. – *Acacia cyclops*, ∇ ▼ –*Acacia longifolia*, ∆ ▲ – *Acacia melanoxylon*, □ ■ – *Acacia saligna*, ◊ ♦ – *Paraserianthes lophantha*.



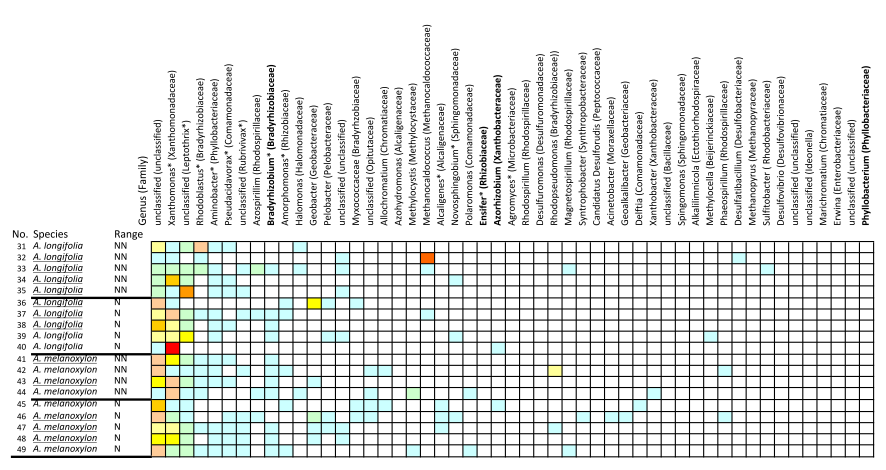
**Figure 1b.** PCA plots for nodule nitrogen fixing bacterial communities based on extracted DNA from nodules associated with four *Acacia* species and *Paraserianthes lophantha*. Open and closed symbols represent native and non-native populations, respectively. – *Acacia cyclops*, ∇ ▼ –*Acacia longifolia*, ∆ ▲ – *Acacia melanoxylon*, □ ■ – *Acacia saligna*, ◊ ♦ – *Paraserianthes lophantha*.



a. **INSERT PDF** WESTERN SPECIES



**INSERT PDF**



b. **INSERT PDF**

Heatmap with relative abundance data for nitrogen fixing bacterial communities in nodules at the genus level.

**Discussion**

The overall aim of this study was to describe and compare the nitrogen fixing bacterial communities in native and non-native ranges from both the rhizosphere and nodules of four *Acacia* spp. and a sister taxon *P. lophantha*. Our original prediction was that the nitrogen fixing bacterial communities in nodules would be more similar across native and non-native ranges for specialist hosts, and less similar for generalist hosts. This expectation was based on the idea that specialist hosts would tend to associate with specific subsets of symbionts (Bever *et al.*, 1997; Grayston *et al.*, 1998; Bever, 2003). For example, a comprehensive study of *Acacia salicina* and *A. stenophylla* across their geographic ranges found that the more generalist *A. salicina* harboured greater rhizobial diversity than the more specialist *A. stenophylla* (Hoque *et al.*, 2011). Consistent with this, Thrall *et al*. (2007) showed that, while *A. salicina* grew equally well in *A. stenophylla* soils as it did in its own, *A. stenophylla* performed best in its own soils. These results further support the idea that generalists may be more successful invaders.

**Nitrogen fixing bacterial composition and diversity in soils**

At the genus level, we found a strong interaction between range and location. This suggests that differences in nitrogen fixing bacterial communities in the rhizosphere between ranges are driven by broad scale differences in nitrogen fixing bacterial communities between eastern and western Australia. Furthermore, these results also indicate that these legumes accumulate different nitrogen fixing bacterial communities in their rhzisospheres across the continent.

Eastern and western populations of these legumes in Australia are divided by the vast Nullarbor Desert which acts as a natural barrier to dispersal of soil micro-organisms across the continent. Thus it is plausible that the geographic isolation and evolutionary divergence may have contributed to the differences in soil bacterial communities between the eastern and western populations (Rout & Callaway, 2012). This is also consistent with our results for the fungal communities found in the rhzisosphere of these legumes across the continent which were also divergent between the eastern and western populations of these legumes (Chapter 5).

Alternatively, it is possible, for instance, that the observed differences in free-living soil nitrogen fixing bacterial communities across east and west in this study may be influenced by differences in soil type and chemistry (Ettema & Wardle, 2002a). This seems unlikely, however, since in an earlier study we did not find substantial differences in soil chemistry between sites of eastern and western populations of the five host plant species (Birnbaum *et al.*, 2012).

It is widely acknowledged that micro-organisms are not randomly distributed in soil, but display spatially predictive and aggregated patterns that are influenced by abiotic and biotic factors (Ettema & Wardle, 2002a), including life-history (Bissett *et al*. 2010) and vegetation (Elgersma & Ehrenfeld, 2011b). For example, Parker (1999) suggested that spatial distribution of bacterial genotypes in legume-rhizobium symbioses follows predictive patterns that are non-random and host dependent. For instance, there are several reports on the spatial distribution of rhizobial communities that follow closely the host plant population distribution patterns across short (i.e., <60 m apart) and long distances (i.e., 1000 km apart) (Spoerke *et al.*, 1996; Parker & Spoerke, 1998). Thus it would be plausible to expect, despite the geographic variation that drives the differences in soil bacterial communities in this study, that host species will also have an effect on the bacterial composition.

Indeed, we found that host species identity had a strong effect on bacterial composition in the rhizosphere. However this effect was driven by the differences of the rhizosphere nitrogen fixing bacterial communities of *A. cyclops* being different from the nitrogen fixing bacterial communities of the other four legume taxa. The diversity estimates were consistent for the two western native species only, *A. cyclops* and *A. saligna*, which both had higher nitrogen fixing bacterial species richness in their rhizospheres in native range populations. *Acacia cyclops* and *A. saligna* had on average almost twice as many bacterial taxa in native range rhizosphere communities than were observed in non-native range populations. Unfortunately, relatively little is known about the free-living nitrogen fixing bacterial communities in the rhizosphere of legumes since the majority of the work so far has targeted and described predominantly the

nodulating taxa. Thus, this study is one of the few ones to describe the free-living nitrogen fixing bacterial community in the rhizosphere of these legumes.

**Nitrogen fixing bacterial composition and diversity in nodules**

We found that, surprisingly, the nitrogen fixing bacterial communities in the nodules at the genus level were not significantly different across the ranges or locations. These results suggest that overall these legumes may be specialists that associate with similar nitrogen fixing bacterial communities across the continent.

Our results are consistent with the findings of several authors that have previously reported that some invasive legumes, including acacias, associate preferentially with the rhizobial communities from their native range that might have been accidentally or intentionally co-introduced with the host plants (Weir *et al.*, 2004; Chen *et al.*, 2005; Rodríguez-Echeverria, 2010; Porter *et al.*, 2011). For example, Weir *et al*. (2004) reported that *A. longifolia* and *A. dealbata* associated in their non-native range in New Zealand with *Bradyrhizobium*, which has been reported to be ubiquitous and predominant in the nodules of legumes in Australia (Barnet *et al.*, 1985). Similar results have been reported for *A. longifolia* in its non-native range in Portugal as well (Rodríguez-Echeverria, 2010). However, for *Bradyrhizobium* in New Zealand it is unknown whether bacterial members of this genus may occur naturally without being involved in symbiotic association with native New Zealand legumes and thus may have not been previously detected. This is plausible, since the strains isolated from New Zealand native legumes’ *Carmichaelia*, *Clianthus*, *Montigena* and *Sophora* nodulescontained predominantly members of *Mesorhziobium* (Weir et al., 2004). Overall, these results and our findings suggest that invasive legumes associate preferentially with the rhizobial communities that are compositionally very similar to the rhizobia from their native range.

Although *Bradyrhizbium* appears to be the dominant taxon found in the nodules of invasive legumes across non-native and native ranges across several countries, recent reports suggest that rhizobial diversity is higher in nodules than previously described. For example, new findings

suggest that legume nodules contain in addition to *Bradyrhizbium* many more taxa e.g. *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Burkholderia*, *Phyllobacterium* and *Devosia* as well as some non-nodulating bacterial endophytes (Muresu *et al.*, 2008; Hoque *et al.*, 2011). Our results support this, as in the present study we also found that bacterial communities in the nodules contained previously less detected nodulating (e.g. *Ensifer*) and non-nodulating (e.g. *Xanthomonas*, *Pseudovorax*, *Rhodoblastus*, *Amorphomonas*, *Novosphingobium*, *Alcaligenaceae* and *Agroyces*) bacterial taxa.

Overall, the diversity of nitrogen fixing bacterial communities in nodules varied across all species. For example, *A. longifolia* had more diverse bacterial communities in its native range nodules, whereas *A. cyclops* had, on the contrary, more diverse bacterial communities in its non-native range nodules. *Xanthomonas*, a genus containing many plant pathogens (Hayward *et al.*, 1993), was found in higher abundance in non-native range nodules of *A. longifolia*, whereas *Bradyrhizobium* was found in slightly higher abundances in its native range populations. Curiously, in a previous study we found that *A. longifolia* grew significantly better in soils from the non-native range than in native soils (Birnbaum *et al.*, 2012). This at least raises the possibility that higher abundances of potentially pathogenic microbes (e.g. *Xanthomonas*) in the non-native range does not constrain the performance of *A. longifolia* and that other biotic and abiotic factors could be more significant in determining the invasion success of this species.

Surprisingly, for *A. cyclops* we found that while nitrogen fixing rhizosphere bacterial communities were more diverse in native range populations, the opposite pattern was observed for nodules where more diverse bacterial communities were found in the non-native range. This indicates that *A. cyclops* may be a generalist host, or it may be experiencing enhanced mutualisms (Chapter 3, Richardson *et al*. 2000) as has been previously suggested for this species

(Birnbaum *et al.*, 2012). Another study of the rhizobial composition of bacteria associating with *A. cyclops* in its non-native range in Libya also found that this host was non-specifically associating with both fast- and slow-growing rhizobia, *Rhizobium* and *Bradyrhizobium*, respectively (Mohamed *et al.*, 2000). Additionally, curiously, we found that the bacterial communities of *A. cyclops’* nodules were different from the bacterial communities in the nodules of the two eastern natives’ *A. longifolia* and *A. melanoxylon*.

**Synthesis of results for soil and nodule bacterial communities associating with legumes**

We found that soil nitrogen fixing bacterial communities across the ranges were significantly different in the rhizosphere of these legumes, plausibly due to differences in geographic location of populations, and thus would expect to find these differences reflected in the nodules as well. This is based on the assumption that plants are more likely to associate through chemical signalling with rhizobia that are found in the vicinity of their roots (Bauer, 1981; Redmond *et al.*, 1986). Additionally, Mantel test showed a significant correlation between the nodule and soil matrices suggesting that these legumes are highly likely to associate with bacteria that are found in their rhizosphere. However, contrary to our expectation, we found that the nitrogen fixing bacterial communities were significantly different across the continents in the rhizospheres of these legumes, but not within the nodules. Thus, there appears to be a certain discrepancy between the results of Mantel test and individual model results for soil and nodule bacterial communities.

There are two plausible explanations for this observation. Firstly, it is likely that the differences in the nitrogen fixing bacterial communities in the rhizosphere were driven by the high number and diversity of putative non-nodulating free-living bacterial communities. Indeed, we found that out of 26 bacterial species that contributed most to the dissimilarity across the continent, 23 were free-living nitrogen fixing bacteria. Secondly, it is plausible that although overall nitrogen fixing bacterial communities in soils are different across the continent there is an abundance of common free-living (i.e. non-nodulating) and rhizobial (i.e. nodulating) taxa that occur on both sides of the continent (Fig. 3).

For example, we found that at the genus level several of the most abundant free-living and nodulating bacterial taxa found in the nodules were indeed the most abundant taxa present in the soils as well (e.g., *Aminobacter*, *Xanthomonas*, *Rhodoblastus and Ensifer).* This suggests that

these hosts form symbiotic associations with similar nitrogen fixing bacterial communities in both native and novel environments that are common across the continent. Interestingly, in a previous study *A. cyclops*, *A. melanoxylon*, *A. saligna* and *P. lophantha* showed no differences in biomass when grown under glasshouse conditions using both non-native and native range soils as inoculum (Chapter 2 & 3). This suggests that they are not affected by these differences in nitrogen fixing bacterial communities across the continent because they are able to attract the same “common” bacteria from both ranges.

**Conclusions**

Overall, our results suggest that, at least at the genus level, nitrogen fixing bacterial communities differ across the Australian continent in the rhizospheres of these legumes, but overall are similar in the nodules. Geographic location (east vs. west) of populations appears to largely drive the observed differences in free-living nitrogen fixing bacterial communities across the ranges for these legumes.

Generally, our findings suggest that invasive legumes associate preferentially with the rhizobial communities that are compositionally very similar to the rhizobia from their native range. Availability and abundance of suitable rhizobia across the continent suggests that these legumes are not constrained by the absence of compatible symbionts in the novel range.

It is important to note that the results here were analysed and presented at the genus level only due to restrictions of the reference taxonomy. Thus our results might be somewhat different with higher taxonomic resolution, as it has been previously documented that soil bacterial communities become less similar at higher resolution (Ramette & Tiedje, 2007; Bissett *et al.*, 2010).

**Acknowledgements**

We would like to thank Carla Harris and Paweł Waryszak for extensive help in the field. We acknowledge Luke Barrett for helpful discussions on the statistical analysis. This work was supported by Macquarie University Research Excellence Scholarship to CB and an Australian Research Council Discovery grant (DP0879494) to ML.

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**Chapter 5: Geographic variation in rhizosphere fungal communities of invasive legumes in non-native and native ranges in Australia**

This manuscript has been prepared for submission to *Soil Biology and Biochemistry* as ‘Birnbaum, C., Bissett, A., Thrall, P.H. and Leishman M.R. ‘Geographic variation in rhizosphere fungal communities of invasive legumes in non-native and native ranges in Australia.’

My contribution to the research and paper: Concept - 90%; Data collection - 70%; Statistical analysis - 90%; Bioinformatic analysis - 50 %; Writing - 80%

**ABSTRACT**

Although acacias are one of the most successful invaders globally, relatively little is known about the role of soil microbial communities, particularly fungal communities including a range of both mutualistic and pathogenic taxa, in their invasion success. The aim of this study was to assess variation in the soil fungal communities in the rhizospheres of four invasive acacias (*Acacia cyclops*, *A. longifolia*, *A. melanoxylon* and *A. saligna*) and a close relative *Paraserianthes lophantha* across their non-native and native ranges in Australia and to determine whether geographic location of host populations contributes to differences in these fungal communities. To characterise soil fungal communities, we collected soil samples from the rhizosphere of each legume species from multiple populations across both their non-native and native ranges within Australia. Soil fungal communities were then amplified from soil DNA using universal fungal primers ITS1-F and ITS4 and sequenced with 454 pyrosequencing. Sequences were clustered at 97% sequence similarity using USEARCH. Multivariate analyses were then performed on fungal community composition to assess variation in fungal communities across all species and between species’ non-native and native populations separately. Additionally, pairwise comparisons were made between host species. Based on relative abundance estimates the fifty most abundant fungal groups were Ascomycota (20 species), Basidiomycota (6 spp.),

Glomeromycota (1 sp.), Zygomycota (3 spp.) and 20 fungal species that could not be classified, indicating a large number of novel fungal taxa. We found significant differences for all plant species in fungal community composition between non-native and native range populations. However, we also found a strong location (i.e. east *vs* west) by range interaction for fungal community composition. This suggests that differences between native and non-native ranges are at least partly driven by fundamental variation in soil fungal communities across the continent. Soil fungal communities in the rhizosphere of these legume species appear to be distinctly different between the east and west of the Australian continent. At the same time, these legume species introduced into novel ranges across the continent, whether east or west, are able to successfully naturalise and spread, indicating that encounters with novel soil fungal communities in non-native ranges does not constrain invasion success, at least within Australia.

**Keywords** biological invasion,ITS, soil microbes, legumes, novel ranges, Fabaceae

**1. Introduction**

Soil biota consists of complex and diverse organisms that can have positive, neutral or negative effects on plant performance and ecosystem function. The effect of soil biota on plants may be positive if the plant accumulates beneficial soil communities such as nitrogen-fixing bacteria and mycorrhizal fungi (Allen & Allen, 1984; Garbaye, 1994) or negative if the plant accumulates soil-borne pathogens and parasites (Van der Putten, 2001). Better characterisation of soil microbial communities in plant non-native compared to native ranges could help resolve the importance of soil communities with regard to predicting the outcome of plant invasions (Reinhart & Callaway, 2004) and determine the extent to which soil microbial communities are modified by the addition of novel plant species to the system.

Several exotic plant species have been shown to modify soil microbial communities in their novel ranges, resulting in differences in the soil microbial community associated with the exotic compared to the co-occurring native plant species (Kourtev *et al.*, 2002; Batten *et al.*, 2006; Elgersma & Ehrenfeld, 2011b; Rout & Callaway, 2012). For example, Batten *et al*. (2006) compared the soil communities (e.g. bacteria and fungi) found in the rhizospheres of two invasive and five native plant species using phospholipid fatty acid analysis profiles and found that invaders changed the soil microbial composition in the areas they invaded compared to the microbial communities found in the native species’ rhizosphere. For instance, invasive species had higher levels of sulfate reducing and sulfur-oxidizing bacteria in their rhizospheres which suggests that invaders may be altering the sulfur cycle in soils (Batten *et al.*, 2006). However, other authors have suggested that legacy effects from the pre-invasion vegetation community could have a stronger effect on soil microbial composition compared to the short-term effects of a newly invaded plant species (Elgersma *et al.*, 2011a).

It is generally agreed that the vast majority of plants rely on one or more soil-borne mutualist (Rudgers *et al.*, 2005; Brundrett, 2009), thus the absence of suitable mutualistic partners can

potentially represent a significant barrier to the invasion success of a species in its novel range. For example, Nuñez *et al.* (2009) reported that mycorrhizal colonization was found to be lower and fewer fungal species were found on pines (*Pseudotsuga menziesii*, *Pinus contorta* and *Pinus ponderosa*) that were further away from the original forestry plantings on Isla Victoria (Argentina). Additionally, these authors found a positive correlation between the presence of mycorrhizae and seedling survival and biomass (Nuñez *et al.*, 2009). This suggests that mutualists are confined to areas close to the plantations and the establishment as well as invasion success of these host species, if the association is obligate, could be constrained by the absence of these symbionts beyond plantations (Nuñez *et al.*, 2009).

Alternatively, host plants may form facultative associations with symbionts and thus are not significantly affected by the absence of compatible organisms in the new range (Pringle *et al.*, 2009). For example, plant dependence on soil symbionts (e.g. mycorrhizae and rhizobia) may decrease along an environmental gradient (Thrall *et al.*, 2008) such as soil fertility (Smith & Read, 1997; Sprent, 2001). In some instances, host plants may evolve to become less dependent on mutualists in invaded ranges (Seifert *et al.*, 2009), form novel associations with native microorganisms in the new range or co-invade together with their mutualists from the native range (Pringle *et al.*, 2009). For instance, Dickie *et al*. (2010) found that the invasion success of *Pinus contorta* (Pinaceae) in New Zealand could be largely attributed to the presence of co-introduced non-native ectomycorrhizal fungi that associate with *P. contorta*. Similar results were also found for *A. longifolia* in its invaded range in Portugal where it was shown that the rhizobia associating with this host in Portugal were genetically very similar to those found in its native range (Rodríguez-Echeverria, 2010).

Legumes in particular have been reported to rely extensively on mutualisms such as rhizobia and mycorrhizal fungi to colonize novel areas (Sprent & Parsons, 2000; Parker, 2001). Overall,

rhizobial communities that associate with invasive legumes have received significantly more attention than the fungal communities they associate with (Thrall *et al.*, 2000; Lafay & Burdon,

2001; Rodriguez-Echeverria *et al.*, 2007; Barrett *et al.*, 2011; Birnbaum *et al.*, 2012), especially the ectomycorrhizal fungi (Duponnois & Plenchette, 2003), and the role they may play in the success of invasive legumes. In Australia, there is some evidence to suggest that *A. cyclops*, *A. longifolia, A. melanoxylon and* *A. saligna* form association predominantly with AM fungi (Brundrett & Abbott, 1991; Jasper et al., 1989). One author has also reported evidence of EM fungi in *A. melanoxylon* roots (Warcup 1980). No study has, to our best knowledge, so far reported the mycorrhizal status, AM or EM fungi or both, for *P. lophantha* in Australia.

Several studies have documented the positive effect of fungi on some acacia species’ growth and performance (Herrera *et al.*, 1993; Duponnois & Plenchette, 2003; Faye *et al.*, 2009). For example, invasive *A. holosericea* growth was significantly enhanced by several AMF fungal species from the genus *Glomus* (Duponnois & Plenchette, 2003 and references therein). In one of the few attempts to describe the impacts of invasive *Acacia* spp. on soil microbes, Lorenzo *et al*. (2010) studied the effects of invasive *A. dealbata* Link. on soil fungi and bacterial communities in three different ecosystems (pine forest, shrubland and grassland) in Northwest Spain. Interestingly, these authors found that *A. dealbata* had an effect on the overall structure of the soil microbial communities, especially the fungal communities that were different in the *A. dealbata* invaded sites in the shrubland (Lorenzo *et al.*, 2010).

Despite our increasing understanding of the biogeographic patterns of invasive plants and soil microbes (Rout & Callaway, 2012), relatively little is known about how geographic isolation may affect the outcome of plant-microbe interactions in native compared to non-native ranges. For instance, fundamental differences of soil microbial communities across ranges may be caused by physical isolation or evolutionary divergence (Rout & Callaway, 2012). The overall aim of this

study was to determine whether the soil fungal communities for four *Acacia* spp. (*A. cyclops*, *A. longifolia*, *A. melanoxylon* and *A. saligna*) and a close relative *Paraserianthes lophantha*

(hereafter collectively termed legumes) are different in their non-native compared to native range soils in Australia. Furthermore, we asked whether the differences in these fungal communities could be due to geographic isolation of eastern and western populations in Australia due to the vast Nullarbor Desert that separates the native and non-native range populations of these legumes. To our knowledge this study represents one of the few attempts to comprehensively describe the diversity of fungal communities across multiple invasive plant taxa and biogeographic ranges.

**2. Materials and methods**

2.1. Target species

The five legumes were chosen to investigate the differences in soil fungal communities of invasive legumes in their non-native compared to native ranges in Australia. *Acacia cyclops* A.Cunn. ex G. Don, *A. saligna* (Labill.) H.L. Wendl and *P. lophantha* (Willd.) I.C. Nielsen are native to Western Australia, but have been introduced and become invasive or naturalised in the eastern states of Australia (New South Wales, South Australia and Victoria), whereas *A. longifolia* (Andrews) Willd. and *A. melanoxylon* R.Br. are native to eastern Australia, but have been introduced and become invasive in the south-west of Western Australia. There are some reports that suggest that A. cyclops, A. saligna

It is unlikely that any of these species have been transported naturally across the continent as the eastern and western ranges of each species are separated by the vast Nullarbor Desert, providing a major barrier to natural seed dispersal between native and introduced ranges (Jacobs & Wilson, 1996). These five species are also considered invasive in a number of regions outside Australia (Impson *et al.*, 2011; Richardson *et al.*, 2011a). Acacias as invaders have received considerable attention recently and many aspects of their invasion ecology have been

covered by several authors in a recent journal special edition (Gallagher *et al.*, 2011; Gibson *et al.*, 2011; Griffin *et al.*, 2011; Le Maitre *et al.*, 2011; Richardson *et al.*, 2011a).

2.2. Study sites and sampling

To describe the fungal communities associated with the five study species we collected cross-continental soil samples from multiple populations of each legume within both non-native and native ranges. Soil samples were collected in 2009 from south-east (31º 51´ S, 115º 45´ N to 33º 48´ S, 121º 49´ N) and south-west (33º 42´ S, 151º 16´ N to 34º 43´ S, 137º 35´ N) Australia (Table 1). For each species, with the exception of *A. melanoxylon*, for which we only had four populations in its non-native range, we sampled five individuals from each of five populations within each range [5 species x 2 ranges (native and non-native) x 5 populations x 5 individuals]. A total of 1000 g of soil was collected beneath each individual as close to roots as possible at a depth of 10-15 cm. Soils were kept in a cooler in the field before being stored at 4oC. Soil sampling and processing equipment was sterilized with 90% ethanol between populations. Soils were sieved with 2 mm sieves to remove leaves and other coarse material and to homogenise the samples. Subsamples of five individuals per population were bulked and placed in sterilized falcon tubes and stored in the freezer at -20oC until further analysis. A final total of 49 soil subsamples were used for five species spanning across both non-native and native range populations within Australia.

**Table 1**. Details of hosts and obtained sequence reads for amplified ITS region from native and non-native soils (n=49) collected across Australia for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) is also presented for each species for native and non-native ranges (mean (±SE)). With asterisk are marked significant differences between the ranges in OTU numbers (*P* < 0.05).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **No.** | **Host plant species, populations** | **Range** | **Obtained sequence reads from soils** | **Final sequence**  **reads from soils** | **State** | **Location** | **Latitude, longitude** | **No. of OTUs** |
| 1. | *A. cyclops* | Native | 1263 | 1038 | WA | Ravensthorpe | 33º 36´ 120º 12´ | 250 (±14)\* |
| 2. | *A. cyclops* | Native | 1086 | 974 | WA | Bremer Bay | 34º 25´ 119º 22´ |  |
| 3. | *A. cyclops* | Native | 2038 | 1915 | WA | D`Entrecasteaux NP | 34º 51´ 116º 1´ |  |
| 4. | *A. cyclops* | Native | 2135 | 1993 | WA | William Bay | 35º 1´ 117º 14´ |  |
| 5. | *A. cyclops* | Native | 1404 | 1284 | WA | Coogee | 32º 7´ 115º 45´ |  |
| 6. | *A. cyclops* | Non-native | 3072 | 2376 | SA | Yorke peninsula | 34º 3´ 137º 45´ | 339 (±21)\* |
| 7. | *A. cyclops* | Non-native | 5086 | 4150 | SA | Yorke peninsula | 34º 43´ 137º 35´ |  |
| 8. | *A. cyclops* | Non-native | 5354 | 4262 | SA | Yorke peninsula | 35º 3´ 137º 43´ |  |
| 9. | *A. cyclops* | Non-native | 3656 | 3020 | SA | McLaren Vale | 35º 14’ 138º 27´ |  |
| 10. | *A. cyclops* | Non-native | 2908 | 2553 | SA | Victor harbour | 35º 32´ 138º 38´ |  |
| **Table 1**. Details of hosts and obtained sequence reads for amplified ITS region from native and non-native soils (n=49) collected across Australia for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) is also presented for each species for native and non-native ranges (mean (±SE)). With asterisk are marked significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | |
| **No.** | **Host plant species, populations** | **Range** | **Obtained sequence reads from soils** | **Final sequence**  **reads from soils** | **State** | **Location** | **Latitude, longitude** | **No. of OTUs** |
| 11. | *A. saligna* | Native | 5506 | 4859 | WA | Wickepin | 32º 37´ 117º 23´ | 310 (±37) |
| 12. | *A. saligna* | Native | 8029 | 7468 | WA | Mordalup | 34º 18´ 116º 44´ |  |
| 13. | *A. saligna* | Native | 5986 | 5153 | WA | Yonderup | 32º 34´ 115º 47´ |  |
| 14. | *A. saligna* | Native | 1708 | 1443 | WA | Toodyay | 31º 33´ 116º 27´ |  |
| 15. | *A. saligna* | Native | 2344 | 2131 | WA | Perth | 31º 51´ 115º 45´ |  |
| 16. | *A. saligna* | Non-native | 986 | 839 | NSW | Tailem Bend | 35º 16´ 139º 27´ | 362 (±80) |
| 17. | *A. saligna* | Non-native | 5212 | 4733 | VIC | Portland-Nelson Rd | 38º 7´ 141º 14´ |  |
| 18. | *A. saligna* | Non-native | 10154 | 8727 | VIC | Surf coast hwy | 38º 16´ 144º 19´ |  |
| 19. | *A. saligna* | Non-native | 4502 | 3379 | SA | Mornington peninsula | 38º 13´ 145º 5´ |  |
| 20. | *A. saligna* | Non-native | 2314 | 2086 | SA | Bega | 36º 39´ 149º 49´ |  |
| **Table 1**. Details of hosts and obtained sequence reads for amplified ITS region from native and non-native soils (n=49) collected across Australia for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) is also presented for each species for native and non-native ranges (mean (±SE)). With asterisk are marked significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | |
| **No.** | **Host plant species, populations** | **Range** | **Obtained sequence reads from soils** | **Final sequence**  **reads from soils** | **State** | **Location** | **Latitude, longitude** | **No. of OTUs** |
| 21. | *P. lophantha* | Native | 7593 | 6776 | WA | Mt. Frankland NP | 34º 4´ 116º 3´ | 538 (±108) |
| 22. | *P. lophantha* | Native | 1054 | 935 | WA | Pemberton | 34º 24´ 116º 5´ |  |
| 23. | *P. lophantha* | Native | 11048 | 9903 | WA | Gingilup Swamps NR | 34º 18´ 115º 24´ |  |
| 24. | *P. lophantha* | Native | 12521 | 10654 | WA | Serpentine NP | 32º 22´ 116º 0´ |  |
| 25. | *P. lophantha* | Native | 12633 | 11557 | WA | Armadale | 32º 9´ 116º 7´ |  |
| 26. | *P. lophantha* | Non-native | 1896 | 1585 | VIC | Port Fairy | 38º 23´ 142º 12´ | 382 (±58) |
| 27. | *P. lophantha* | Non-native | 4839 | 3775 | VIC | Surf coast hwy | 38º 18´ 144º 19´ |  |
| 28. | *P. lophantha* | Non-native | 5597 | 4351 | VIC | Toora | 38º 38´ 146º 17´ |  |
| 29. | *P. lophantha* | Non-native | 4055 | 3366 | NSW | Eden | 37º 04´ 149º 54´ |  |
| 30. | *P. lophantha* | Non-native | 2000 | 1738 | NSW | Scarborough | 34º 15´ 150º 57´ |  |
| **Table 1**. Details of hosts and obtained sequence reads for amplified ITS region from native and non-native soils (n=49) collected across Australia for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) is also presented for each species for native and non-native ranges (mean (±SE)). With asterisk are marked significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | |
| **No.** | **Host plant species, populations** | **Range** | **Obtained sequence reads from soils** | **Final sequence**  **reads from soils** | **State** | **Location** | **Latitude, longitude** | **No. of OTUs** |
| 31. | *A. longifolia* | Native | 6442 | 4452 | VIC | Portland-Nelson Rd | 38º 11´ 141º 20´ | 271 (±61) |
| 32. | *A. longifolia* | Native | 3582 | 2994 | VIC | Cape Otway | 38º 51´ 143º 30´ |  |
| 33. | *A. longifolia* | Native | 1911 | 1664 | VIC | Wilsons Promontory | 38º 56´ 146º 16´ |  |
| 34. | *A. longifolia* | Native | 2539 | 2303 | NSW | Croajingalong NP | 37º 29´ 149º 35´ |  |
| 35. | *A. longifolia* | Native | 619 | 508 | NSW | Lake Tabourie | 38º 51´ 150º 9´ |  |
| 36. | *A. longifolia* | Non-native | 908 | 837 | WA | Lake Powell NR | 35º 1´ 117º 45´ | 270 (±62) |
| 37. | *A. longifolia* | Non-native | 2248 | 2053 | WA | Mt Barker | 34º 39´ 117º 33´ |  |
| 38. | *A. longifolia* | Non-native | 2456 | 2246 | WA | Gracetown | 33º 51´ 115º 01´ |  |
| 39. | *A. longifolia* | Non-native | 2056 | 1861 | WA | Watkins Road NR | 32º 18´ 116º 0´ |  |
| 40. | *A. longifolia* | Non-native | 3543 | 3218 | WA | Gidgegannup | 31º 47´ 116º 11´ |  |
| **Table 1**. Details of hosts and obtained sequence reads for amplified ITS region from native and non-native soils (n=49) collected across Australia for four *Acacia* species and *Paraserianthes lophantha*. Number of OTUs (richness) is also presented for each species for native and non-native ranges (mean (±SE)). With asterisk are marked significant differences between the ranges in OTU numbers (*P* < 0.05) (cont.). | | | | | | | | |
| **No.** | **Host plant species, populations** | **Range** | **Obtained sequence reads from soils** | **Final sequence**  **reads from soils** | **State** | **Location** | **Latitude, longitude** | **No. of OTUs** |
| 41. | *A. melanoxylon* | Native | 2147 | 1938 | SA | Mt Lofty Summit | 34º 58´ 138º 42´ | 315 (±28) |
| 42. | *A. melanoxylon* | Native | 4610 | 4051 | VIC | Port Fairy | 38º 17´ 142º 1´ |  |
| 43. | *A. melanoxylon* | Native | 3355 | 2794 | VIC | Apollo Bay | 38º 45´ 143º 39´ |  |
| 44. | *A. melanoxylon* | Native | 3054 | 2529 | VIC | Toora | 38º 4´ 146º 23´ |  |
| 45. | *A. melanoxylon* | Native | 2310 | 2009 | NSW | South East Forest NP | 37º 8´ 149º 28´ |  |
| 46. | *A. melanoxylon* | Non-native | 1785 | 1670 | WA | Albany | 35º 1´ 117º 53´ | 275 (±31) |
| 47. | *A. melanoxylon* | Non-native | 3205 | 2809 | WA | Elleker | 35º 00´ 117º 43´ |  |
| 48. | *A. melanoxylon* | Non-native | 3161 | 2783 | WA | Quinninup | 34º 25´ 116º 15´ |  |
| 49. | *A. melanoxylon* | Non-native | 1903 | 1797 | WA | Perth | 31º 51´ 115º 45´ |  |

2.3. Molecular analysis

DNA from 49 soil samples was isolated using a PowerSoil DNA isolation kit following manufacturer’s protocols (MO Bio Laboratories, Inc. Carlsbad, CA). Internal transcribed spacer (ITS) was then amplified from soil DNA using fungal primers: ITS1-F 5’ *CTTGGTCATTTAGAGGAAGTAA* 3’ (Gardes & Bruns, 1993) and ITS4 5’ *TCCTCCGCTTATTGATATGC* 3’(White *et al.*, 1990). We chose ITS as it is the most commonly sequenced region of the nuclear ribosomal repeat unit in mycology (Hibbett *et al.*, 2005; Nilsson *et al.*, 2009). The ITS region is a convenient target region for molecular identification of fungi because it is easily amplified from small or dilute DNA samples (Gardes & Bruns, 1993), for example from environmental samples like soils (Nilsson *et al.*, 2009), and is often highly variable among morphologically distinct fungal species (Gardes *et al.*, 1991). Additionally, the large number of ITS copies per cell (Vilgalys & Gonzalez, 1990) makes this region a good target for pyrosequencing (Nilsson *et al.*, 2009). PCR reactions were performed by the Research and Testing Laboratory (Lubbock, Texas, USA) following the protocol in Lucero *et al.* (2011) and Dowd *et al*. (2008b). DNA was amplified using the HotStarTaq Plus Master Kit (Qiagen, Valencia, CA) with the primer pair ITS1F and ITS4 with the annealing temperature of 55°C (Lucero *et al.*, 2011). Initial PCR products were either directly sequenced or cloned into pCR2.1 cloning vector using the TA Cloning kit® (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s protocol (Lucero *et al.*, 2011).

2.4. Fungal (fTEFAP) tag-encoded FLX amplicon pyrosequencing

Samples were analysed by the Research and Testing Laboratory (Lubbock, Texas, USA) using fungal tag-encoded FLX amplicon pyrosequencing (fTEFAP) to determine the fungal communities present (Lucero *et al.*, 2011) according to the protocol in Dowd *et al*. (2008b). Following Dowd *et al*. (2008b) PCR amplicon products from different samples were mixed in equal volumes, and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). In preparation for FLX sequencing (Roche, Nutley, New Jersey), the DNA fragments’

size and concentration were accurately measured by using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380 Fluorometer (Turner Biosystems, CA, USA). A 9.6 E+06 sample of double-stranded DNA molecules/μl were combined with 9.6 million DNA capture beads, and then amplified by emulsion PCR. After bead recovery and bead enrichment, the bead-attached DNAs were denatured with NaOH, and sequencing primers were annealed. A two-region 454 sequencing run was performed on a 70×75 GS PicoTiterPlate (PTP) by using a Genome Sequencer FLX System (Roche, Nutley, New Jersey). All FLX related procedures were performed following Genome Sequencer FLX System manufacturers instructions (Roche, Nutley, New Jersey) (Dowd *et al.*, 2008b).

2.5. Bioinformatic analyses

Downstream sequence analyses were performed on ITS sequences from soils in mothur version 1.22.0 (Schloss *et al.*, 2009) following the adapted sequence quality-control pipeline analysis described in detail in Schloss *et al.* (2011), until sequence fragments that were part of larger sequences were clustered together into separate groups. Fungal ITS sequences were grouped into OTUs (Operational Taxonomic Units) at 97% sequence similarity using the USEARCH OTU pipeline (Edgar, 2010), using the following parameters: MINSIZE=2; PCTID error=98%, OTU=97%, BIN=97%; ABSKEW=2; chimera check against “self” only. Additionally, the relative abundance of fungal OTUs in soil data was calculated for each host species and population.

2.6. Statistical analyses

Principal coordinates analysis (PCA) based on a Bray-Curtis dissimilarity matrix was carried out on presence/absence transformed data generated from the OTU species matrix for soil fungal communities, which allowed visual inspection to identify differences between non-native and native range populations for each species and for eastern and western populations separately.

PERMANOVA (Anderson, 2001) was then used to test for overall differences between legumes, across all legumes between their native and non-native and between eastern and western populations. A similarity percentage analysis (SIMPER) based on a Bray-Curtis index of dissimilarity was used to determine the dissimilarity in soil fungal communities within eastern and western populations and to identify fungal species that contributed strongly to that dissimilarity.

One-way ANOVA was used to test for differences in the number of OTUs between the non-native and native range for each species separately. All analyses and ordinations were performed in the R programming language (version R2.14.2) using vegan 2.0-3 (Oksanen *et al.*, 2011) package.

**3. Results**

A total of 192526 sequences were obtained (Table 1) for the amplified ITS region from soil DNA (49 samples) with a mean fragment length of 355 bp (max 717 bp, median 368 bp). Following sequence quality control in mothur, the total number of remaining ITSsequencing reads was reduced to 163539 (i.e., 84% of all sequences). Blast search of sequences in the USEARCH OTU pipeline revealed 4448 distinct fungal species groups at 97%. The fifty most abundant fungal species were Ascomycota (20 species), Basidiomycota (6 sp.), Glomeromycota (1 sp.), Zygomycota (3) and 20 fungal species that could not be classified (Table 2).

The five most common and abundant fungi based on the relative abundance estimates were *Penicillium* spp*.,* *Podospora dimorpha* ([Sordariales](http://www.uniprot.org/taxonomy/42302)) and several distinct uncultured fungal groups (Fig. 1, Table 2). Additionally, several abundant mycorrhizal fungi species were commonly identified from the legume rhizospheres. Arbuscular mycorrhizal *Glomus* sp. and widely distributed ectomycorrhizal *Inocybe* sp. (Ryberg *et al.*, 2008) were found in the soils from all species’ rhizospheres with the exception of *A. cyclops*. Interestingly, *Sebacina*, which according to some reports could include species that are ectomycorrhizal (Glen *et al.*, 2002), was found in

almost all native and non-native populations of *A. cyclops* and in one native *P. lophantha* population but was absent from the other four species’ soils (Fig. 1). Curiously, *Sepedonium chalcipori*, a mycoparasite which parasitizes the fruiting bodies of other fungi (Neuhof *et al.*,

2007) was identified exclusively from the non-native range soils of the western native species *A. cyclops*, *A. saligna* and *P. lophantha* whereas this mycoparasite was absent in the soils of eastern native species *A. longifolia* and *A. melanoxylon* (Fig. 1). Nine plausibly pathogenic fungal taxa were found among the fifty most abundant fungal species identified from the rhizosphere of these legumes (Fig. 1, Table 2).

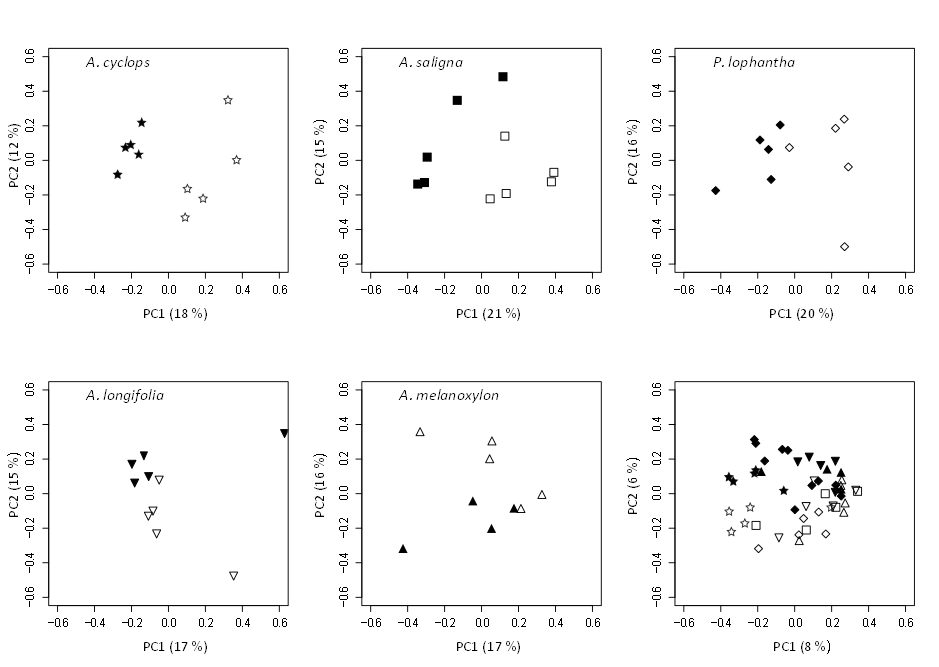
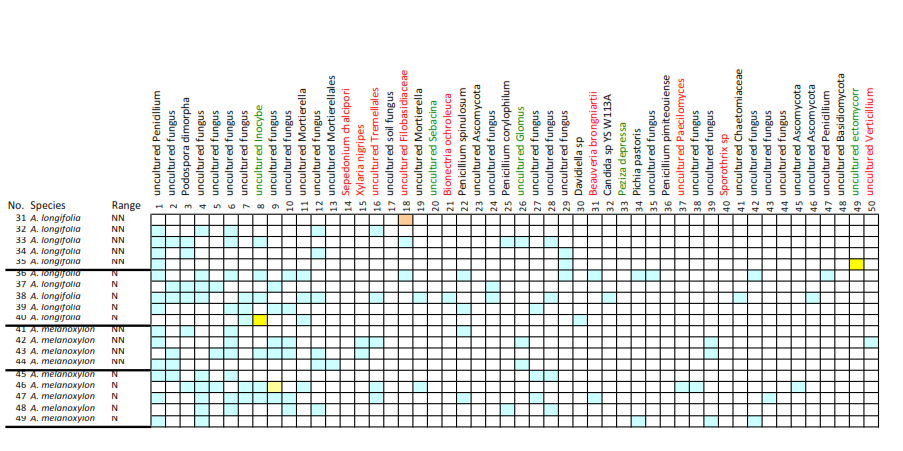
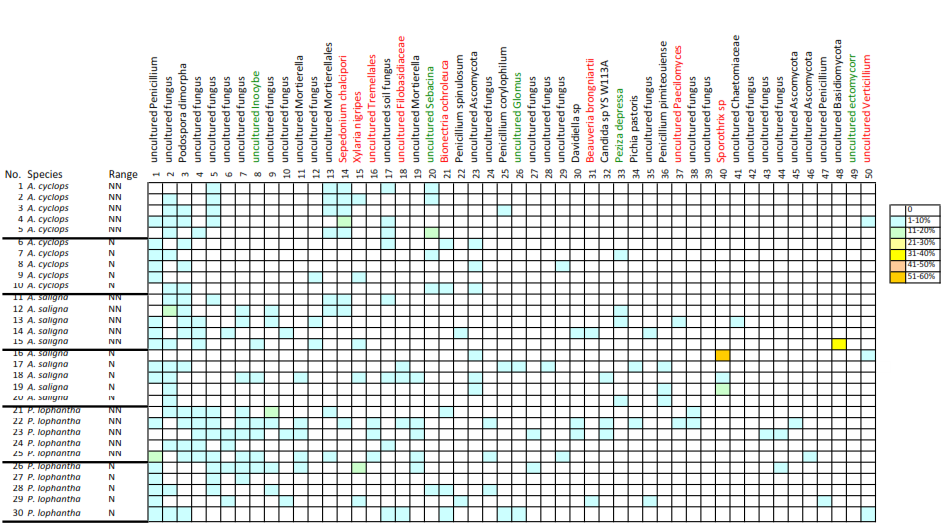
The overall model for fungal community composition revealed a significant interaction between geographic location (i.e. east vs west) and range (non-native vs native) which suggests that differences in fungal communities between ranges are associated with differences between the fungal communities of south-east compared to south-west Australia (Fig. 2, Table 3). We also found significant differences in fungal communities between the five study species (Table 3). Fungal communities were different between all species with an exception of two eastern native species, *A. longifolia* and *A. melanoxylon*, that had similar fungal communities (Table 3). Notably, fungal communities of *A. cyclops* were very different from the other four legume fungal communities (Table 3). Fungal species richness was significantly higher in *A. cyclops* non-native range populations (F1,8 = 12.42, *P* = 0.0078, Table 1). Overall, species richness was very similar for the other four legumes between their non-native and native populations (Table 1). SIMPER analysis revealed that 771 fungal species (17%) contributed up to 50% of the average Bray-Curtis dissimilarity for location (east vs west).

**Table 2**. Fifty most abundant fungal taxa found in the rhziosphere of four *Acacia* species and *Paraserianthes lophantha*. Additional information is given for each OTU, its placement in phylum and its description, if known, and reference. NA – not available.

|  |  |  |  |
| --- | --- | --- | --- |
| **No.** | **Name** | **Phylum** | **Description** |
| 1 | uncultured *Penicillium* sp*.* | Ascomycota | mould1 |
| 2 | uncultured fungus | NA | NA |
| 3 | *Podospora dimorpha* | Ascomycota | coprophilous fungi2 |
| 4 | uncultured fungus | NA | NA |
| 5 | uncultured fungus | NA | NA |
| 6 | uncultured fungus | NA | NA |
| 7 | uncultured fungus | NA | NA |
| 8 | uncultured *Inocybe* sp*.* | Basidiomycota | ectomycorrhizae3 |
| 9 | uncultured fungus | NA | NA |
| 10 | uncultured fungus | NA | NA |
| 11 | uncultured *Mortierella* sp*.* | Zygomycota | P-solubilizing fungus4 |
| 12 | uncultured fungus | NA | NA |
| 13 | uncultured *Mortierellales* | Zygomycota | P-solubilizing fungus4 |
| 14 | *Sepedonium chalcipori* | Ascomycota | mycoparasite5 |
| 15 | *Xylaria nigripes* | Ascomycota | obligate pathogenic fungus6 |
| 16 | uncultured *Tremellales* | Basidiomycota | mycoparasites, saprobes7 |
| 17 | uncultured soil fungus | NA | NA |
| 18 | uncultured *Filobasidiaceae* | Basidiomycota | mycoparasite8 |
| 19 | uncultured *Mortierella* sp*.* | Zygomycota | P-solubilizing fungus4 |
| 20 | uncultured *Sebacina* sp*.* | Basidiomycota | ectomycorrhizae9 |
| 21 | *Bionectria ochroleuca* | Ascomycota | plant pathogen10 |
| 22 | *Penicillium spinulosum* | Ascomycota | mould1 |
| 23 | uncultured Ascomycota | Ascomycota | NA |
| 24 | uncultured fungus | NA | NA |
| 25 | *Penicillium corylophilum* | Ascomycota | mould1,11 |
| 26 | uncultured *Glomus* | Glomeromycota | arbuscular mycorrhizae12 |
| 27 | uncultured fungus | NA | NA |
| 28 | uncultured fungus | NA | NA |
| 29 | uncultured fungus | NA | NA |
| 30 | *Davidiella sp.* | Ascomycota | teleomorph of *Cladosporium* s.str.13 |
| 31 | *Beauveria brongniartii* | Ascomycota | entomopathogenic fungus14 |
| 32 | Candida sp YS W113A | NA | NA |
| 33 | *Peziza depressa* | Ascomycota | ectomycorrhizae15 |
| 34 | *Pichia pastoris* | Ascomycota | methylotrophic yeast16 |
| 35 | uncultured fungus | NA | NA |
| 36 | *Penicillium pimiteouiense* | Ascomycota | NA |
| 37 | uncultured Paecilomyces | Ascomycota | entomopathogenic fungi17,18 |
| 38 | uncultured fungus | NA | NA |
| 39 | uncultured fungus | NA | NA |
| 40 | *Sporothrix* sp*.* | Ascomycota | endophytic pathogen19,20 |
| **Table 2**. Fifty most abundant fungal taxa found in the rhziosphere of four *Acacia* species and *Paraserianthes lophantha*. Additional information is given for each OTU, its placement in phylum and its description, if known, and reference (cont.). NA – not available. | | | |
| **No.** | **Name** | **Phylum** | **Description** |
| 41 | uncultured Chaetomiaceae | Ascomycota | NA |
| 42 | uncultured fungus | NA | NA |
| 43 | uncultured fungus | NA | NA |
| 44 | uncultured fungus | NA | NA |
| 45 | uncultured Ascomycota | Ascomycota | NA |
| 46 | uncultured Ascomycota | Ascomycota | NA |
| 47 | uncultured *Penicillium* sp*.* | Ascomycota | mould1 |
| 48 | uncultured Basidiomycota | Basidiomycota | NA |
| 49 | uncultured ectomycorrhizae | Basidiomycota | ectomycorrhizae |
| 50 | uncultured Verticillium | Ascomycota | wilt pathogen21 |

**1** - (Hunter *et al.*, 1988); **2** - (Bell, 2004); **3** - (Ryberg *et al.*, 2008); **4** - (Zhang *et al.*, 2011); **5** - (Neuhof *et al.*, 2007); **6** - (Wood & Thomas, 1989); **7** - (Zugmaier *et al.*, 1994); **8** - (Watson & Dallwitz, 2008); **9** - (Glen *et al.*, 2002); **10** - (Tondello *et al.*, 2012); **11** - (Bok et al., 2009); **12** - (Morton & Benny, 1990); **13** - (Braun *et al.*, 2003); **14** - (Neuvéglise *et al.*, 1997); **15** - (Buée *et al.*, 2007); **16** - (Clare *et al.*, 1991); **17** - (Liu *et al.*, 1998); **18** -(Shamsilawani A. B. *et al.*, 2009); **19** - (Wingfield *et al.*, 1993); **20** - (Wen *et al.*, 2009); **21** - (Nazar *et al.*, 1991).

**Figure 1**. Heatmap with relative abundance data for fungal communities detected in the rhizosphere of four *Acacia* species and *Paraserianthes lophantha* across non-native and native range populations. OTUs that had ≤ 1% abundance score were not included in the heatmap. Colour codes: red – pathogens, parasites and saprobes; green – mycorrhizae (i.e. AM and EM).



**Figure 2**. PCA plots for soil fungal communities based on extracted DNA from soils associated with four *Acacia* species and *Paraserianthes lophantha*. Open and closed symbols represent native and non-native populations for species plots, respectively. – *A. cyclops*, □ ■ - *A. saligna*, ◊ ♦ - *P. lophantha*, ∇ ▼- *A. longifolia*,∆ ▲ - *A. melanoxylon*. Additionally a summary plot showing species fungal communities based on geographic location is shown with open and closed symbols indicating populations sampled in south-east and south-west Australia, respectively.



|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Table 3**. Summary of PERMANOVA results for variation in fungal communities in the rhizosphere across all species, native and non-native range, geographic location (east or west) and between species pairs. | | | | | |
| Model | df | SS | MS | *F* | *P* |
|  |  |  |  |  |  |
| Species | 1 | 0.5415 | 0.5414 | 1.8105 | **0.006** |
| Range | 1 | 0.4276 | 0.4276 | 1.4298 | **0.035** |
| Location | 1 | 0.8220 | 0.8220 | 2.7485 | **0.0001** |
| Species x range | 1 | 0.4100 | 0.4099 | 1.3708 | 0.054 |
| Species x location | 1 | 0.3460 | 0.3459 | 1.1568 | 0.173 |
| Range x location | 1 | 1.0710 | 1.0709 | 3.5809 | **0.0001** |
| Species x range x location | 1 | 0.3580 | 0.3579 | 1.1969 | 0.138 |
| Residuals | 41 | 12.2622 | 0.2990 |  |  |
| Total | 48 | 16.2382 |  |  |  |
|  |  |  |  |  |  |
| *A. cyclops* x *P. lophantha* | 1,19 | 0.9372 | 0.9372 | 3.3426 | **<0.0001** |
| *A. cyclops* x *A. saligna* | 1,19 | 0.6204 | 0.6204 | 2.1028 | **<0.0001** |
| *A. cyclops* x *A. longifolia* | 1,19 | 1.0275 | 1.0275 | 3.3104 | **<0.0001** |
| *A. cyclops* x *A. melanoxylon* | 1,18 | 1.0737 | 1.0736 | 3.7391 | **<0.0001** |
| *A. saligna* x *P. lophantha* | 1,19 | 0.5797 | 0.5796 | 1.8886 | **0.0038** |
| *A. saligna* x *A. longifolia* | 1,19 | 0.5642 | 0.5642 | 1.6746 | **0.0079** |
| *A. saligna* x *A. melanoxylon* | 1,18 | 0.6438 | 0.6438 | 2.0424 | **0.0036** |
| *P.* *lophantha* x *A. melanoxylon* | 1,18 | 0.4208 | 0.4208 | 1.4041 | **0.0304** |
| *P. lophantha* x *A. longifolia* | 1,19 | 0.4370 | 0.4370 | 1.3562 | **0.0305** |
| *A. longifolia* x *A. melanoxylon* | 1,18 | 0.3406 | 0.3406 | 1.0275 | 0.3712 |

**4. Discussion**

4.1. Differences in soil fungal communities between the non-native and native ranges

The aim of this study was to determine whether legumes in Australia accumulate or associate with similar or distinct rhizosphere fungal communities across their non-native and native ranges. Our results showed that fungal communities were indeed significantly different between the non-native and native range populations across all study species. However, interestingly, we found a very strong geographic location by range interaction for fungal community composition which suggests that the observed differences between ranges are strongly influenced by geographical location. This also implies that although fungal communities are fundamentally different across the continent, at least in the populations that we sampled, these differences are unlikely to have constrained the invasion success of these legumes when introduced to novel sites.

The spatial structure and composition of soil microbial communities, as well as broader geographic patterns of distribution are likely to be strongly dependent on both abiotic and biotic factors (Ettema & Wardle, 2002b), including organism life-history (Bissett *et al.*, 2010). Additionally, there is some evidence to suggest that perceived microbial composition varies greatly depending on sampling strategies, spatial variation and land-use (Osborne *et al.*, 2011). Furthermore, vegetation structure prior to the arrival of invasive species may also influence soil microbial composition and plausibly has stronger effect on below-ground microbial communities compared to the short-term effects of newly arrived invasive species (Elgersma *et al.*, 2011a). However, invasive plants, including legumes have been also often reported as potential modifiers of chemical composition and microbial structure of newly invaded sites (Callaway *et al.*, 2008; Mangla & Callaway, 2008; Marchante *et al.*, 2008a; Lorenzo *et al.*, 2010; Rascher *et al.*, 2012).

It is possible that the differences we observed in the fungal communities across the south-eastern and south-western populations in our study were due to differences in the chemical characteristics of the soils we sampled. However, this is unlikely since in an earlier study we found little or no differences in soil chemistry (e.g. pH, total N, P, C, organic matter, ammonium and nitrate) between the non-native and native range populations of these legumes (Birnbaum *et al.*, 2012). However we cannot exclude other abiotic factors such as disturbance, rainfall and fire-regimes affecting the observed patterns in the soil fungal communities. For example, Pattinson *et al*. (1999) reported that fire significantly disturbed *Glomus* species (AMF) propagules and hyphae on the soil surface, thus suggesting that frequent fires could affect the fungal composition near the soil surface. Furthermore, Anderson *et al*. (2007) suggested that basidiomycete fungal communities are similarly altered in Australian sclerophyll forests by repeated prescribed burning which contributes to more uniform basidiomycete communities in soils.

However, given the consistent differences in microbial communities between eastern and western Australia across a range of soil types and conditions, it is most likely that geographical isolation is responsible for the evolution of different soil microbial communities in these regions. The eastern and western parts of Australia are separated by the vast Nullarbor Desert, providing a significant geographic barrier to the exchange of organisms across the continent (Jacobs & Wilson, 1996). Thus it is plausible that different soil microbial communities have evolved between the eastern and western states in the same way that we see consistent evolutionary divergences in plant lineages between east and west Australia (Crisp & Cook, 2007). This result is also in accord with our previous findings of consistent differences in the free-living soil nitrogen fixing bacterial communities between eastern and western populations of the same five host plant species (Chapter 4). In that study we also found a significant range by location interaction effect on these bacterial communities suggesting that the geographic barrier between the eastern and western populations could have contributed to the differences

in nitrogen fixing bacterial communities found in the rhizosphere of these legumes in their native vs non-native ranges as well (Chapter 4).

Overall, our results suggest that these legumes are not hindered by the absence of the fungal communities from their native range as they are plausibly able to form new associations with novel fungal taxa in the introduced range. All five species have formed naturalised or invasive populations within Australia, and are successful invaders on other continents where the soil microbial community is likely to be even more different. This result is consistent with the results from a glasshouse plant-soil feedback experiment described in Chapter two, where soil origin (native vs non-native range) had no effect on plant growth. However, in the experiment described in Chapter three *A. longifolia* did grow significantly bigger in its non-native range soils suggesting that this species may be released from harmful soil pathogens in its non-native range. Yet, the soil fungal community analysis described here overall did not show a greater abundance of pathogenic fungi in the native range soils of *A. longifolia*.Interestingly, though, we did find two pathogenic fungal species only in the native range of *A. longifolia* (*Bionectria ochroleuca,* a reported plant pathogen (Tondello *et al.*, 2012) and *Beauveria brongniartii* entomopathogenic fungus (Neuvéglise et al., 1997)), but not in its non-native range soils.

Other pathogenic fungi in the rhizosphere of the other four host legume taxa were also detected. For example, *Xylaria nigripes*, an obligate pathogenic fungus (Wood & Thomas, 1989), was found in both non-native and native range soils of *A. cyclops* and *A. saligna*, however was absent from *P. lophantha* non-native range soils. Furthermore, *X. nigripes* was absent from all *A. longifolia* soils, however present in *A. melanoxylon* non-native population soils. Despite this, the fungal communities of *A. longifolia* and *A. melanoxylon* were similar overall which suggests that the two eastern native species associate with fundamentally similar fungi across the continent.

4.2. Mycorrhizal fungi in the rhizosphere of acacias

Our results imply that these legumes harbour significantly different soil fungi in their non-native compared to native ranges, at least in Australia. Although in this study we did not specifically sample roots from the host plants, it is likely that some fragments of hyphae as well as spores could be found in the soil samples and could serve as an indication of the presence of some mycorrhizal species.

Acacias are known to form associations with both arbuscular mycorrhizal (AM) and/or ectomycorrhizal (EM) fungi (De La Cruz & Garcia, 1991; Founoune *et al.*, 2002; Brundrett, 2009) and in controlled conditions it has been demonstrated that mycorrhiza can significantly improve the growth of acacias (Duponnois *et al.*, 2001), particularly in the presence of rhizobia (Requena *et al.*, 1997; Marques *et al.*, 2001; Chalk *et al.*, 2006; Moreira *et al.*, 2010). Thus, the presence of compatible mycorrhizal fungi in the rhizosphere, particularly when appropriate rhizobia are also present, could have a significant effect on the establishment success of acacias in novel environments as has been shown for other legumes (Pacovsky *et al.*, 1986). In this study several common mycorrhizal fungi were found in all species’ rhizospheres which suggest that these legumes are not constrained by lack of access to mycorrhizae in their novel ranges.

Previous studies have suggested that in northern and eastern Australia acacias associate with both EMF and AMF (Warcup, 1980; Bellgard, 1991), while in south-west Australia they have only been reported to associate with AMF (Jasper *et al.*, 1989; Brundrett & Abbott, 1991). In this study, we found evidence for the presence of several AM and EM fungi in soils sampled from the rhizosphere of acacias across non-native and native ranges. Curiously, widely distributed *Glomus* sp. (AMF) and *Inocybe* sp. (EMF) were detected in both native and non-native soils of *A. saligna*, *A. longifolia*, *A. melanoxylon* and *P. lophantha*, but were not found in *A. cyclops*, whereas another mycorrhizal fungus (*Sebacina*)was found in *A. cyclops* native and non-native soils but was almost entirely absent in the soils of the other legume species (except in one instance in a native *P. lophantha* population). Interestingly, members of the genus *Sebacina*

(e.g. *S. vermifera)* have been described to function as growth-promoting endophytes on some hosts, however they have also been linked to decreased herbivore resistance by impairing accumulation of defence proteins in plants (Barazani *et al.*, 2007). In Australia, members of the genus *Sebacina* have been found in the roots of *Eucalyptus marginata* (jarrah) and described as ectomycorrhizal, although generally *Sebacina* has not been widely recognized as an EMF genus (Glen *et al.*, 2002). Overall these results suggest that mycrorrhizal communities associated with *A. cyclops* are significantly different compared to the other four legumes in our study. Indeed, we found that, generally, the fungal communities of *A. cyclops* were fundamentally different from the other four legume taxa.

4.3. Conclusions

We found that the fungal communities associating with invasive Australian legumes in our study are different between the non-native and native ranges. However, these differences are likely to be due to fundamental differences in the soil fungal communities between south-east and south-west Australia. Thus, it is unlikely that these legumes are constrained by novel fungal communities in their non-native ranges in Australia. The invasive success of all five species in areas outside Australia where soil microbial communities are likely to be even more different, suggests that these legumes are not constrained by soil fungal communities in novel ranges generally.

**Acknowledgements**

We would like to thank Carla Harris and Paweł Waryszak for extensive help in the field. This work was supported by Macquarie University Research Excellence Scholarship to CB and an Australian Research Council Discovery grant (DP0879494) to ML.

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**Chapter 6: Thesis discussion**

This thesis explored the role of the soil biota, with an emphasis on nitrogen fixing bacteria and soil fungal communities, in the establishment success of invasive legumes in novel sites in Australia utilizing a variety of techniques. Five legumes, four Australian *Acacia* species (*A. cyclops*, *A. longifolia*, *A. melanoxylon* and *A. saligna*) and their sister taxa *Paraserianthes lophantha*,were selected as study species because they represent an excellent model system. Generally, legumes are species that require mutualists (rhizobia and mycorrhizae), they have been introduced and become naturalised or invasive both within Australia and elsewhere, and relatively little is known about the roles of beneficial and detrimental elements of soil microbial communities in determining plant invasion success in novel habitats. In this final thesis Chapter, key results from Chapters 2-5 are synthesised and placed into the broader context of plant invasion biology. In addition, I highlight some promising directions for future research.

**The role of soil biota in plant invasion biology**

Soil microbial communities are increasingly recognised as important determinants of plant diversity-productivity patterns (Schnitzer *et al.*, 2011) and the abundance and rarity of plant species within communities (Klironomos, 2002; Smith & Reynolds, 2012). Soil microbes have been also shown to drive succession in plant communities (Van der Putten *et al.*, 1993; Koide & Dickie, 2002; Kardol *et al.*, 2006). For example, Kardol *et al*. (2006) observed in model systems of plants and soils from different successional stages that early-successional plants were more sensitive to pathogens compared to species from more stable plant communities, indicating that soil pathogens contribute to ecosystem development.

In the context of invasive plants, the absence of detrimental pathogenic and parasitic microbial communities when introduced to novel environments has been found to facilitate invasion success (Mitchell & Power, 2003; van der Heijden *et al.*, 2008; Reinhart *et al.*, 2010), while the

absence of compatible mutualists may constrain invasion success (Parker, 2001; Parker *et al.*, 2006; Dickie *et al.*, 2010). Some authors have suggested that mutualisms may be relatively more beneficial in the non-native range where the role of enemies is reduced compared to the native range (Reinhart & Callaway, 2004). Thus, the presence of suitable symbionts may be of great importance for the establishment success of viable populations in the novel range for an invasive species, especially for specialist hosts that require particular subsets of symbionts in order to grow successfully (Pringle *et al.*, 2009).

Although soils contain much of the biodiversity of terrestrial ecosystems (Torsvik & Øvreås, 2002) and soil microbes influence a large number of significant ecosystem processes such as nitrogen and carbon cycling (Tiedje, 1988; Hogberg *et al.*, 2001; Ehrenfeld, 2003), research in invasion biology has largely focussed on aboveground flora and fauna (Levine *et al.*, 2003). This disparity in knowledge between belowground and aboveground processes is mainly due to the difficulties inherent in studying soil microbial communities (Wolfe & Klironomos, 2005). Nevertheless, recent rapid advances in technological and methodological approaches have made it now more feasible to assess soil microbial diversity and composition and incorporate this new knowledge into our understanding of below-ground plant-soil processes and their role in invasion success.

**Plant-soil feedbacks and invasive species**

One of the most widely used techniques to study reciprocal interactions between plants and their associated soil microorganisms in the rhizosphere is the plant-soil feedback method (Bever *et al.*, 1997; Klironomos, 2002; Kulmatiski *et al.*, 2008). In summary, the plant-soil feedback approach is of great value because it informs how plants, via root exudation, deposition and susceptibility to enemies and symbionts, can alter soil microbial communities and whether these alterations subsequently increase or decrease plant growth (Kulmatiski *et al.*, 2008). In the context of invasion biology, positive plant-soil feedbacks are expected to increase local plant abundance and the ability to invade (Kulmatiski *et al.*, 2008), while negative plant-soil feedbacks

decrease plant abundance and may therefore limit invasibility. Negative feedbacks also contribute to more diverse plant communities (Bever, 2003; Bever *et al.*, 2010).

Substantial evidence exists to show that invasive plant species experience more positive feedback in their non-native range due to release from native range soil pathogens (Reinhart *et al.*, 2003; Callaway *et al.*, 2004; Smith & Reynolds, 2012) and more negative feedback in their native range because their abundance and persistence is mediated and held in check by antagonistic components of the soil microbial community (Klironomos, 2002; Bonanomi *et al.*, 2005; Zuppinger-Dingley *et al.*, 2011). However these general plant-soil feedback patterns do not necessarily apply to every invader in its non-native range (Inderjit & van der Putten, 2010). Thus, several authors have recently reported that some invaders do not experience reduced belowground enemy attack in introduced range soils (te Beest *et al.*, 2009; Andonian *et al.*, 2011a). For example, te Beest *et al*. (2009) suggested that highly invasive shrub *Chromolaena odorata* (Siam weed) in South Africa did not experience a decreased load of soil pathogens in its introduced range soils. Nevertheless, these authors showed that seedlings from the non-native range when grown in non-native range soils were bigger compared to the native range seedlings, suggesting that selection could have taken place during the invasion event, although soil origin did have significant effect on root/shoot allocation responses for this species (te Beest *et al.*, 2009). In another recent study, Andonian *et al*. (2011a) reported that a globally invasive weed, *Centaurea solstitialis* (yellow starthistle), experienced negative feedback in expanding populations within its introduced range, suggesting that enemy release from soil-borne pathogens is not sufficient to explain the invasion success of this species either. Furthermore, these authors suggested that biogeographic variation in soil-microbe effects may be responsible for different mechanisms that may operate on this species in distinct regions which contribute to overall variation in this species’ invasion success (Andonian *et al.*, 2011a).

In Chapter 2, I showed using a glasshouse plant-soil feedback experiment that soil origin did not explain biomass differences of the five study species across their non-native and native range

soils, in contrast to previous findings. Instead my results indicated that seed origin (native compared with non-native range) was an important determinant of plant biomass for two of the five species and this was associated with rhizobial nodulation. Thus the results from Chapter 2 suggest that there is no net effect of the soil microbial community on establishment success of these legumes in their native versus non-native ranges. Furthermore, the greater biomass of *A. longifolia* and *A. saligna* seedlings grown from non-native range seeds suggests that other processes may be important in determining the success of these species outside their native range, such as for example genetic changes and human imposed artificial selection (van Kleunen & Schmid, 2003). Human activity in agricultural and horticultural systems has been reported to facilitate intentional introduction of particular plant genotypes into the introduced range as well as the creation of new genotypes during plant breeding programmes and via processes such as admixture (Blumenthal & Hufbauer, 2007; Prentis *et al.*, 2008). This is highly likely for Australian *Acacia* species that have been extensively introduced by humans across the world and within Australia for ornamental, forestry and rehabilitation reasons and have subsequently escaped the original plantings and become naturalised and invasive (Carruthers *et al.*, 2011; Richardson *et al.*, 2011a; Richardson & Rejmánek, 2011b).

A study by Harris and colleagues on the same five study species (Harris *et al*., 2012) found that three species out of five, *A. longifolia*, *A. saligna* and *P. lophantha*, had lower genetic diversity in their introduced range populations while, surprisingly, *A. cyclops* had no difference across the ranges and *A. melanoxylon* had greater genetic diversity in the non-native range in Australia. Lower genetic diversity in the introduced range suggests that genetic bottlenecks and founder events may have occurred during the introduction and subsequent invasion process, whereas higher genetic diversity in the introduced range indicates that multiple introductions from distinct native sources may have occurred (Le Roux *et al.*, 2011). Unfortunately, for these legumes relatively little is known regarding their introduction history between different parts of Australia (Harris *et al.*, 2012). However it is apparent that reduced genetic diversity in the non-native range for *A. longifolia*, *A. saligna* and *P. lophantha* has not resulted in a reduction in plant

viability, and thus the introduction of particular phenotypes in the non-native range is a more plausible explanation of greater seedling biomass for *A. longifolia* and *A. saligna.*

It is noteworthy that out of the five study species examined in this thesis, one species showed consistently stronger responses to manipulations across experiments. Seedlings of *A. longifolia* grownfrom seeds collected in the non-native range grew significantly larger when grown in non-native range soils (Chapters 2 and 3). This result is consistent with the findings of Harris *et al*. (2012) and Harris *et al*. (unpub.) who found that *A. longifolia* plants in their non-native range in Western Australia were (i) larger in the field and (ii) grew larger from seed collected from the non-native range under glasshouse conditions using standard potting soil mix. These results for *A. longifolia* further support the notion that overall, soil microbial communities do not constrain this species’ invasion success in the novel range in Australia, and that other processes related to variation in genetic diversity across the ranges may be more important.

**Plant-soil feedback does not explain the invasion success of the five study species in Australia**

The observed lack of a net soil origin effect on the growth of these legumes is intriguing and to date such findings have been little reported in the plant-soil feedback literature, especially for legumes. One of the plausible explanations for this might be the difficulty associated with characterising the spatial distribution of microorganisms in soil, which is known to be heterogeneous and non-random (Ettema & Wardle, 2002a) and may vary seasonally. Although thorough care was taken when collecting soil samples in the field and subsequent handling in the glasshouse, it is important to acknowledge that extrapolating plant-soil feedback effects from the glasshouse experiment to field conditions should be done with caution (Andonian *et al.*, 2011b). For example, by sampling only a fraction of the soil community, it is possible that other taxa may have been missed that are important contributors to soil community composition and exert an effect on host plant species in the field (Andonian *et al.*, 2011b).

Despite the overall lack of a significant soil microbial effect, either positive or negative, on these legumes’ growth and biomass in their introduced ranges in Australia, I found that nitrogen fixing bacterial and fungal communities in the rhizosphere of these species were different across non-native and native host ranges (Chapters 4 and 5). This indicates that despite differences in nitrogen fixing bacterial and fungal community composition between the ranges, the presence or absence of particular microbial taxa does not appear to result in either negative or positive consequences. Therefore, this suggests that these five legume species may be overall generalists and able to plausibly form mutualisms with a range of rhizobial bacteria and mycorrhizal fungi. Although, to determine this for mycorrhizal fungi, root colonisation assays are needed to inform whether the mycorrhizal fungi found in the rhizosphere are indeed similar to the ones found within roots.

In fact I found a high number of common bacterial and fungal taxa that were present across the eastern and western populations of all five study species, suggesting that although there were overall significant community compositional differences, subsets of the soil microbial communities occurred on both sides of the continent. This is highly likely since the sites that were sampled were similar in their aboveground vegetation composition (i.e. woodland dominated by *Eucalyptus* species in the overstorey and Fabaceae species in the mid-storey). Mitchell *et al*. (2010) suggested that vegetation structure can serve as a good predictor of soil microbial composition and in the long term may be a more useful tool than measurements of soil characteristics at a single time point as these are often prone to short-term changes in response to abiotic variation. Thus it is plausible that I did not observe an overall soil origin effect on these legumes’ growth because soil microbial communities associating with these legumes across the continent are functionally similar, despite some compositional differences.

**Rhizobial communities of invasive legumes in Australia**

Rhizobial communities and free-living nitrogen fixing bacteria, associated with the five study species were analysed in Chapters 3 and 4 of this thesis using multiple approaches. This is one of the first studies to comprehensively compare the nitrogen fixing bacterial communities in both the rhizosphere and in the nodules of legumes across a broad geographic scale.

Legumes have been reported to rely extensively on rhizobia to successfully establish and colonize new areas (Sprent & Parsons, 2000; Parker, 2001). Therefore, absence of compatible soil symbionts such as rhizobia in the novel range could serve as a significant constraint to invasion success (Parker, 2001) unless invaders encounter compatible soil mutualists (Reinhart & Callaway, 2004; Chen *et al.*, 2005; Parker *et al.*, 2007). Similarly to pathogenic interactions, some mutualisms may be highly specific among some taxa, however overall it has been suggested that the majority of mutualisms do not constitute tight coevolutionary relationships (Richardson *et al.*, 2000; Bronstein, 2003; Callaway *et al.*, 2011). Generally relatively little is known about the evolutionary dynamics of plant-soil mutualisms (Simms & Taylor, 2002).

In Australia, legumes, including *Acacia* spp., are reported to vary in their specificity towards rhizobial symbionts (Burdon *et al.*, 1999; Thrall *et al.*, 2000), although there is some evidence to suggest that more generalist *Acacia* spp. harbour more diverse rhizobia (Thrall *et al.*, 2007; Hoque *et al.*, 2011). However, studies from Portugal and New Zealand have found predominantly the slow-growing *Bradyrhizobium* in invasive acacias’ nodules (Weir *et al.*, 2004; Rodríguez-Echeverria, 2010). Thus it appears that there is substantial variation in legume-rhizobia symbiosis depending on the geographic location.

In Chapter 3 of this thesis I showed that rhizobial abundance was similar across the non-native and native ranges for all host species, whereas rhizobial community composition (based on T-RFLP analysis of nodules) was significantly different between non-native and native ranges for the three western native species, *A. cyclops*, *A. saligna* and *P. lophantha*. The opposite pattern was observed for the two eastern natives, *A. longifolia* and *A. melanoxylon,* which had similar

rhizobial communities between the ranges but different community composition between the species. The study described in Chapter 4 further examined soil and nodule community composition in the native and non-native ranges of the five study species, using pyro-sequencing. This analysis showed that the rhizobial communities in the nodules were not different between the ranges for any of the five study species. In contrast, the free living

nitrogen fixing bacterial communities from the rhizosphere were different between native and non-native ranges, which was strongly associated with differences between eastern and western sample locations rather than the range *per se*.

It is important to note that the molecular studies described in Chapters 3 and 4 targeted different genes. For instance, in Chapter 3 *nifD* and *nodA* genes were amplified from the nodule DNA, whereas in Chapter 4 only the *nifH* gene was amplified from the nodule DNA. *Nod* genes have been reported to be unique to rhizobia and in some cases the phylogenies of *nod* genes may correlate with the host plant (Dobert *et al.*, 1994; Lindström *et al.*, 1995). In contrast, *nif* genes are found in many other non-rhizobial bacteria (Haukka *et al.*, 1998). Indeed, in Chapter 4 I reported on the high number of free-living nitrogen fixing bacterial species in both soils and nodules. Therefore, different results from Chapters 3 and 4 need to be interpreted in the light of different methodologies used (e.g. due to targeting different genes in these two studies).

Although T-RFLP analysis gives a high-resolution community compositional overview of targeted organisms (Anderson & Cairney, 2004) it provides less information on the communities compared to pyro-sequencing. Nevertheless, it is likely that the results observed in Chapter 3 reflect the “true” rhizobial community composition in the nodules of these legumes. This is because the sequencing data presented in Chapter 4 was analysed at the coarse genus level only, due to the reference taxonomy restriction which yielded the maximum highest resolution of rhizobial species at this level. Therefore depending on the taxonomic level of the data that is being analysed it is plausible that interpretation of the results of Chapter 4 may change if analysis was performed at a higher resolution such as at species-level (Ramette & Tiedje, 2007;

Bissett *et al.*, 2010). Thus the results from Chapter 4 may confirm the results from Chapter 3 if we (i) only analysed the rhizobial communities found in nodules and omitted the free-living nitrogen fixing bacteria and (ii) analysed the rhizobial communities from Chapter 4 at the higher taxonomic level. This is obviously an area for future work.

Overall, the results from Chapters 3 and 4 suggest that absence of rhizobia in the non-native range would be unlikely to constrain the invasion success of these legumes. This result further supports the findings from the plant-soil feedback experiment described in Chapter 2 and indicates that a lack of compatible rhizobial symbionts is unlikely to constrain these species in their novel ranges, at least within Australia.

**Fungal communities of invasive legumes in Australia**

In Chapter 5 the soil fungal communities, both pathogenic and mycorrhizal, in the rhizosphere were analysed across the non-native and native ranges of the five study species. Although the role of soil pathogens in plant invasions has been generally well documented for a range of species (Mills & Bever, 1998; Klironomos, 2002; Mangla & Callaway, 2008; Reinhart *et al.*, 2010), relatively little is known about the role of soil fungal pathogens in the success of legume species introduced into novel ranges, either within or outside Australia.

The importance of mycorrhizal fungi in legume growth has been rarely addressed compared to that of rhizobia (Lafay & Burdon, 1998; Thrall *et al.*, 2007), yet legumes are widely acknowledged to rely on both types of symbionts (i.e. mycorrhizae and rhizobia) for their successful establishment and growth (Pacovsky *et al.*, 1986). For example, the beneficial effects of dual inoculation of mycorrhiza in conjunction with rhizobia on legume growth in controlled conditions has previously been documented (Marques *et al.*, 2001; Chalk *et al.*, 2006). This indicates that the presence of compatible symbionts might be of great importance for establishment in the non-native range and also serve as a substantial constraint for growth if either of these mutualisms is absent.

The results from Chapter 5 suggest that the fungal communities in the rhizospheres of the five study species are different between native and non-native ranges within Australia. This reflects the findings for nitrogen-fixing bacterial communities (Chapter 4) and confirms that geographic variation contributes strongly to these differences in symbiont assemblages across the continent. For example, I observed some variation in parasitic and mycorrhizal fungi across both host species and ranges. Thus, *Sepedonium chalcipori*, a mycoparasite which parasitizes the fruiting bodies of other fungi (Neuhof *et al.*, 2007) was identified exclusively from the non-native range soils of the western native species *A. cyclops*, *A. saligna* and *P. lophantha* whereas this mycoparasite was absent in all soils of eastern native species *A. longifolia* and *A. melanoxylon*. This indicates that the western native species may experience indirect effects from novel range biota (e.g. mycoparasites) that attack plausibly beneficial fungi (e.g. mycorrhizae) found in the rhizosphere of these hosts.

Furthermore, interestingly, arbuscular mycorrhizal *Glomus* sp. was only found in the rhizosphere in western populations of *A. saligna*, *P. lophantha*, *A. longifolia* and *A. melanoxylon*. This result is consistent with previous reports that have suggested that acacias associate in south-west Australia exclusively with arbuscular mycorrhizal fungi (Jasper *et al.*, 1989; Brundrett & Abbott, 1991). In northern and eastern Australia acacias have been reported to associate with both arbuscular and ectomycorrhizal fungi (Warcup, 1980; Bellgard, 1991). However, I found evidence for ectomycorrhizal fungi in both eastern and western populations. Nevertheless, to support these findings, root colonization assays are needed to assess which mycorrhizal fungi from the rhizosphere indeed associate with these acacias via roots.

Despite the substantial variation in fungal communities across the continent there was a high number of common fungal taxa in the legume rhizospheres in both native and non-native ranges. These were predominantly unknown and uncultured fungi, but also included some mycorrhizal and pathogenic taxa. For instance, widely distributed ectomycorrhizal *Inocybe* sp. was present in the non-native and native range populations of all legumes with the exception of

*A. cyclops* which appears to accumulate somewhat different fungi in its rhizosphere compared to the other four legume taxa (e.g. ectomycorrhizal *Sebacina* sp.).

Overall, though, my results show that despite substantial differences across the ranges in soil fungal communities these legumes do not appear to be constrained by lack of compatible mutualists (e.g. rhizobia and mycorrhizae) in their novel ranges nor released from soil pathogens in their novel ranges in Australia.

**Conclusions and future directions**

It is becoming increasingly apparent that to understand the complex mechanisms that drive the invasion success of a plant species introduced to a novel environment it is necessary to assess interactions with other taxa both above- and belowground in the introduced and native ranges. Recent studies of the role of soil microbial communities in the invasion success of plant species (Klironomos, 2002; Van Grunsven *et al.*, 2007; te Beest *et al.*, 2009; Zuppinger-Dingley *et al.*, 2011) and the results from this thesis are providing important insights into the role of reciprocal plant-soil effects on invasive success of introduced plants. The general picture that is emerging is that generalisations regarding the mechanisms underlying successful plant invasions are elusive. For some invaders, abiotic conditions and propagule pressure are the key factors, for others interactions with aboveground taxa are crucial, and for some plant invaders interactions with the soil microbial community underpin invasion success.

This thesis has comprehensively assessed the role of soil biota in the invasion success of five legume species in Australia. Overall, the results of this research support the view that these species are not constrained by soil microbial communities in their introduced range populations despite differences in soil bacterial and fungal composition across the continent.

There is still much research to be done on understanding the role of soil biota in the invasion success of legumes at larger cross-continental spatial scales. Thus, it is likely that Australian

legumes introduced to different continents experience quite different soil microbial communities that may significantly influence invasion success. Plant-soil feedback experiments incorporating non-native range seed and soil from outside Australia compared to native range seed and soil within Australia could address this. Furthermore, studies comparing community composition of the rhizospheres of co-occurring native and invasive species would shed light on the effect of invaders on soil microbial composition. With improved laboratory methods, more accessible novel culture-independent techniques (e.g. sequencing) and more rapid data processing software, it is now possible to conduct comprehensive surveys and analyses of soil microbial communities in relatively short time scales which was not feasible just a decade ago. Thus it is likely that future studies will be able to increasingly describe many novel soil microbial taxa and thus improve our understanding of the unseen belowground soil microbial communities and their role in mediating plant growth and abundance.

A better understanding of above- and belowground interactions and their role in invasion success of plants introduced to novel environments is essential for better informed management and restoration strategies of invaded native ecosystems both in Australia and elsewhere. The results of this thesis suggest that plant species that are generalists or are introduced to broadly similar environments are unlikely to be constrained by the soil microbial community in the recipient environment. This suggests that in these cases, management strategies that target other aspects of the invasion pathways of these species are more likely to be successful.

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**Appendix A: Paper accepted for publication during candidature**