Mycorrhizal associations in the Fabaceae: are they really needed?

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Abstract

Mycorrhizal associations and root specialisations are beneficial to plants found in low nutrient soils, particularly those characteristic of Australian heathlands and woodlands. Legumes are characterised by the universal presence of N-fixing nodules, but some species have also been reported to have mycorrhizal associations and cluster roots. Seven species of native legumes commonly found in low nutrient heathy woodland ecosystems in south eastern Australia were grown in pot-culture to determine the relative importance of different nutrient strategies in growth, nodulation and nutrient content. The first pot-culture experiment used soil collected either from the field (unburnt sites with low nutrient capital) or white sand (negligible nutrient content) and with or without a soil P supplement (rock phosphate). All species produced significantly greater above- and belowground biomass, nodulation was higher (greater number and larger nodules) and N and P content was greater when grown in field soil amended with P. *Viminaria juncea* benefitted more from the presence of cluster roots compared to ectomycorrhizal associations while the remaining species did not produce cluster roots but had ectomycorrhizal associations suggesting a greater contribution from this root specialisation. The second pot-culture experiment investigated the effect of P supply on plant growth, nodulation and nutrient content. Apart from *Acacia verticillata*, there was no significant difference in above- and belowground plant biomass with increasing P supply. In contrast, nodule number and weight for *Acacia verticillata* and *A. pulchella* increased significantly with increasing P supply. Phosphorus concentration and content in leaves increased significantly with increasing P in all species but N content did not show the same

pattern. Phosphorus supply had a greater effect on the extent of nodulation and presumably, N-fixation than on host plant growth. From this study it is clear that mycorrhizal associations are required for increased growth and P uptake, and, putative enhancement of N-fixation in the majority of the species studied. Further investigations are required to determine how widespread the occurrence of cluster roots and mycorrhizal associations are in native Australian legumes and if there is a taxonomic and/or ecological basis to this distribution.

Introduction

Heathland ecosystems are widespread throughout southern and eastern Australia. These areas have some of the most nutrient impoverished soils of all terrestrial ecosystems, particularly in terms of nitrogen (N) and phosphorus (P) (Adams *et al*. 1994). It has long been established that to survive in low nutrient soils, plants may have different N and P requirements (Chapin 1980), growth rates (Grime 2001) and mechanisms for nutrient use (Vitousek 1982, Schlesinger *et al*. 1989, Aerts 1990). In addition, research over the last three decades has shown that low nutrient soils are the most conducive to development of roots specialised for nutrient acquisition (Marschner 1995, Michelson *et al*. 1996, 1998). Heathlands, particularly those in Australia, have given rise to a unique combination of plant species and nutrient acquisition strategies (e.g. Lamont 1982, Pate 1994, Specht and Specht 2002, Lamont 2003, Hopper and Gioia 2004). Early studies of heathlands in Australia concentrated on providing descriptions of various components of the ecosystem (e.g. Specht and Rayson 1957; Specht *et al*. 1958). More recent studies have built on these studies by providing further evidence of the diversity of heathland species in their physiognomy, anatomy, physiology and ecology (e.g. Specht and Rundel 1990; Adams *et al*. 1994; Turnbull *et al*. 1996; Thomas *et al*. 1999).

Symbioses such as mycorrhizal associations (arbuscular, ericoid-, orchid- and ectomycorrhizal, Smith and Read 1997) and N-fixing nodules (associations with *Rhizobium* and *Frankia* for example, Vance 2001) rely on the mutualistic interchange of carbon and N or P between the plant and its microsymbiont. Other specialisations such as cluster roots (including proteoid, capillaroid and dauciform roots, Playsted *et al*. 2006, Shane *et al*. 2006) and high density fine roots (Bakker *et al*. 2006) function by increasing the absorptive surface of roots and/or releasing organic acids and phenolics to promote uptake of P (Dinkelaker *et al*. 1995, Adams *et al*. 2002, Shane and Lambers 2005; Denton *et al*. 2007). At the community level, variability in nutrient uptake and use among species is the key to efficient nutrient cycling. This variability is compounded by complementary exploration of soil by

roots with distinct distributions within the profile (Pate 1994; Pate and Bell 1999), marked seasonality of fine root production (Gill and Jackson 2000), variable exploitation of nutrient-rich patches in the soil (Palmer and Dixon 1990; Jackson and Caldwell 1996; Hodge 2004) and 'sharing' of nutrients by mycorrhizal links between individuals of the same or different species (Francis *et al.* 1986; Newman 1988; Selosse *et al.* 2006). It seems self-evident that mechanisms for enhanced nutrient acquisition and utilisation are critical to survival of plant species and plant communities (Kahmen *et al.* 2006), particularly in highly infertile soils.

Legumes have been reported to have a range of root specialisations for acquiring nutrients, including N-fixing nodules, cluster roots and mycorrhizal associations (Lamont 1982, Brundrett *et al.* 1996, Adams *et al.* 2010). The set of adaptations is particularly well demonstrated in white lupin (Schulze *et al.* 2006). High levels of root plasticity related to P-supply have also recently been demonstrated (Adams *et al.* 2002, Denton *et al.* 2006, Pang *et al.* 2010, Suriyagoda *et al.* 2010), as has changes to root morphology (Kerley 2000). In general, low levels of N and P promote production of cluster roots (Dinkelaker *et al.* 1995, Racette *et al.* 1990). A number of species, including several Australian native legumes, can form cluster roots alongside nodules capable of N-fixation (Sprent 1995, Skene 1998). From this arrangement it has been hypothesised that cluster root formation has evolved as a means of supplying P for nodule formation and N-fixation but also as a mechanism for coping with reduced growth as a consequence of the carbon drain on host plants for N-fixation (Robson 1983). However, the relationships among N- and P-supply with nodulation and cluster root formation is complex with conflicting experimental evidence arguing the importance of P in N-fixation (e.g. Israel 1987, Sanginga *et al.* 1989, Louis *et al.* 1990, Racette *et al.* 1990, Reddell *et al.* 1997 a, b).

The presence of nodules capable of N-fixation is often also associated with ecto- and arbuscular mycorrhizal fungi and the general consensus is that the improved nutrition due to the mycorrhizal association stimulates N-fixation (Smith and Read 1997, Sprent 2001, Scheubin and van der Heijden 2006). This arrangement can be found in many pioneer species such as *Alnus* and *Allocasuarina* and it has been suggested that their success and use in phytoremediation is due to these root specialisations (Roy *et al.* 2007, Orfanoudakis *et al.* 2010). An area that has not yet been considered in detail is the role of cluster roots alongside mycorrhizal fungi, but it is again thought that the improved nutrient supply from cluster roots would enhance nodule formation and N-fixation (Diem 1996, Hurd and Schwintzer 1997). As

P (and N) supply increases, it is likely that the proportion and importance of root specialisations will decrease.

Aim of the study

Following the hypothesis that the relative proportions of root specialisations of legumes varies according to supply of P, the aim of this project was to (1) confirm the presence of root specialisations in a number of native legumes, (2) compare the relative importance of each of these strategies for growth and nutrient uptake and (3) investigate the importance of P supply on legume growth and production of nodules.

Materials and methods

Experimental species included representatives from the Fabaceae that are relatively common in heathy Stringybark woodland located within the southwest region of the Glenelg-Hopkins catchment areas near Casterton, Victoria (37º 34' S, 141º 23' E). This particular area was chosen since the vegetation and soil of this area have recently been characterised for other research purposes (York 2010, unpublished report). The vegetation type is associated with deep uniform aeolian sands and tertiary sandy clay soils which have been altered to form quartzite gravel (Department of Sustainability and Environment (DSE) 2004). Soils are acid (pH 5.7-5.9), low in total C (3.2-3.6%) and N (0.04-0.08%) and are particularly low in extractable P (1.36-1.55 mg P g DW soil^{-1}). The mean annual rainfall in the area is 653 mm and falls mainly in the period from July to September (Bureau of Meteorology 2010). The annual maximum and minimum mean temperatures are 29.9 °C and 8.3 °C, respectively.

The vegetation of the area is described as heathy Stringybark woodland with *Eucalyptus arenacea* Marginson & P.Ladiges (Desert stringybark) and *E. baxteri* (Benth.) Maiden & Blakely ex J. Black (Brown stringybark) as the dominant overstorey tree species. The understorey layer consists of medium, small and prostrate shrubs with about 65% cover. The shrub layer contains grass trees (*Xanthorrhoea australis* R.Br. and *X. caespitosa* D.J.Bedford), Silver banksia (*Banksia marginata* Cav) and several species of heaths (Epacridaceae) and woody peas (Fabaceae). Geophytes and annuals can be quite common but the ground cover is normally fairly sparse (DSE 2004).

Experimental species included *Acacia myrtifolia* (Sm.) Willd., *A. pulchella* R.Br., *A. suaveolens* (Sm.) Willd., *A. verticillata* (L'Hér.) Willd., *Kennedia prostrata* R.Br., *Pultenaea*

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stricta Sims and *Viminaria juncea* (Schad.) Hoff. Seed was prepared for germination in January 2007 using heat and scarification treatments as recommended by seed suppliers (D Phillips, Portland Seed Bank, pers. comm.). Treated seed was distributed over the surface of seed trays filled with white sand, covered with a fine layer of sand and watered. Seedlings (about 6 weeks-old) were transplanted from seed trays to 10 L black plastic pots in mid-March 2007 which had been prepared two weeks earlier to allow full saturation and settling of the growing media. For both experiments, a layer of gravel was placed at the bottom of the pots for drainage.

Experiment 1 – Interaction of phosphorus and nutrient uptake strategy

Pot-culture for the first experiment was prepared using the factorial combination of white sand or field soil mixed with or without rock phosphate (RP) to produce the four treatments hypothesised to promote different expression of each root specialisation:

1. white sand only (no P) – to promote nodulation but limited mycorrhizal infection or formation of cluster roots (referred to as 'White sand')

2. white sand, RP (high P) – to promote formation of cluster roots but limited mycorrhizal infection or nodulation (referred to as 'White sand+RP')

3. field soil only – (low P) to promote good mycorrhizal infection and nodulation but limited cluster root formation (referred to as 'Field soil')

4. field soil, RP (high P) – to promote good mycorrhizal infection and cluster root formation but limited nodulation (referred to as 'Field soil+RP')

Field soil was collected from undisturbed heathy Stringybark woodland near Casterton and sieved (2 mm) on-site before transporting to the glasshouse facility. Field soil was used to provide a source of fungal and bacterial propagules for mycorrhizal infection and nodule formation while contributing very low nutrient input. White quartz sand used in commercial bricklaying (sieved to 2 mm) was considered to contain no additional N or P available for plant uptake and to have no capacity for P-fixation. In addition, white sand contained limited fungal and microbial propagules for mycorrhizal infection and nodule formation. 'Hill Rock' rock phosphate was added at a rate of 10 g per pot to provide slow release of P (approximately 50 μg P g DW soil^{-1}) to plant roots over the period of growth (G Emerson, Emfert Pty. Ltd., pers. comm.) as described in Adams *et al*. (2002). All soil and sand combinations were mixed with a cocktail of four commercially available moist peat inoculants (*Rhizobium* inoculants normally used for faba bean, subclover, field pea and lupin)

to promote nodule formation. After 4 weeks of growth in pots, seedlings of *Acacia myrtifolia*, *A. suaveolens*, *Kennedia prostrata*, *Pultenaea stricta* and *Viminaria juncea* were thinned to one individual per pot. Each of the four treatments were replicated five times for each of five species (total n = 100).

Plants were grown in a glasshouse under natural light and maintained at an ambient daytime temperature of approximately 20-24 ˚C. Seedlings received regular weekly aliquots of modified Hoaglands solution (¼ strength) to provide all essential mineral elements apart from N and P (see Adams *et al*. 2002). The nutrient solution contained $CaCl_2$ (0.1 mM), K_2SO_4 (0.05 mM), $MgSO_4$ (0.1 mM) and Fe-Na-EDTA (0.05 mM) and micronutrients as salts containing B, Mn, Zn, Cu, Mo and Co (Hoagland and Arnon 1950). Water was added as required to maintain pots at or near field capacity between nutrient applications and pots were flushed with water the day prior to nutrient addition to prevent accumulation of salts. Pots were rotated regularly to reduce edge effects.

Harvesting commenced in December 2007 after 9 months of growth in pot-culture. Shoots were removed, dried at 60 ˚C for 48 hours and dry weights determined. Roots were carefully washed free of sand or soil and nodules were removed by hand and counted. The presence or absence of cluster roots was noted but were not removed. Sub-samples of fine roots (approximately 0.5-1.0 g wet weight) were collected from each plant and stained to determine the presence of infection by mycorrhizal fungi. Fine roots were stained with 1% trypan blue in lactic acid:glycerol:water (3.25:3:4) for 24 hours and examined microscopically for evidence of mycorrhizal associations. Remaining roots were dried at 60 ˚C for 48 hours and dry weights were determined. Once sub-samples of roots had been examined for mycorrhizal associations, they were washed free of storage solution (lactic acid:glycerol), dried and added to the main root sample.

Experiment 2 – Effect of phosphorus supply on legume growth and nodulation
Pot-culture for the second experiment consisted of white quartz sand mixed with a cocktail of four commercially available moist peat inoculants to promote nodule formation (see above). After 4 weeks of growth in pots, seedlings of *A. myrtifolia*, *A. verticillata*, *A. pulchella* and *Viminaria juncea* were thinned to one individual per pot.

One of three P treatments was applied to each pot. Phosphorus was supplied as either: no P (referred to as '0P'), 0.06 mM KH_2PO_4/K_2HPO_4 (referred to as '½P') or 0.125 mM KH_2PO_4/K_2HPO_4 (referred to as '1P'). Phosphorus was added as part of a once-weekly aliquot of 200 ml of ¼ strength modified Hoagland's solution (see above). No additional N was added. Each species was replicated 10 times for each P treatment (total n = 120). Pots were maintained using the same temperature and watering regimes described for Experiment 1. All 40 pots of each P treatment were kept together during the experimental period but the positions of pots were rotated regularly within each treatment to reduce edge effects.

Unfortunately many of the replicate plants used in this experiment died during a particularly hot summer period and unavoidable temperature fluctuations in the glasshouse. At least five replicates survived for each species and treatment apart from representatives of *Viminaria juncea* in the '1P' treatment for which all replicates died. The resulting replication used for descriptive presentation and statistical analyses are indicated (generally n = 5).

Plants were harvested in March 2008 when they were about 12 months-old. Roots were separated from aboveground shoots and washed free of adhering sand. Washed root systems were laid in trays of clean water and nodules were separated by hand, then counted and weighed. Shoot, root and nodule material was dried at 60 ˚C for 48 h and reweighed.

Analysis of plant material
Dried shoot or phyllode material from both experiments was finely ground to 2 mm (Culatti Micro Hammer Mill, Zurich, Switzerland) and analysed for total N using a LECO CHN 2000 analyser (Leco Corporation, St Joseph, MI) and for extractable P using an ICP-AES after rapid block digestion in sulphuric acid.

Statistical analysis
One-way analysis of variance (ANOVA) was tested using shoot, root and nodule dry weight and total N and extractable P as dependent variables and treatment as the fixed factor for each species. When treatments were significantly different for each dependent variable, LSD multiple comparisons of means were used to determine which treatments were different at the 95% confidence level. Student's t-tests were used for comparison of the same range of variables measured for *Viminaria juncea* in Experiment 2.

Results

Experiment 1 – Interaction of phosphorus and nutrient uptake strategy

Plant growth varied considerably among treatments and species (Fig. 1). All species grew poorly in white sand or field soil (Fig. 2) and apart from *Viminaria juncea*, were also significantly smaller when grown in white sand+RP. The field soil+RP treatment yielded the greatest above- and belowground biomass for all species and in most cases, 10- to 20-fold more shoot and root biomass compared to the other treatments. The exceptions were that the root biomass of *Pultenaea stricta* grown in RP treatments were not significantly different and *Acacia suaveolens* produced significantly greater root biomass when grown in field soil and field soil+RP than when grown in white sand or white sand+RP (Fig. 2).

All species produced nodules regardless of treatment (Fig. 3) but numbers of nodules ranged from 0 to 600 nodules on individual plants. It was hypothesised that white sand and field soil (low P treatments) would generate the greatest nodulation but this clearly was not the case. All species produced significantly more and larger nodules when grown in field soil amended with RP (high P treatements). For three species (i.e. *A. myrtifolia*, *A. suaveolens* and *Pultenaea stricta*), the biomass of nodules was not significantly different among white sand, white sand+RP and field soil+RP treatments, but for *Kennedia prostrata* there was significant differences for treatments with high P. In contrast, *Viminaria juncea* had fewer but much larger nodules on plant roots grown in white sand+RP compared to plants grown in field soil+RP (Fig. 3).

Cluster roots were formed by *Viminaria juncea* growing in all treatments, but were only produced by one other plant of *Acacia myrtifolia* growing in white sand (Table 1). Contrary to what was expected, cluster roots were found on all individuals of *Viminaria juncea* growing in white sand or field soil and on fewer individuals grown in treatments with RP added. Arbuscular mycorrhizal associations were rare, only occurring in one individual of *Acacia myrtifolia* grown in field soil (data not shown). In contrast, evidence of ectomycorrhizal associations were found in most species and treatments (Table 1), the only exception being *Viminaria juncea* for which ectomycorrhizal roots only occurred in treatments with RP. Generally, plants grown in substrate with rock phosphate had the greatest number of individuals with mycorrhizal roots. One exception was *Pultenaea stricta*, in which ectomycorrhizal associations were not only found in the roots of all plants grown in RP treatments, but also in all of the plants grown in white sand.
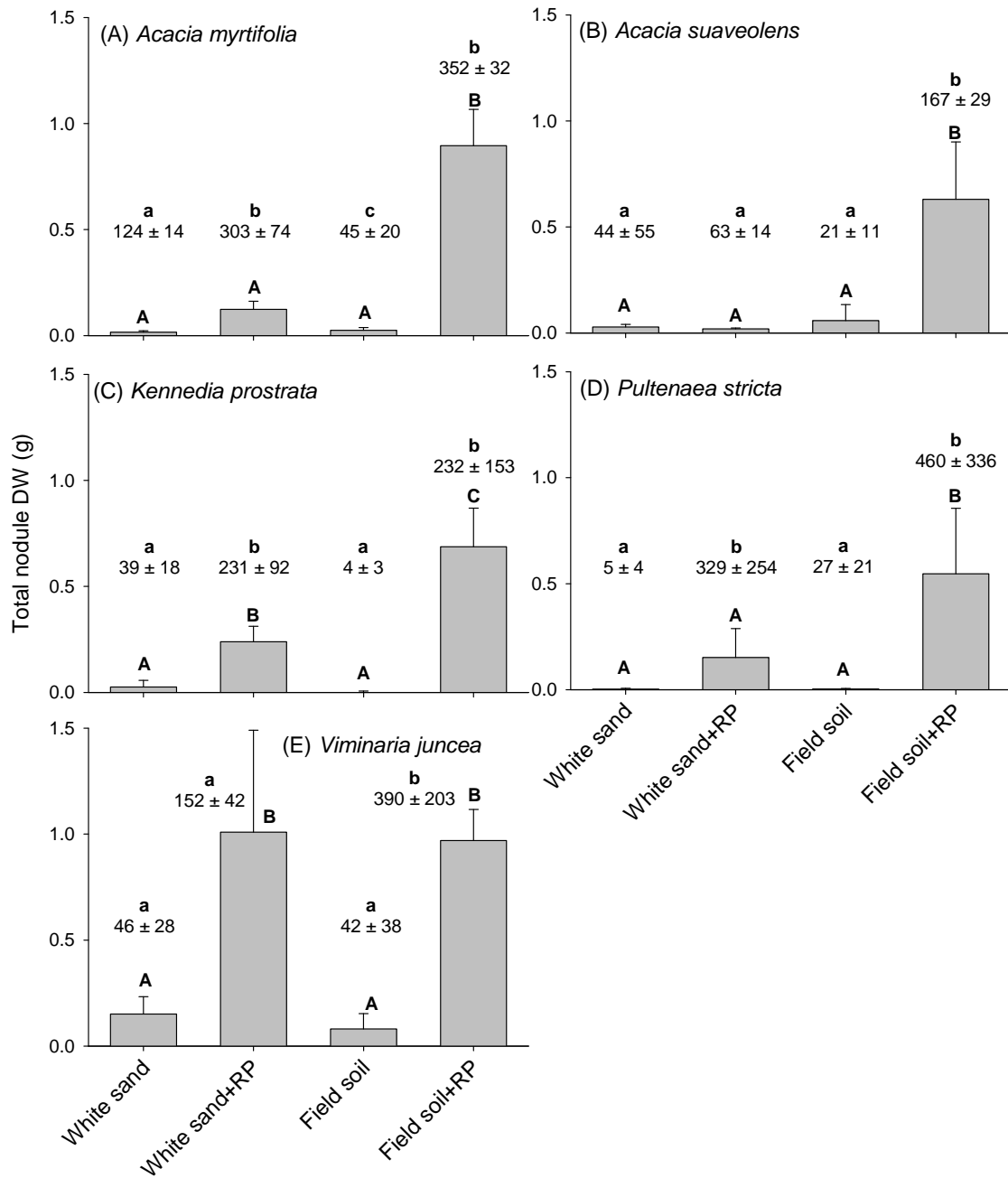
Fig. 1 Pot-culture of five species of native woody legumes (A) *Acacia myrtifolia*, (B) *A. suaveolens*, (C) *Kennedia prostrata*, (D) *Pultenaea stricta* and (E) *Viminaria juncea* grown in either white sand (WS) or field soil (FS) with or without rock phosphate (RP) to manipulate formation of nodules, mycorrhizal associations and cluster roots depending on availability of P and fungal inoculate.
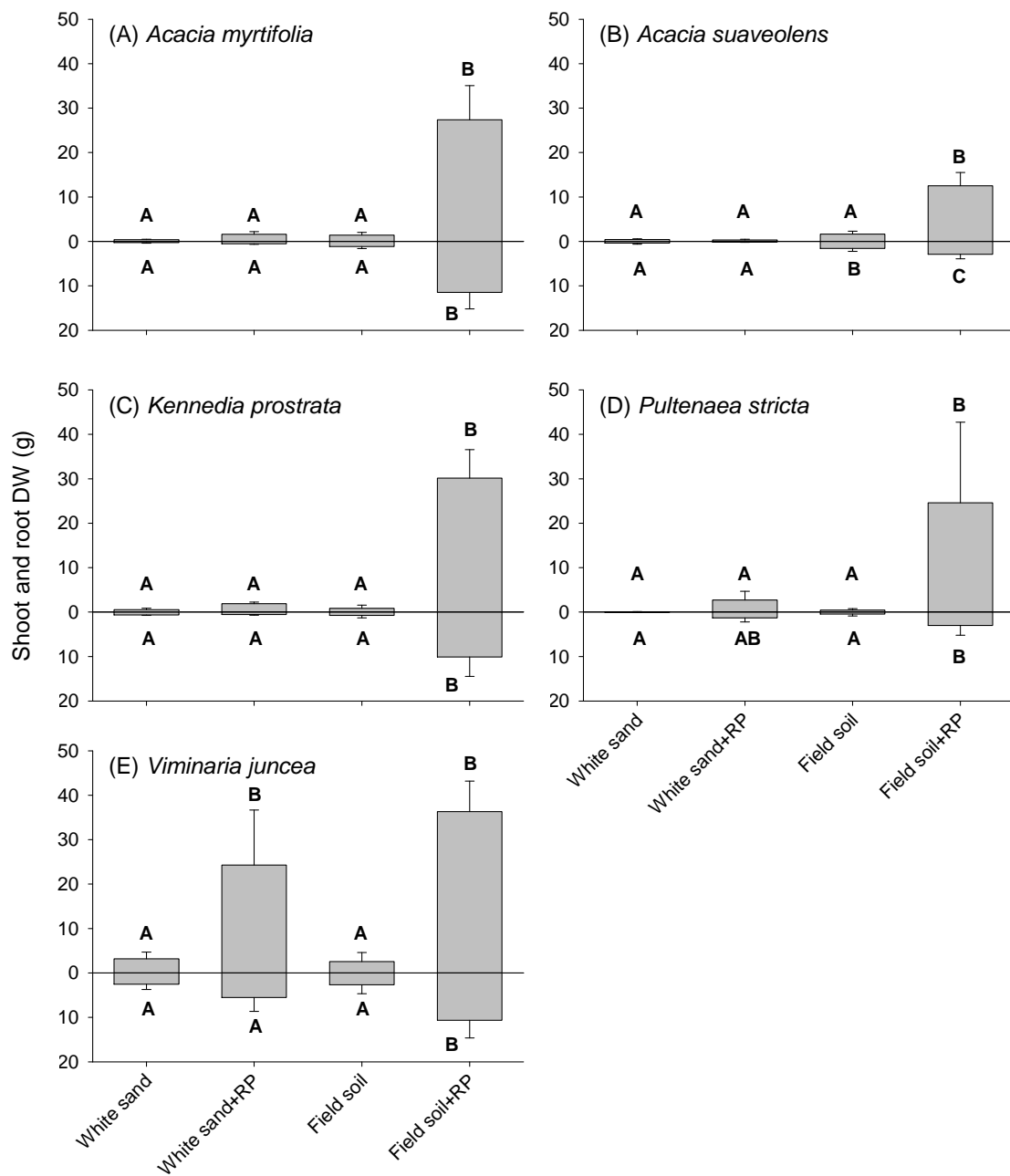
Fig. 2 Shoot and root dry weight (DW) of (A) *Acacia myrtifolia*, (B) *A. suaveolens*, (C) *Kennedia prostrata*, (D) *Pultenaea stricta* and (E) *Viminaria juncea* grown in four different pot-cultures. Bars represent mean values (n = 5) and error bars are standard deviation. Different upper case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).
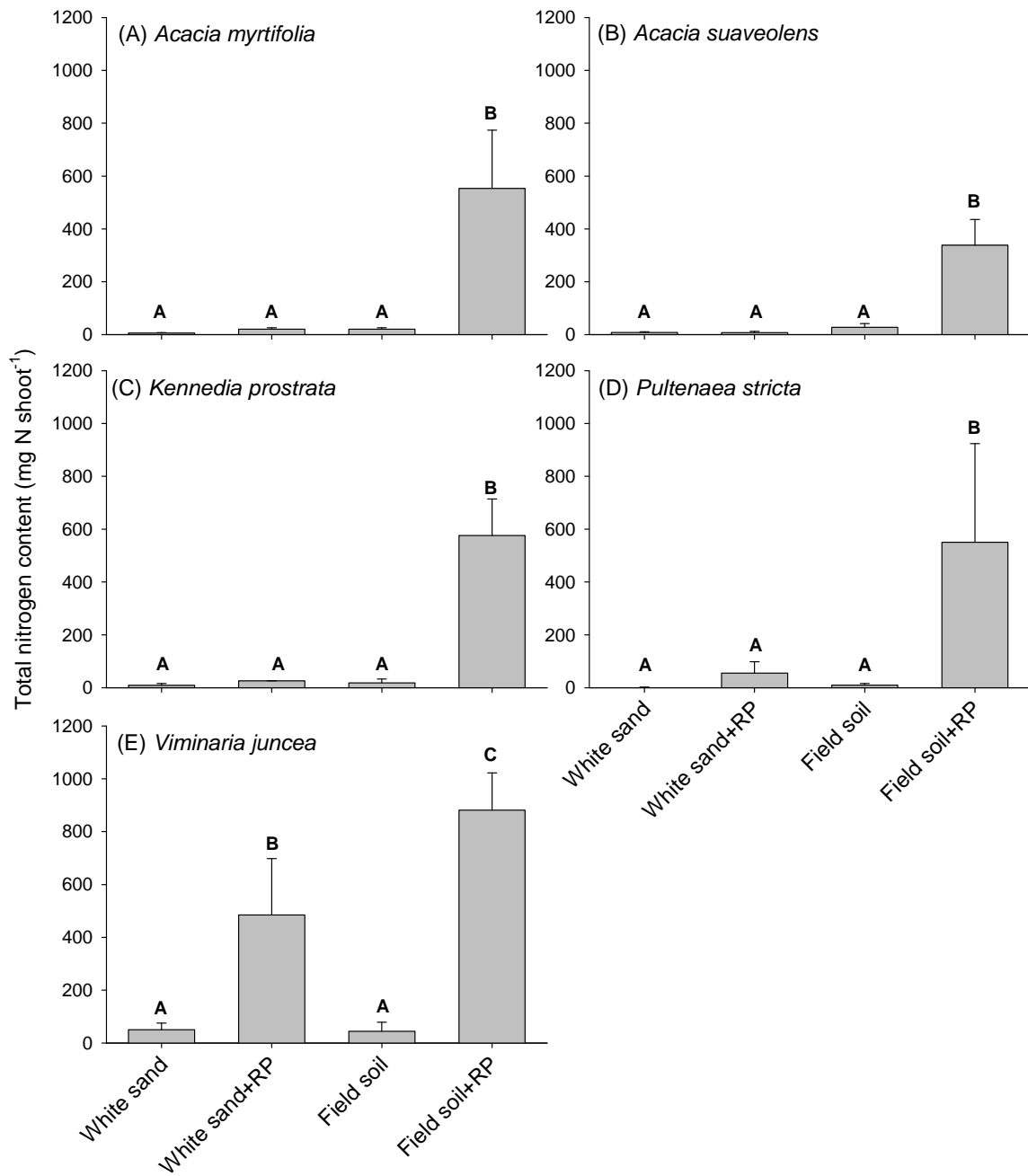
Fig. 3 Nodule dry weight (DW) and number of nodules from (A) *Acacia myrtifolia*, (B) *A. suaveolens*, (C) *Kennedia prostrata*, (D) *Pultenaea stricta* and (E) *Viminaria juncea* grown in four different pot-cultures. Bars represent mean nodule DW (n = 5) and error bars are standard deviation, numbers above bars are mean nodule number ± standard deviation. Different upper and lower case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).

Table 1 Formation of cluster roots (CR) and ectomycorrhizal associations (ECM) of five native legume species grown in four different pot-cultures. Numbers of plants with CR or ECM associations are indicated with the number of root systems investigated in brackets.

Species	White sand		White sand+RP		Field soil		Field soil+RP	
	ECM	CR	ECM	CR	ECM	CR	ECM	CR
Acacia myrtifolia	1 (5)	1 (5)	1 (5)		2 (5)		5 (5)	
Acacia suaveolens	1 (5)		3 (5)		3 (5)		3 (5)	
Kennedia prostrata	1 (5)		3 (3)		2 (5)		4 (5)	
Pultenaea stricta	4 (4)		3 (3)		2 (4)		5 (5)	
Viminaria juncea		5 (5)	3 (5)	2 (5)		5 (5)	1 (5)	1 (5)

Total N content in aboveground (shoot) biomass ranged between 2 and 930 mg N shoot^{-1} (Fig. 4) and N concentration in shoot tissues varied from 1.5 and 3.0% (data not shown). There were no significant differences in total N content among plants grown in white sand, white sand+RP and field soil treatments for all species apart from *Viminaria juncea*. Total N content of aboveground biomass was consistently and significantly different for all plants grown in the field soil+RP treatment. For *Viminaria juncea*, treatments with high P (white sand+RP and field soil+RP) were significantly associated with higher N content (Fig. 4) and N concentration (data not shown).

A similar pattern can be reported for extractable P content in aboveground (shoot) biomass (Fig. 5). All species, apart from *Viminaria juncea* had significantly less P in aboveground biomass when grown in white sand, white sand+RP and field soil treatments compared to field soil+RP. For *Viminaria juncea*, both treatments with high P (white sand+RP and field soil+RP) resulted in significantly greater P content in shoots. Phosphorus concentration in shoot tissue (mg P g DW shoot^{-1}) was also significantly greater in plants grown in white sand or field soil amended with RP compared to plants without (Fig. 6), but significantly greater P concentration was found in shoot tissue of plants grown in white sand+RP compared to field soil+RP. Phosphorus concentration was often double that measured for plants of the same species grown in field soil+RP. The only exception was *Viminaria juncea* which had similar concentrations of P in shoot tissue when grown with RP.

Fig. 4 Total nitrogen content of aboveground biomass (mg N shoot^{-1}) of (A) *Acacia myrtifolia*, (B) *A. suaveolens*, (C) *Kennedia prostrata*, (D) *Pultenaea stricta* and (E) *Viminaria juncea* grown in four different pot-cultures. Bars represent mean N content (n = 5) and error bars are standard deviation. Different upper case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).
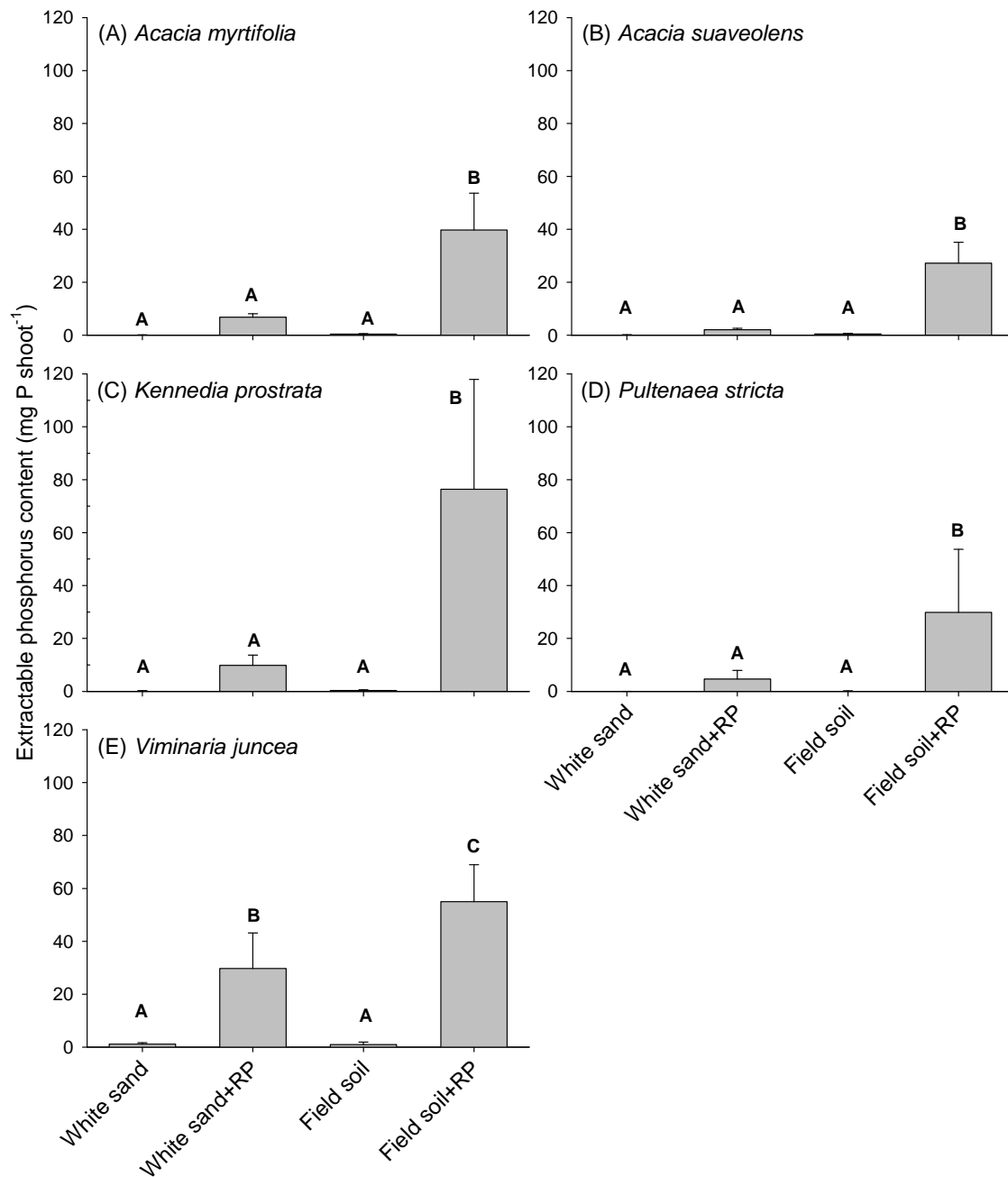
Fig. 5 Extractable phosphorus content of aboveground biomass (mg P shoot^{-1}) of (A) *Acacia myrtifolia*, (B) *A. suaveolens*, (C) *Kennedia prostrata*, (D) *Pultenaea stricta* and (E) *Viminaria juncea* grown in four different pot-cultures. Bars represent mean P content (n = 5) and error bars are standard deviation. Different upper case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).
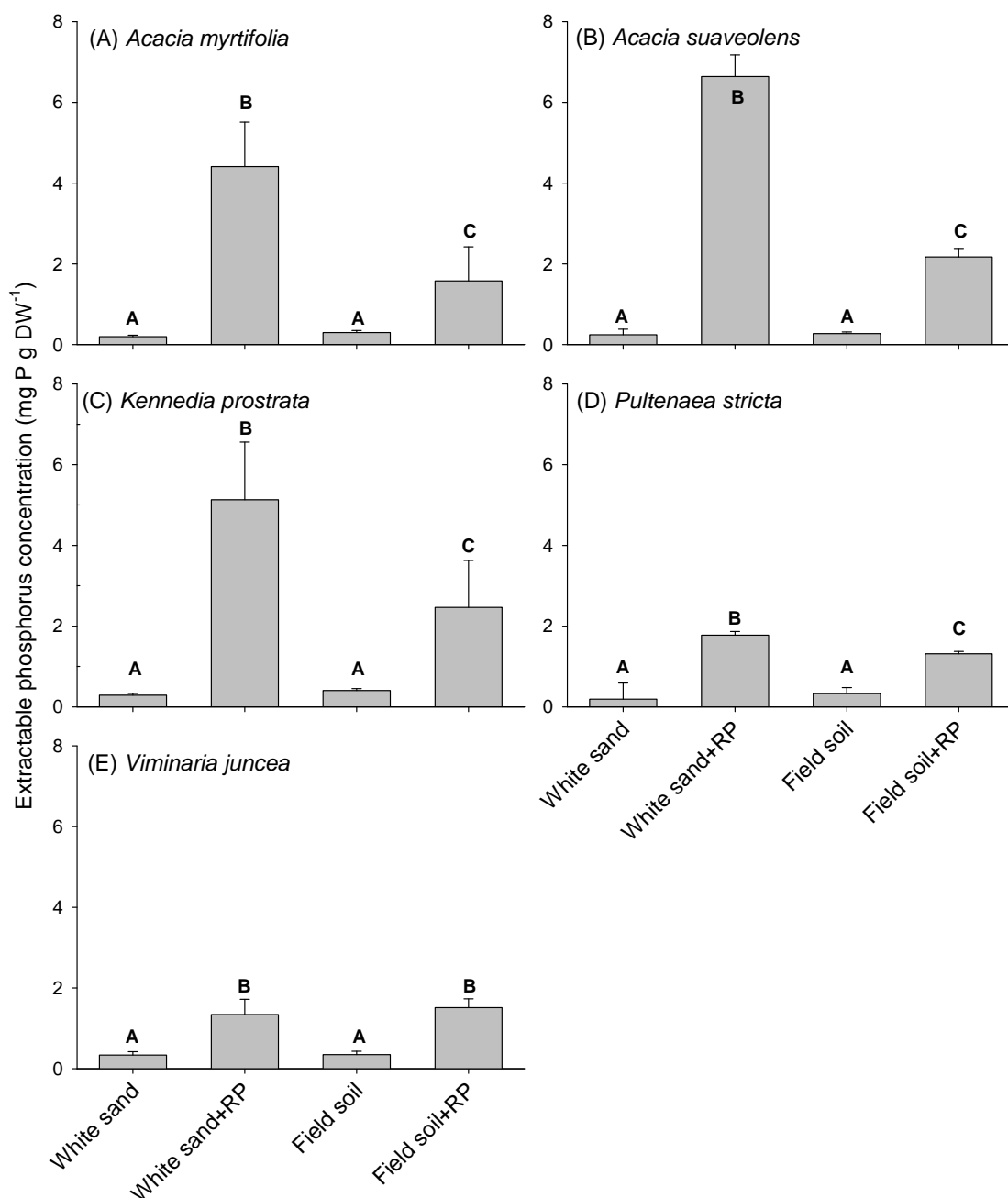
Fig. 6 Concentration of extractable phosphorus in aboveground shoot biomass (mg P g DW^{-1}) of (A) *Acacia myrtifolia*, (B) *A. suaveolens*, (C) *Kennedia prostrata*, (D) *Pultenaea stricta* and (E) *Viminaria juncea* grown in four different pot-cultures. Bars represent mean P concentration (n = 5) and error bars are standard deviation. Different upper case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).

*Experiment 2 – Effect of phosphorus supply on legume growth and nodulation*

Shoot and root biomass of *Acacia verticillata* and *Viminaria juncea* were three to four times greater than for *A. myrtifolia* and *A. pulchella* (Figs. 7, 8). For the *Acacia* species, shoot and

root biomass of plants supplied with 0P had the smallest biomass but differences were only significant for *A. verticillata* (Fig. 8). Shoot and root biomass of *A. myrtifolia* and *A. verticillata* grown in the 1P treatment was equivalent or lower than the ½P treatment. Shoot and root biomass of *Viminaria juncea* was greatest for the 0P treatment but unfortunately, no data was available for the 1P treatment for *Viminaria juncea* (see Materials and methods). Root biomass of all species generally reflected patterns of shoot growth but with much greater variability as indicated by relatively large error bars (Fig. 8).


Fig. 7 Pot-culture of four species of native woody legumes (A) *Acacia myrtifolia*, (B) *A. pulchella*, (C) *A. verticillata* and (D) *Viminaria juncea* grown in white sand with or without addition of weekly aliquots of P to investigate the growth and formation of nodules depending on availability of P.
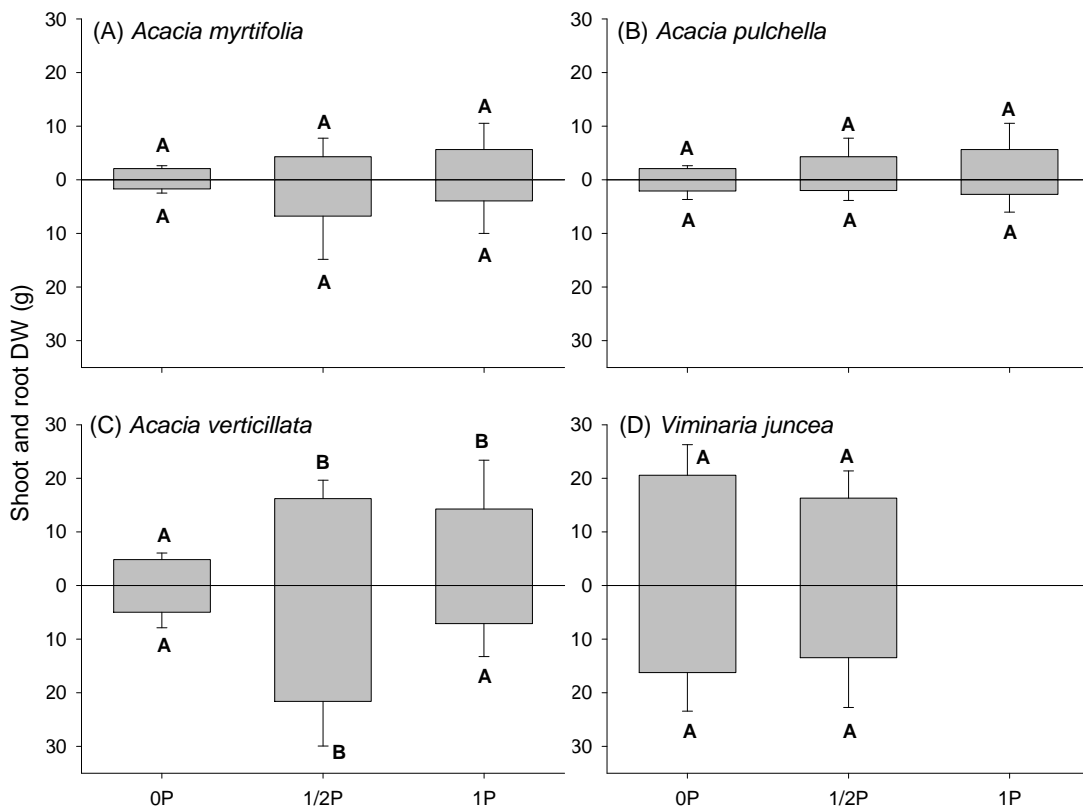
Fig. 8 Shoot and root dry weight (DW) of (A) *Acacia myrtifolia*, (B) *A. pulchella*, (C) *A. verticillata* and (D) *Viminaria juncea* grown in pot-culture supplied with three different levels of P. Bars represent mean values (n = 5) and error bars are standard deviation. Different upper case letters represent significant difference among treatments for each species (one-way ANOVA, LSD multiple comparisons, $p < 0.05$).

The number of nodules on individual plants ranged from no nodules for *A. pulchella* and *A. verticillata* grown in the 0P treatment to over 250 nodules for *A. myrtifolia* grown in the 1P treatment (values in brackets, Fig. 9). Total nodule dry weight increased with increasing addition of P and nodule number followed a similar pattern. Significant differences among total nodule dry weight were only found for *A. pulchella* and *A. verticillata*. Significant differences in mean nodule number were found among all treatments for the three species of *Acacia* except *A. myrtifolia* where nodule numbers were not significantly different from 0P compared to ½P (Fig. 9).
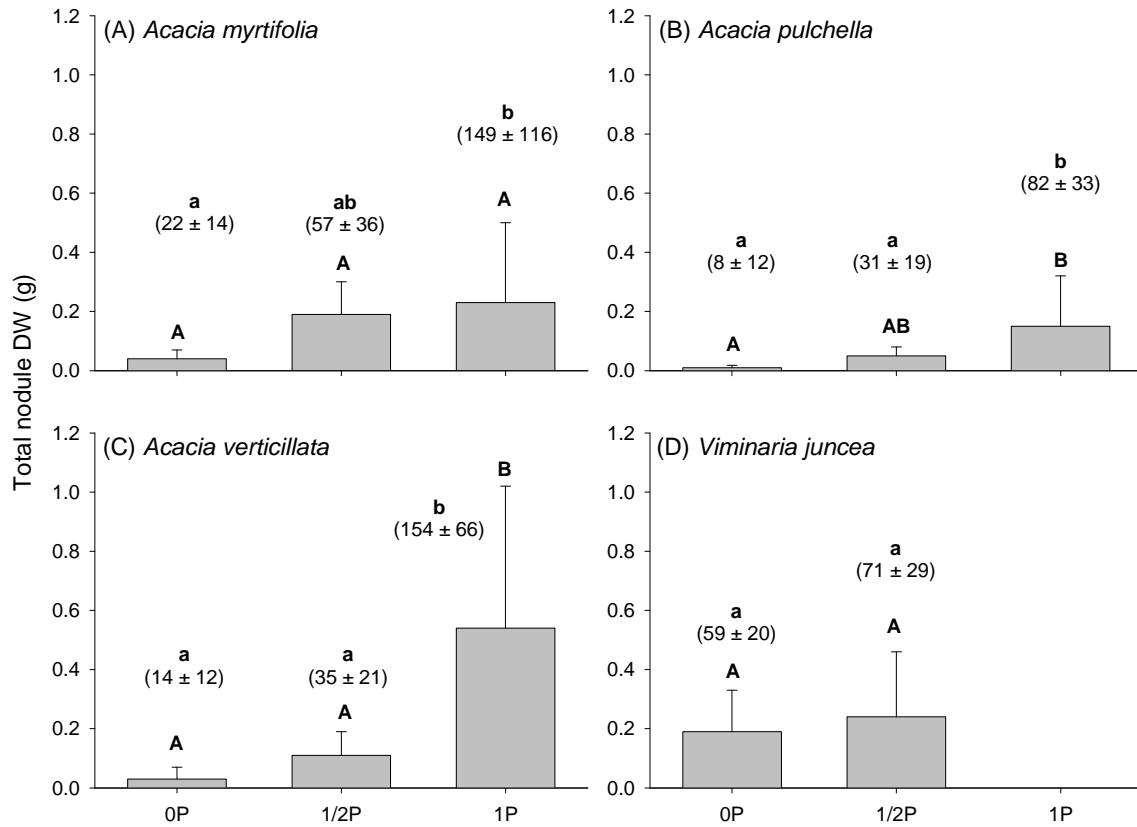
Fig. 9 Nodule dry weight (DW) and number of nodules from (A) *Acacia myrtifolia*, (B) *A. pulchella*, (C) *A. verticillata* and (D) *Viminaria juncea* grown in pot-culture supplied with three different levels of P. Bars represent mean total nodule DW (n = 5) and error bars are standard deviation, numbers above bars are mean nodule number ± standard deviation. Different upper and lower case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).

The mean percentage of nodule biomass contributing to the total belowground biomass was calculated for each treatment (Table 2). The percentage of nodule was lowest for *A. verticillata* grown in the 0P and ½P treatments (less than 1%) and greatest for the same species grown in 1P treatments (greater than 8%).

Table 2 Mean percentage of nodule biomass (± standard deviation) contributing to belowground biomass for four native legume species grown in the glasshouse with different P treatments.

Species	0P (%)	½P (%)	1P (%)
Acacia myrtifolia	2.3 ± 1.4	7.2 ± 4.9	7.8 ± 4.4
Acacia verticillata	0.7 ± 0.3	0.6 ± 0.5	8.5 ± 4.6
Acacia pulchella	0.4 ± 0.1	3.3 ± 2.4	5.7 ± 2.5
Viminaria juncea	1.2 ± 0.7	1.7 ± 0.7	-

Nitrogen content of aboveground biomass was generally similar among treatments for each species and ranged from 25 to 470 mg N shoot^{-1} (Fig. 10). Nitrogen concentration in aboveground biomass ranged from 0.9 to 2.7% N (data not shown). The only significant increase was for *Acacia verticillata* grown in the 0P treatment compared to higher levels of P (½P and 1P). Total N in leaves (%) increased, but not significantly, with increasing addition of P for all species except *A. myrtifolia* (data not shown). Total N in leaves of *A. myrtifolia* remained at about 1.5% regardless of how much P was available and total N for all species and treatments were all within a small range of 1.0 to 2.5% (data not shown).

Extractable P content of shoots increased with increasing supply of P (Fig. 11). This increase was significant for no or low application of P (0P and ½P) for *Acacia myrtifolia* and *A. verticillata* and for low to high applications (½P and 1P) for *A. pulchella.* There was no significant increase in shoot P content for *Viminaria juncea.* Similarly, P concentration in shoots increased with increasing availability of P in all four species (Fig. 12). These increases were significant for *A. myrtifolia* and *A. verticillata* across all three treatments and for 0P compared to ½P and 1P for *A. pulchella. Acacia pulchella* had the greatest concentration of P in leaves (up to 1100 mg P g DW leaves^{-1}).
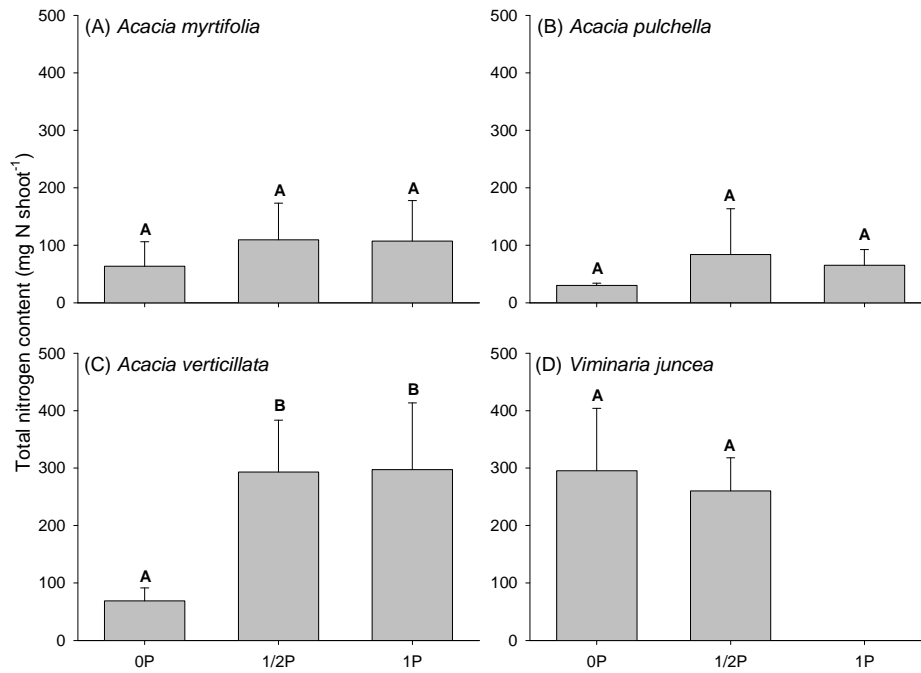
Fig. 10 Total nitrogen content of aboveground biomass (mg N shoot^{-1}) of (A) *Acacia myrtifolia*, (B) *A. pulchella*, (C) *A. verticillata*, and (D) *Viminaria juncea* grown in pot-culture supplied with three different levels of P. Bars represent mean N content (n = 5) and error bars are standard deviation. Different upper case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).
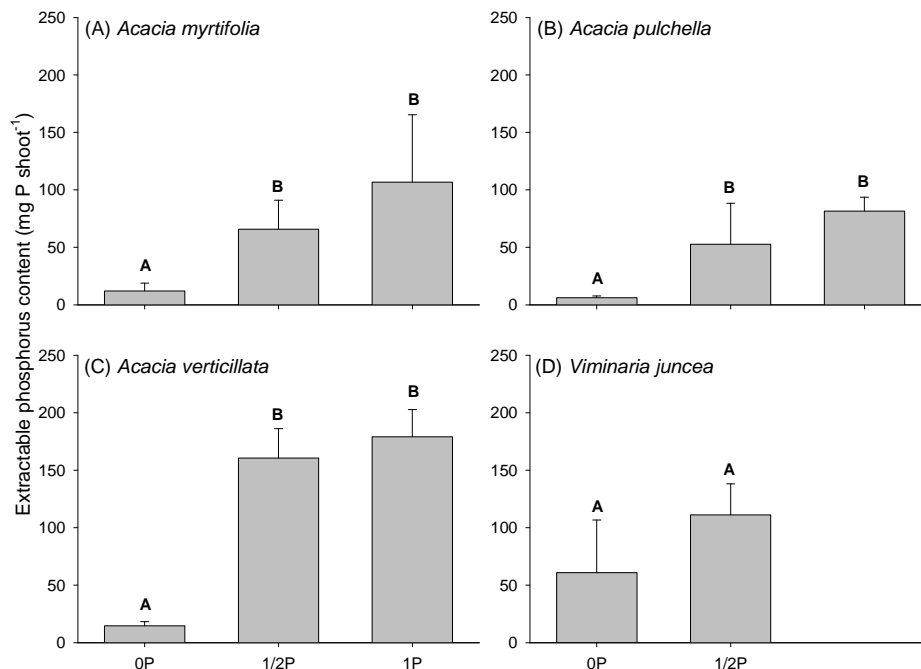
Fig. 11 Extractable phosphorus content of aboveground biomass (mg P shoot^{-1}) of (A) *Acacia myrtifolia,* (B) *A. pulchella,* (C) *A. verticillata,* and (D) *Viminaria juncea* grown in pot-culture supplied with three different levels of P. Bars represent mean P content (n = 5) and error bars are standard deviation. Different upper case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).
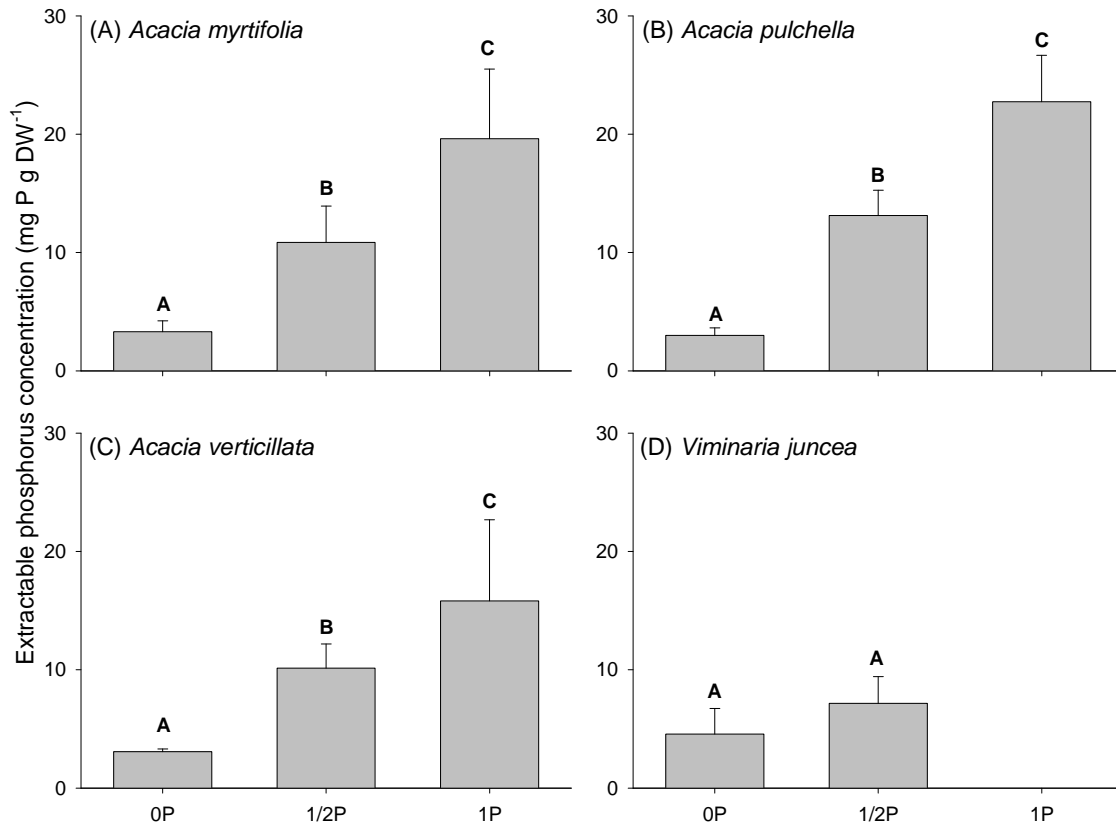
Fig. 12 Concentration of extractable phosphorus in aboveground shoot biomass (mg shoot^{-1}) of (A) *Acacia myrtifolia*, (B) *A. pulchella*, (C) *A. verticillata*, and (D) *Viminaria juncea* grown in pot-culture supplied with three different levels of P. Bars represent mean P concentration (n = 5) and error bars are standard deviation. Different upper case letters represent significant differences among treatments for each species (one-way ANOVA, LSD multiple comparisons, p <0.05).

Two species, *Acacia myrtifolia* and *Viminaria juncea* were common to both experiments. When grown in white sand with no added P (white sand+RP in Experiment 1 or 0P in Experiment 2), both species had 10-fold smaller mean dry weight (total above- plus belowground biomass) in Experiment 1 compared to biomass accumulation in Experiment 2. Mean nodule dry weight and number were similar for *Viminaria juncea* in both experiments (~0.2 g plant^{-1} and ~50 nodules respectively), but there was a five-fold difference in mean nodule number for *Acacia myrtifolia* in Experiment 1 compared to Experiment 2 (124 compared to 22 nodules) and these nodules weighed half that (0.02 g) of the total nodule dry weight collected in Experiment 2 (0.04 g). Foliar N and P content was lower for both species in Experiment 1 compared to Experiment 2. The reason for this is unknown as both experiments were set up and the same time and in the same manner. The only difference was

that plants in Experiment 1 were grown for 9 months prior to harvesting and for 12 months in Experiment 2.

Discussion

The legumes investigated here are native to low nutrient soils of woodlands in south-east Australia. For the four species of *Acacia* and *Pultenaea stricta*, this study is the first report of their ability to form nodules and their ectomycorrhizal status. Only one species, *Acacia myrtifolia* is a new account of formation of cluster roots. There are only two published reports of formation of cluster roots by *Acacia* (Sward 1978, Adams *et al*. 2002) so this is perhaps not a surprising result. Nodule and cluster root formation for *Viminaria juncea* was confirmed (Lamont 1972, Dinkelaker *et al*. 1995, Adams *et al*. 2002) but the mycorrhizal association for this species has not previously been published. In previous studies, *Kennedia prostrata* formed cluster roots (Trinick 1977, Adams *et al*. 2002, Pang *et al*. 2010 a, b) but did not in this study. The reason for this remains unknown.

Interaction of phosphorus and nutrient uptake strategy

The combination of a good source of mycorrhizal innocula (field soil treatments) and P (+RP treatments) promoted greater above- and belowground biomass accumulation, greater nodule formation and greater N and P concentration and content in shoots. For all species apart from *Viminaria juncea*, this coincided with the greatest incidence of ectomycorrhizal infection. Contrary to the original hypotheses posed, equal numbers of plants of *A. myrtifolia*, *A, suaveolens*, *Kennedia prostrata* and *Pultenaea stricta* showed evidence of ectomycorrhizal fungal infection regardless of whether they were grown in high or low P soils and with or without a source of native inoculum. This did not however equate to increased growth, nodulation or nutrient content, even when P was available in soil. The extent of mycorrhizal infection was not measured (e.g. number of infected root tips per root system) so it is possible that even though fungal structures were present in roots, the incidence in some treatments was too low to produce any marked effect on nutrient uptake and growth. As plants grown in white sand were not supplied with a source of native fungal inoculum, it is further possible that the colonising fungal species were different for this treatment and less effective for P uptake. This hypothesis has some credence because when P was present in the growing medium without native inoculum (white sand+RP) there was no significant increase in plant biomass or P content compared to soil with native fungal inoculum (field soil). Phosphorus concentration in shoots and nodulation was greater for plants grown in the presence of P

which indicates that P uptake did occur but not due to any particular root specialisation that was investigated here. This suggests that soil type played some role (Gardener *et al*. 1982, Adams *et al*. 2002) or native fungal inoculants are needed to promote effective mycorrhizal associations for increased P uptake.

If cluster roots were present, they were expected to be profuse in low P treatments. This was the case for *Viminaria juncea*, however, this pattern was not associated with the greatest biomass, nodulation or nutrient content. Plants with access to high levels of P were larger, showed greater nodulation and increased N and P content in shoots compared to plants that did not, regardless of the presence of root clusters or mycorrhizal infection. *Viminaria juncea* grown in pot culture with the combination of field soil and high P had significantly greater P content in shoots compared to white sand+RP. This suggests that other forms of P present in the field soil, such as organic P, may have been available for uptake, possibly by mycorrhizal fungi or cluster roots or both (Adams and Pate 1992).

Plants in all treatment were exposed to a cocktail of commercially available legume bacterial inoculants providing the same potential for nodule formation. Clearly, access to P was important for nodule formation and even more essential for N-fixation as indicated by equivalent numbers of nodules for white sand+RP and field soil+RP treatments but not for plant biomass, nodule weight or N content or concentration. This result is consistent with other leguminous agricultural species including soybean (Israel 1987, Ribet and Drevon 1995, Drevon and Hartwig 1997), common bean (Pereira and Bliss 1987), pea (Jakobsen 1985), clover (Almeida *et al*. 2000, Hogh-Jensen *et al*. 2002) and alfalfa (Drevon and Hartwig 1997).

Effect of phosphorus supply on legume growth and nodulation
In this experiment, *A. verticillata* was the only species to show a significant increase in biomass with addition of P. The other species tested had only small increases in biomass, and in the case of *Viminaria juncea*, a decrease in biomass with addition of P was measured. In many published studies, an increase in host plant growth has been recorded (e.g. Robson *et al*. 1981, Reinsvold and Pope 1987, Yahiya *et al*. 1995), but in equally as many others, no increases in host plant growth were recorded (e.g. Sanginga *et al*. 1991). Robson *et al*. (1981) suggested that P increased N-fixation in subterranean clover by stimulating host plant growth rather than by exerting an effect on rhizobial growth and survival or on nodule formation and

function. This seems logical as an increase in host plant growth would allow greater allocation of nutrients and carbohydrates to roots and nodules. This does not seem to be the case for the legumes tested in this study but it should be kept in mind that they differ from clover by being perennial and woody (Pate *et al.* 1993, 1998) and native to an inherently nutrient-poor ecosystem.

An increase in supply of P generally translated to an increase in N concentration and content of shoots. However, N concentration and content of shoot systems did not differ significantly between P treatments for *A. verticillata* and *A. pulchella* suggesting that P does not have a limiting role in nodule formation, at least for these two species. In contrast, whole plant N content and/or N concentration in leaves increased as result of P fertilisation in studies with common bean (Pereira and Bliss 1987), pigeon pea (Itoh 1987) and cowpea (Cassman *et al.* 1981). Again, these are all herbaceous species compared to the perennial woody species tested.

Nodule biomass was stimulated by increase in P supply but the pattern was not the same for root dry weight, particularly in *A. myrtifolia* and *A. pulchella*. This indicates that the effect of external P on nodulation was independent of host plant growth as proposed by Gentili and Huss-Danell (2003). As shown in Experiment 1, the relatively greater effect of P on nodulation than on host plant growth suggests that nodule development is reliant on P supply and is therefore sensitive to P deficiency.

Total dry weight of nodules was low when plants were supplied with no P. Phosphorus deficiency limits nodulation as well as nodule growth and function, thus reducing N-fixation capacity (Cadisch *et al.* 1989). Early studies suggested that P deficiency reduces root growth of the host plant and limits photosynthetic carbohydrate supply to nodules (Jakobsen 1985). As a consequence of the host plant being P-deficient, the demand for N by the host plant is also reduced and it is likely that the proportion of photosynthate partitioned to nodules decreases (Yang 1995). Nodules are also known to be the strong sinks for P, particularly under conditions of P-deficiency (Al-Niemi *et al.* 1997, Tang *et al.* 2001, Hogh-Jensen *et al.* 2002).

There was a consistent increase in foliar P concentration with increased P application in the native legumes investigated. In legumes, application of P has been found to increase N

concentration in host plant tissues by stimulating rates of N-fixation (Knight 1986, Israel 1987, Schulze 2004, Schulze *et al*. 2006). For other species, such as *Acacia* (Witkowski 1994) and various native heaths (Specht and Groves 1966, Grundon 1972), an increase in growth has been recorded with addition of low concentrations of nutrients but a decline in growth was found at higher concentrations. Other studies have found no difference in the growth of Australian grassland species (Bennett and Adams 2001) or heathland Epacridaceae (Bell *et al*. 1994) following the addition of P despite the presence of mycorrhizal associations.

In this study and others that have been made reference to, only a small number of species have been investigated experimentally. It is likely that a wider spectrum of native legumes will have even greater diversity of root specialisations, distributions and morphologies than currently documented and the interactions among these and nutrient supply are expected to be complex. Regardless, this potential puts Australian native legumes at the forefront of research for agricultural application (Denton *et al*. 2006, Robinson *et al*. 2007, Pang *et al*. 2010b, Suriyagoda *et al*. 2010) and possibly soil remediation (Reichman 2007) and salinity (Manchanda and Garg 2008) and for developing our understanding of plant functioning and biodiversity in low nutrient environments (Pate and Hopper 1993, Hopper and Gioia 2004).

From this study it is clear that mycorrhizal associations are required for increased growth and P uptake, and, putative enhancement of N-fixation in all but one of the species studied. The presence of cluster roots on *Viminaria juncea* conferred a greater advantage than mycorrhizal associations to plant growth, N-fixation and P uptake. Simply increasing the supply of P does not necessarily increase plant growth, nodulation and nutrient content. Further investigations are required to determine how widespread the occurrence of cluster roots and mycorrhizal associations are in native Australian legume species and if there is a taxonomic and/or ecological basis to this distribution.

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