final report

Project code: B.PUE.0104 (AWI Project WP 564)

Prepared by: *Richard Simpson, Rebecca Haling, Graeme Sandral, Megan Ryan et al.*
CSIRO Agriculture, NSW Department of Primary Industries, University of Western Australia

Date published: 16 January 2017

PUBLISHED BY
Meat and Livestock Australia Limited
PO Box 991
NORTH SYDNEY NSW 2059

Phosphorus-efficient legume pasture systems

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Phosphorus (P) fertiliser is a critical input for productive pasture systems in southern Australia but the cost of fertiliser has risen substantially. There is a major opportunity to counter rising costs by improving the efficiency of P-use on farms. This project determined the critical soil test P requirements (i.e. soil test levels needed for near maximum growth) of alternative pasture legumes to establish soil fertility benchmarks for pasture management. Two species of serradella (*Ornithopus sativa, O. compressus*) were found to have very low critical P requirements relative to subterranean clover (*Trifolium subterraneum*) and it was estimated that their use in fertilised pastures could reduce P fertiliser costs by ~30% annually. When grown in moderately P-deficient soils the serradellas also achieved about twice the yield of subterranean clover. Low-P pasture systems can be developed immediately in areas where serradellas are already grown. Wider adoption hinges on understanding how widely serradellas can be grown in permanent pasture areas. High P efficiency was associated with long, fine roots that have long root hairs. This allows a plant to forage effectively for nutrients in soil. This knowledge is being used to identify more P-efficient lines of subterranean clover to improve yields in moderately fertilised paddocks and to push subterranean clover closer to the very high P efficiency of the serradella species.

Executive summary

Background:

Phosphorus (P) fertiliser is essential for high production and profitability in Australian agriculture because most soils are deficient in P. However, the cost of P fertiliser has doubled over the last 15 years and the efficiency with which P is used in Australia is very low. For example, beef/sheep enterprises must typically apply 5-9 units of P to produce only 1 unit of P in animal products. Low P-use efficiency represents a major opportunity for farmers because improvements in the efficiency with which P is used will help to counter the threats to farm profitability and global competitiveness that are posed by tightening global P supply and an increasing P-fertiliser price.

The main reason for low P efficiency is the accumulation of P in the soil when fertiliser is applied (sometimes termed "P-fixation"). The rate of P accumulation is positively correlated with the soil test P (STP) concentration at which the soil is maintained by applications of P fertiliser. The STP concentration needed for maximum pasture production is determined by the high P requirements of pasture legumes such as *Trifolium subterranean* (subterranean clover), the most widely-used pasture legume in southern Australia. The STP concentration needed for near maximum yield by the legume is termed its 'critical P requirement'.

Pasture legumes that achieve equivalent herbage yields at lower STP concentrations (i.e. lower critical P requirements) than *T. subterraneum* may reduce the amount of P fertiliser needed for productive pastures because fertilising soils to lower STP concentrations is expected to reduce the rate at which P accumulates in the soil. Since the late 1990s numerous alternative pasture legumes have been developed in Australia, mainly to address gaps in areas of the agricultural landscape and in farming systems where subterranean clover cannot be used reliably. Little is known about the P requirements of these species.

This project was undertaken to benchmark the STP requirements of alternative pasture legumes relative to *T. subterraneum* with the aims of: (a) identifying legumes with lower critical P requirements, and (b) providing guidelines for optimal management of soil P fertility for pastures. Parallel research was initiated to understand the reason(s) why *T. subterraneum* has a high critical P requirement and, by using this knowledge, to examine the feasibility of developing more P-efficient *T. subterraneum* cultivars.

Field experiments to benchmark the critical P requirements of alternative pasture legumes

Field experiments (Chapter 4) were undertaken concurrently with more detailed glasshouse experiments to benchmark the critical external P requirement of a range of alternative legume species relative to *T. subterraneum*. Field sites were established on the tablelands at Yass (2012-2014) and Burrinjuck (2013-2013), and in the Riverina at Belfrayden and Beckom (2014), NSW. The latter two sites were especially necessary to test the critical P requirement of some legume species that were found not to be well-adapted to the tablelands environment. The critical external P requirement was determined as the Colwell- or Olsen-extractable P concentration at 90% or 95% of maximum yield. The legume species were grouped into either a low, intermediate, high or very high P requirement category. After considering the repeatability of species STP benchmarks among growing seasons and sites,

the data was further distilled to three categories of P-requirement for practical, P-efficient management of pastures (see Conclusions).

The *Ornithopus* spp. had consistently and substantially lower critical P requirements than *T. subterraneum,* but only *O. sativus* (cv. Margurita) achieved similar dry matter yields. The results indicate that *Ornithopus* spp. are potentially suitable for developing low-P grazing systems, but further work will be required to test the adaptation and persistence of these species in a wider range of farming districts.

Detailed investigations of the root morphology traits among the alternative pasture legumes and their role in achieving a low critical P requirement for maximum growth

An initial screen of root traits associated with soil phosphorus acquisition was undertaken for a range of pasture legumes and two grass species (Chapter 5), many of which had been included in the field benchmarking experiments (Chapter 4). Up to a 3.6-fold range in specific root length (79–281 m root g^{-1} root) and 6-fold range in root hair length (0.12–0.75 mm) was found among the species. This translated into large differences in the effective volume of soil explored and, consequently, the nutrient foraging potential among the species. The commonly used *T. subterraneum* (cv. Leura) and *M. sativa* (cv. SARDI 10) had relatively low specific root lengths, short root hairs and low nutrient foraging potentials. *O. compressus*, *O. sativus* and *B. pelecinus* had root hair lengths, specific root lengths and consequently nutrient foraging potentials more similar to those of the two grass species. It was hypothesized that differences among the species in root morphology result in significant differences in their critical P requirements.

In Chapters 6 and 7, five legume species and one grass species that had previously been shown to differ in in their nutrient foraging capacity (Chapter 5) were grown in an experiment to investigate how root morphology influenced nutrient foraging and the critical P requirement of the pasture legumes. The experiment mimicked results from the field experiments in which *O. compressus* and *O. sativus* had critical P application requirements that were about half that of *T. subterraneum*. Importantly, in these controlled-environment experiments the *Ornithopus* spp. were able to yield as well as *T. subterraneum*, which suggested potential for them to be used as productive legumes in low P-input grazing systems. *B. pelecinus* and *T. hirtum* had critical external P requirements that were also lower than that of *T. subterraneum* but they did not yield as well.

It was demonstrated that species with lower critical P requirements were able to take up more P per unit root dry mass than those with higher critical P requirements because they deployed a combination of long, fine roots with long root hairs to maximize the volume of soil explored. This enabled them to capture more P at lower soil extractable-P concentrations. *T. subterraneum* (cv. Leura) achieved a very high root length density in response to low soil P concentrations but failed to capture P efficiently due to its very short root hairs.

The critical external P requirement and root morphological traits of a much wider range of legumes was characterised in a glasshouse experiment (Chapter 8). The experiment confirmed results of Chapters 6 and 7 that significant variation in the critical external P requirement exists among potential pasture legumes, and that this is driven by differences in the ability of a legume to explore soil effectively with its root system. Again, *O. compressus* and *O. sativus* were found to yield as well as *T. subterraneum* but with less than half the amount of applied P. Also of interest, *B. pelecinus* has consistently been reported as having

root characteristics suited to P-efficiency (Chapters 5 to 8), and indeed has a low critical external P requirement when characterised in controlled-environment studies (Chapters 6 and 8), but its critical P requirement in field trials has not reflected this (Chapter 4). The reason(s) for this should be investigated further with the objective of capitalising on the anticipated P efficiency of this species. Likewise, some *Trifolium* spp. (e.g. *T. michelanium* balansa clover) were found to have a lower critical external P requirement than *T. subterraneum*, which could prove useful in the niche farming environments to which they are suited.

The root system features of subterranean clover that result in its high P requirement and potential for improving its P efficiency.

Root morphology: By this stage of the project it had been established that long, fine roots (i.e. high specific root length) with long root hairs enables some legume species to achieve a low critical external P requirement. Conversely, lack of these root morphology attributes was behind the high critical P requirement of *T. subterraneum*. Intra-specific variation in these key root morphology traits, and other traits that could potentially confer P acquisition efficiency, was examined using a collection of heritage and modern subterranean clover cultivars and the 'core' collection of subterranean clover lines (representing ~80% of the genetic variation in *T. subterraneum*) (Chapters 9 and 10.). The ranges in root traits was generally similar for the collection of cultivars and the core collection. Root hair length of the core collection ranged two-fold from 0.21 to 0.43 mm. Specific root length of the core collection' ranged two to three-fold; from 36-105 m g^{-1} for 3 week-old plants to 86-161 m g^{-1} for 6-week old plants. Specific root length was negatively correlated with average root diameter. Lateral root angles (i.e. degrees from vertical) that reflect shallow rooting are a potential indicator of the ability of plants to explore nutrient-rich topsoil layers ranged from 41 to 68°(core collection) at 0-5 cm depth and 54 to 68° at 5-10 cm depth, and were highly correlated between depths. The ability of subterranean clover lines to achieve high root length density (i.e. to proliferate roots) in response to P-enriched soil patches also varied among the clover lines. Despite the wide range in root hair lengths of *T. subterraneum*, the longest root hair lengths did not approach the lengths of root hairs in the *Ornithopus* species which were 2- to 3-fold longer than those of *T. subterraneum.* The specific root length (fineness of roots) of the *T. subterraneum* lines also fell short of that observed in the highly P-efficient *Ornithopus* spp. However, the *T. subterraneum* lines with the finest roots were within 70%-80% of the specific root length of the *Ornithopus* species.

Large (2-fold) differences in herbage yield among the cultivars of *T. subterraneum* were observed in moderately P-deficient soil in controlled-environment experiments (Chapters 10 and 11). About half of the variation in P uptake from low P soil was associated with variation in root proliferation (nutrient foraging) among the cultivars. This observation must be checked in future field experiments but, if confirmed, will prove important for increasing the productivity of farm paddocks that are managed with moderate soil P fertility.

Whilst there are clear gains to be made by selecting in favour of more P-efficient *T. subterraneum* cultivars, it must be recognised that the yields of even the most P-efficient clover lines were well behind the yield achieved by the *Ornithopus* spp. in moderately P-deficient soil. The differences between the species in their P acquisition efficiency was mainly a result of differences in root morphology. Root proliferation was comparable for both species, but *T subterraneum* only achieved high root length densities by allocating high proportions of

plant dry mass to its nutrient foraging roots. This was costly for herbage production. The *Ornithopus* spp. did not need to do this because of their finer roots and achieved much greater soil exploration in low P soil because they also had very long root hairs (Chapter 7).

Inter-specific variation in root traits associated with nutrient foraging (i.e. long root hairs and/ or high specific root length) could provide a potentially useful source of genetic variation for improving the P-efficiency of *T. subterraneum*. An initial examination was made of root morphology traits in seven *Trifolium* spp. that are genetically-allied to, and can potentially be hybridised with *T. subterraneum* (Chapter 14). While significant variation in nutrient foraging traits were observed among the *Trifolium* spp., the range in root traits did not generally exceed that measured for *T. subterraneum*. However, *T. meduseum* and *T. pauciflorum* had relatively long root hairs (~0.4 cf. 0.25 mm in *T. subterraneum*). This preliminary investigation indicated that further work should be conducted to quantify the range in variation within, and among these species and to determine whether successful inter-specific crosses can be made.

Mycorrhizal colonisation of clover roots: Arbuscular mycorrhizal fungi (AMF) associations with roots are an important for P acquisition in many species, and it has been reported that their contribution to P nutrition might be more pronounced in species with short or no root hairs. Chapter 12 reports the intra-specific variation in colonisation of *T. subterraneum* roots. Colonisation level ranged from ~12 to 68% of root length among *T. subterraneum* lines. It was also established that key root traits for enhancing plant P-uptake may change in the presence of AMF, but not sufficiently to warrant deliberate inclusion or exclusion of AMF when screening germplasm for such traits.

Carboxylate exudation from roots: Exudation of carboxylates into the rhizosphere is a strategy which can enable plants to access sparingly-available forms of P. Chapter 13 reports the quantity and composition of carboxylates in the rhizosphere of a range of the alternative legume species, *T. subterraneum* and two grasses. There was significant variation in both the quantity and composition of carboxylates measured in the rhizosphere of the pasture legumes species. Citrate was the dominant carboxylate in the rhizosphere of most species, including *T. subterraneum*. Malonate, followed by citrate, were the dominant carboxylates in the rhizosphere of the *Ornithopus* spp. The *Ornithopus* spp. were found to exude high amounts of carboxylates per unit root dry matter but, due to their high specific root length, this did not translate into a high quantity per unit root length or a high concentration in the rhizosphere. *T. subterraneum* released small quantities of carboxylates into its rhizosphere and it was concluded that this was not an important P acquisition trait for subterranean clover.

Aligned PhD research: Chapter 15 reports progress in an aligned PhD project titled “Root morphology and P-efficiency impacts of root disease organisms” (undertaken by Robert Jeffery) that is presently underway. The experiments investigated the shoot and root growth of *T. subterraneum* and *O. compressus* in response to P, root disease and/ or mycorrhizal colonisation. A range of experimental conditions and soil microbial factors that may modify the legume responses to P were examined in detail. The experiments demonstrated the importance of simulating pasture sward conditions in P nutrition studies and the complex interactions that can exist between AMF or root pathogens and legume P nutrition. This informed the experimental methods used throughout the project.

Conclusions

Three STP benchmark groups for a range of alternative pasture legumes are proposed for practical and efficient soil P management:

Very high P requirement (Olsen P >17; Colwell P[1] >50 mg/kg): *Medicago sativa* (lucerne)

High P requirement (Olsen P = 15, Colwell P = 35 mg/kg): *Biserrula pelecinus* (biserrula), *M. truncatula* (barrel medic), *Trifolium glanduliferum* (gland clover), *T. hirtum* (rose clover), *T. incarnatum* (crimson clover), *T. spumosum* (bladder clover), *T. subterraneum,* (subterranean clover)

Low P requirement (Olsen P = 10; Colwell P = 25 mg/kg): *Ornithopus compressus* (yellow serradella), *O. sativus* (French serradella), *Lotus corniculatus* (birdsfoot trefoil), *T. purpureum* (purple clover)

Substantially more P-efficient pasture systems can be developed for temperate southern Australia by using either yellow serradella or French serradella in place of subterranean clover. If serradella pastures are fertilised to the critical STP requirement of serradella, rather than to the higher STP concentrations recommended for subterranean clover or white clover pastures, it is estimated that they may require up to 30% less P fertiliser for equivalent production. In P-deficient soils, the serradellas can also substantially out-yield subterranean clover.

However, wide adoption of P efficient pasture systems will depend on how widely serradellas can be used as the key legume in mixed, permanent pastures. There is a major gap in knowledge about the adaptation range for these species.

Investigation of root morphology among *T. subterraneum* lines indicated there was useful variation in subterranean clover root morphology. It was concluded that selecting for P-efficient root traits can substantially improve the yield of subterranean clover grown in moderately P-deficient soils, and this will potentially make a large difference to pasture yields in moderately P-deficient soils. The performance of P-efficient clover cultivars is yet to be confirmed in field experiments.

Breeding for improved P-acquisition efficiency in *T. subterraneum* should focus on selecting lines with: (i) higher specific root lengths (small root diameters with low root tissue densities) because this will increase the effectiveness and persistence of root proliferation, and (ii) substantially longer root hairs.

The way forward for improving the P efficiency of temperate pastures presents an interesting dilemma. The *Ornithopus* spp. have the root systems necessary for high P efficiency and P-fertiliser savings, but are presently restricted in their adaptation range. Research and development may overcome this limitation. The yield of some lines in areas where serradella has not been used were very encouraging, but possible issues of legume persistence remain to be explored. In contrast, *T. subterraneum* cultivars with improved P-efficiency have been tentatively identified and may potentially deliver production benefits. However, there is a substantial step between the most P-efficient lines of *T. subterraneum* and the *Ornithopus* spp. To span this P-efficiency gap, clover lines with roots that have higher specific root length and considerably longer roots hairs will need to be developed. Higher specific root lengths are possible with the resources of the *T. subterraneum* genome, but more radical genetic steps

[1] Colwell P benchmarks are rounded to the nearest 5- or 10-unit equivalent and are applicable to soils with PBI = 40-80.

(inter-specific hybridisation, directed mutagenesis) may be required to achieve the lengths of root hairs needed to emulate the high P-efficiency of the serradellas.

Table of contents

1 Background

1.1 Phosphorus use efficiency of pasture systems

Phosphorus (P) fertiliser is essential for high production and profitability in Australian agriculture because most soils are deficient in P. However, the efficiency with which P is used is very low. Data collated for sheep and beef enterprises in southern Australia indicate that the median P-balance efficiency (PBE)[2] of grazing farms is only 10-20%. This means that 5-9 units of P is applied as fertiliser to produce 1 unit of P in animal products (McLaughlin *et al*. 1992; Weaver and Wong 2011). Under ideal circumstances, P inputs would equal P removals indicating maximum P-use efficiency (i.e. no P surplus).

Inefficient use of P in sheep and beef grazing systems can be the result of P losses from a field. For example, large losses occur when soils have a very low P-sorption capacity (e.g. sandy soils in SA, WA and coastal areas; Russell 1960; Ozanne *et al*. 1961) or are eroded. However, on the majority of Australian farms, P-inefficiency is associated with accumulation of phosphate in sparingly-available forms in the soil (Simpson *et al*. 2014; Simpson *et al*. 2015). This is the result of "P-sorption"[3] reactions between phosphate and the soil particles (Barrow 1999; McLaughlin *et al*. 2011). Some P is also incorporated into and then accumulated in soil organic materials that resist mineralisation (Barrow 1969; Turner *et al*. 2005)[4]. In addition, grazed fields also accumulate P in stock camp areas as a result of disproportionate deposition of excrement in these areas (Williams and Haynes 1992).

1.2 The P-efficiency opportunity

Low P-use efficiency represents a major opportunity for Australian farmers because improvements in the efficiency with which P is used will help to counter the threats to farm profitability and global competitiveness that are posed by the increasing price of P-fertiliser and the likelihood of tightening global supply.

Simpson *et al*. (2015) have examined the impact of soil P management practices on the magnitude of P accumulation in a long-term grazing experiment near Canberra. They also assessed where P was accumulated in fertilised paddocks. The major sink for P was accumulation in the topsoil layers of non-camp areas in grazed fields (Fig. 1.1). Only ~6% or less of the accumulated P was accounted for in sheep camps.

[2] P-balance efficiency (PBE) is a useful measure for summarising the overall P efficiency of an agricultural system. It is defined as the ratio of P outputs in products, to P inputs in fertiliser and feed (Syers *et al*. 2008; Weaver and Wong 2011). The median PBE of dairy farms is slightly higher (~30%) than that of sheep and beef farms (10-20%). Inputs of P to cows in supplementary feed that initially bypass the soil,and high outputs in milk are likely reasons for the marginally higher P-use efficiency in dairy systems. The PBE of grain production on similar soil types is also low (45-54%) but substantially better than that of grazing systems.

[3] Here, we use the term "P sorption", as proposed by Barrow (1999), to represent the net process of phosphate movement from soil solution to the solid phase of the soil and the continuing slow reactions between phosphate and soil particles that ultimately result in phosphate being only sparingly available for plant uptake.

[4] The net result of all of these these P accumulation reactions is sometimes incorrectly termed "P-fixation".

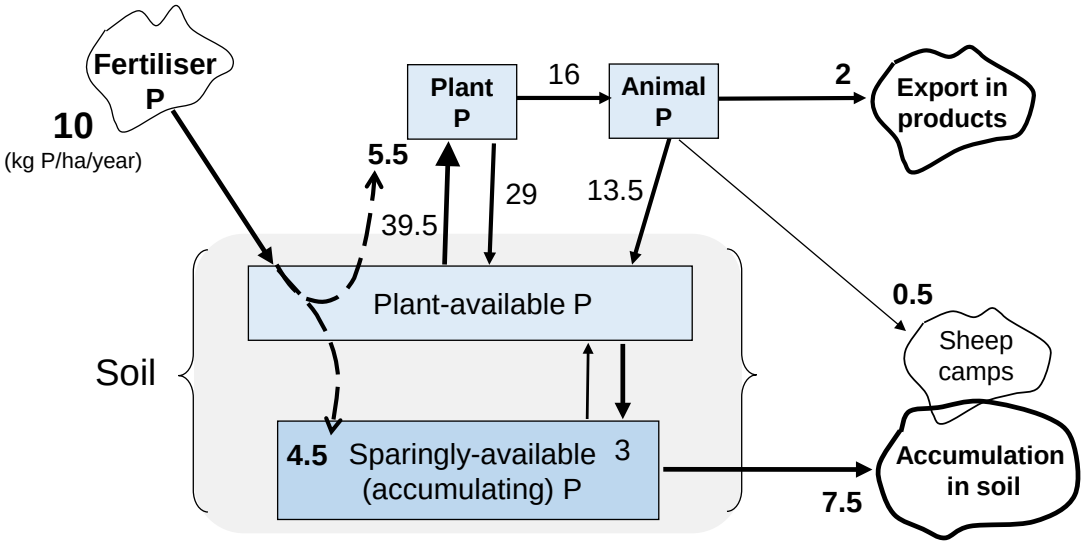


Figure 1.1 Net annual flows of P in the P cycle of a continually-grazed, subterranean clover-based pasture. The data are derived from a long-term soil P fertility x grazing experiment near Hall, ACT that was being maintained close to the critical soil test P concentration for maximum production by annual P fertiliser applications (Simpson *et al*. 2015; McLaren *et al*. 2015a). Values in bold are measured P flows. Other values were deduced.

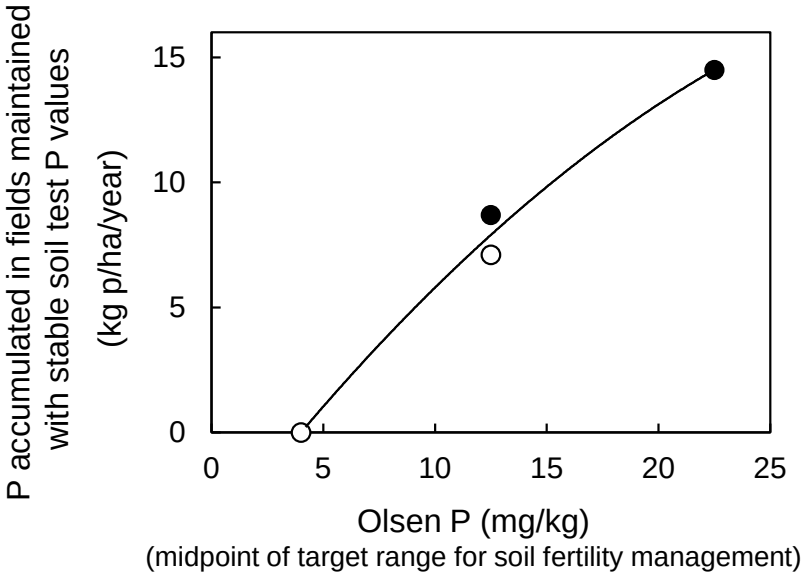


Figure 1.2 Amounts of P accumulated annually in grazing systems maintained for six years with relatively stable soil P fertility. Closed symbols (●) indicate pastures grazed with 18 sheep/ha, open symbols (○) indicate pastures grazed with 9 sheep/ha (data from Simpson *et al*. 2015).

The grazing system treatments in this experiment were maintained at three levels of soil P fertility and from this it was shown that the P accumulated in grazed fields (i.e. the component of P associated with fertiliser inefficiency) was greater when paddocks were maintained at higher extractable-P (i.e. soil test P [STP]) concentrations (Fig. 1.2). The immediate implication of this finding for P-efficiency is that farmers should avoid over-fertilising because higher STP concentrations are associated with more P accumulation in the soil. The result also indicated that if grazing systems could be managed productively at lower STP concentrations, less P would be accumulated in fields and this should translate into a lower P fertiliser requirement (Simpson *et al*. 2014).

Pasture legumes have the highest P requirements of the species used in temperate grass-legume pastures (Ozanne *et al*. 1969; Ozanne *et al*. 1976; Hill *et al*. 2006) and their requirements set the STP concentration to which a pasture is fertilised for optimum production. On this basis, Simpson *et al*. (2014) argued that using pasture legumes with lower "critical" P requirements than subterranean clover (i.e. legumes that achieve equivalent maximum yields but at lower STP concentrations), would reduce inefficiency in P use in pasture systems and that this would translate into a reduction in the P-fertiliser cost of production.

1.3 Estimated savings in the P-fertiliser cost of pasture production

The on-going, annual savings in P fertiliser that might accrue from using a low-P, alternative legume to subterranean or white clover can be calculated using data from Simpson *et al*. (2015). About 2 kg P/ha was exported annually in animal products from optimally-fertilised paddocks in the experiment. Optimal P fertility for a subterranean clover-based pasture is considered to be an Olsen STP (Olsen *et al*. 1954) concentration (top 10 cm soil layer) of ~15 mg P/kg (*Gourley et al. 2007*; Moody 2007); the equivalent Colwell STP (Colwell 1963) value for soil at this site (Phosphorus Buffering Index = 50) was ~30 mg P/kg. When the pasture soil was maintained at this STP concentration, approximately 10 kg P/ha was accumulated in the grazing system each year (Fig. 1.3). About 95% of this was accumulated in the soil. Given that 2 kg P/ha was exported each year and other losses of P from the system were negligible, it can be estimated that the grazing system requires about 12 kg P/ha/year for soil P fertility to be maintained at a level that ensures near-optimum production.

Assume that subterranean clover in this system can be replaced by a novel alternative legume that yields as well as the clover but achieves its maximum yield at a lower Olsen P concentration; e.g. at ~10 mg P/kg (equivalent Colwell P will be ~20 mg P/kg). On this basis, it can be predicted from Fig. 1.3 that the amount of P accumulated in the system each year will be ~6 kg P/ha. P export from the field will be unchanged because the two pasture types are assumed to have equivalent yields. Consequently, the P input for maintenance of production at the new optimum STP concentration will be ~8 kg P/ha/year. This represents a ~33% reduction in P-fertiliser costs as a result of using the novel pasture legume.

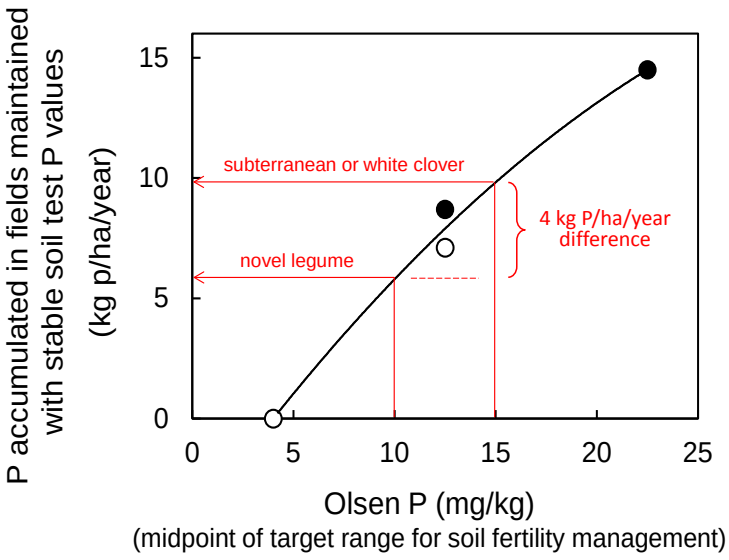


Figure 1.3 Method for estimating the amount of P that will be accumulated annually in a grazed field when it is maintained with a relatively stable STP concentration (based on data from Simpson *et al*. 2015). The amount of P exported annually from the fields receiving near-optimum P inputs was ~2 kg P/ha. The amount of P fertiliser required to maintain production at the nominated the STP concentration is calculated as: 'P exported' plus 'P accumulated in the field'.

1.4 Significance for the sheep/beef industries

1.4.1 Global phosphorus supply

P-fertilisers are manufactured from high-grade phosphate rock "reserves". These P reserves are the phosphate rock deposits that can be mined economically at "today's price" and are considered to be a finite resource. Predictions of imminent global shortages (e.g. Cordell *et al*. 2009, Cordell and White 2011) are now considered unlikely. Present estimates indicate that the known P reserves may last ~300-400 years at current rates of use (Van Kauwenburgh 2010). However, there is much debate about the longevity of P reserves and some analysts continue to predict a much shorter period of P availability at current prices (Reijnders 2014).

The world also has vast lower-grade phosphate rock "resources", but they are classed as uneconomic to mine at today's price. They will only become available for use at a considerably higher cost than is presently experienced (Van Kauwenburgh 2010).

There is potential for significant instability in the cost of P. An example of how unstable P prices can be is shown by the transient 6-fold increase in the price of P that was experienced during 2007/08 (Fig. 1.4). Instability such as this is regularly discussed by many analysts as a significant global risk because phosphate rock reserves underpin global food security. Price instability would potentially impact food production and contribute to social unrest.

There is no doubt that price instability would also adversely impact the profitability and stability of production for Australian farms. Australian farmers are very sensitive to changes in P-fertiliser costs. During the price spike of 2007/08, many stopped applying P. Fortuitously, this occurred at a time when drought had encouraged farmers to reduce stock numbers and the temporary halt to fertiliser use had little impact on production. However, Lean *et al*. (1997) document the situation that can arise when inadequate P-fertiliser investments push a grazing business into a downward production and profitability cycle.

The main reason for the continuing discussion about P supply and price stability is the very uneven distribution of P resources across the world. Morocco presently controls 85% of the global phosphate rock reserves with China the next largest P source (6% of global supply). Within 30 years, USA reserves will be depleted and Morocco and China will control P supply (Van Kauwenburgh 2010).

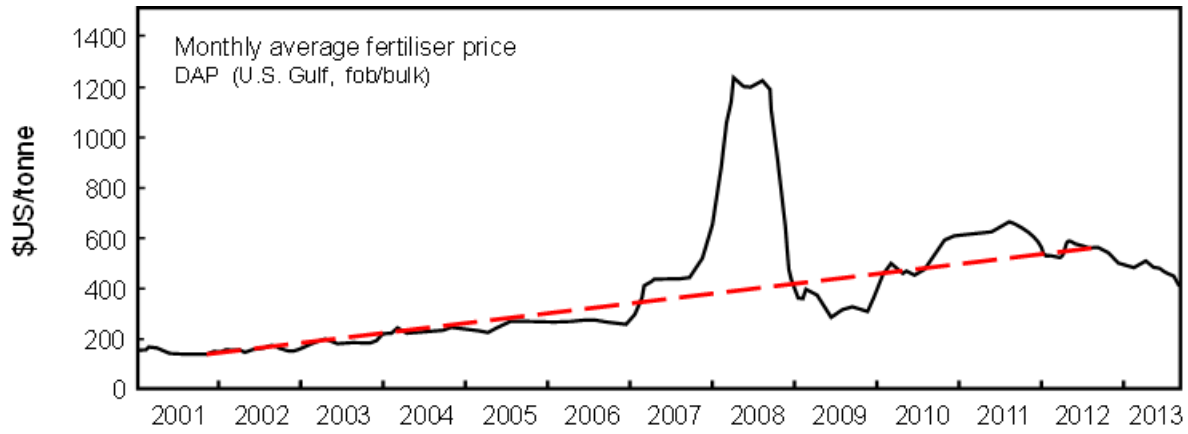


Figure 1.4 The global phosphorus price trend since 2000 (source: IFDC 2014)

1.4.2 Increasing phosphorus prices place a disproportionate burden on the terms of trade for Australian farms

Of immediate concern for Australian farms, is the rising cost of P which has more than doubled since 2000 (Fig. 1.4; IFDC 2014). This is more than twice the rate of inflation in Australia. The current trend in the underlying P price was predicted by Frantel *et al*. (1985;1988) who analysed the likely increase in costs of production (for 1990-2010) as new phosphate mines are opened to maintain global supply. Global population growth into the future and responses to climate change that will see land sown to biomass energy crops are expected to drive even higher global reliance on the world's P reserves (Sutton *et al*. 2013). It is hard to escape the conclusion that it is highly likely that the price of P will continue to rise.

P is a significant component of production costs in grazing enterprises. For example it accounts for 20-25% of the annual variable costs in sheep/beef enterprises (McEachern *et al.* 2010) and is often the largest cost after labour and debt-servicing. Consequently, a rapidly increasing P price places a disproportionate burden on the terms of trade for Australian farms and on the competitiveness of their products on world markets because of the high dependence of Australian agriculture on P fertiliser inputs. Improvements in the P efficiency of farming will mitigate against the impacts of rising P costs.

1.5 Overarching aims of the project

The overarching objectives of the project were to:

(i) Benchmark the P requirements of alternative pasture legumes relative to subterranean clover with the aim of (a) identifying legumes with lower critical P requirements than subterranean clover, should they exist, and (b) providing guidelines for optimal management of soil P fertility of pastures.

(ii) Understand the reason(s) why subterranean clover has a relatively high critical P requirement and, using this knowledge, to examine the feasibility of developing more P-efficient cultivars of subterranean clover.

2 Project objectives

- Prove that highly productive pasture systems can be operated with substantially less phosphorus (P)-fertiliser by using plants with low 'critical' P requirements.
- Quantify (benchmark) the critical P requirements of key pasture legume species relative to subterranean clover. P-fertiliser management guidelines, strategies for targeted fertiliser use and objective information concerning the P-fertility requirements of emerging, novel, alternative pasture.
- Identify the root morphology traits that have the largest influence on the critical P requirements of subterranean clover and alternative legume species.
- Assess the variation in P-efficient root traits of subterranean clover and quantify the potential for breeding P-efficient clovers.
- Clear decision point for breeding improved subterranean clovers, and/or evaluation of alternative legume species for P-efficient farming systems.
- Improved environmental credentials for grazing industries with respect to efficiency of fertiliser use, reduced over-applications, and less loss of P to the wider environment.

3 The research team

Richard J. Simpson, Rebecca E. Haling, Adam Stefanski, Zongjian Yang, Richard A. Culvenor

CSIRO Agriculture, GPO Box 1600, Canberra ACT 2601. Contact: richard.simpson@csiro.au

Graeme A. Sandral, Andrew Price, Shane Hildebrand, Chris Fuller

Department of Primary Industries, Wagga Wagga Agricultural Institute, Pine Gully Road, Wagga Wagga, NSW, 2650. Contact: graeme.sandral@industry.nsw.gov.au

Megan H. Ryan, Daniel R. Kidd, Hans Lambers, Robert Jeffery

School of Plant Biology and Institute of Agriculture, The University of Western Australia, Crawley, WA, 6009. Contact: megan.ryan@uwa.edu.au

Phillip G.H. Nichols,

Department of Agriculture and Food Western Australia, 3 Baron-Hay Court, South Perth, Western Australia and the Institute of Agriculture, The University of Western Australia, Crawley, WA, 6009.

4 Critical external phosphorus requirements of pasture species grown under field conditions

Graeme A. Sandral, Richard J. Simpson, Andrew Price, Shane Hildebrand, Chris Fuller, Rebecca E. Haling, Adam Stefanski, Zongjian Yang, Richard A. Culvenor, Megan H. Ryan, Daniel R. Kidd, Hans Lambers

4.1 Introduction

The external phosphorus (P) concentration of soil drives pasture legume growth and nitrogen fixation in most Australian pastures. Consequently, P is a critical input for grazing enterprises. However, the cost of P has risen steeply since 2000 and there is every expectation that global demand for P will continue to increase and price pressures will persist into the future (Sutton *et al*. 2013). The P balance efficiency of fertilised pastures in Australia is very low with about 5-9 units of P applied as fertiliser in sheep-beef enterprises to produce 1 unit of P in animal products (McLaughlin *et al*. 1992; Weaver and Wong 2011). If P efficiency can be improved there is an opportunity for Australian farners to reduce their P costs even in the face of a rising global price. The most common reason for low efficiency is accumulation of P in soil (McLaughlin *et al*. 2011) although other P losses (erosion, leaching, runoff) can also occur, particularly from sandy soils with low P sorption capacity. Pasture legumes that achieve equivalent yields at lower soil test P concentrations (i.e. lower critical P requirements) than *Trifolium subterraneum* (the most widely-used pasture legume) may reduce the amount of P fertiliser needed for productive pastures because fertilising soils to lower soil test P concentrations is expected to reduce the rate at which P accumulates in the soil (Simpson *et al*. 2014). Since the late 1990s numerous alternative pasture legumes have been developed, mainly to address gaps in the areas of the agricultural landscape and farming systems where subterranean clover cannot be used reliably (Nichols *et al*. 2012). Little is known about the P requirements of these species. Here we report results from field experiments examining the growth during spring of a number of alternative legumes and two perennial grasses in response to six P fertility levels. This research aimed to determine the external critical P requirement (i.e. the soil extractable-P concentration required to achieve 90% of maximum yield) of a range of pasture legume species under field conditions. The critical P requirement of the legume can be used as a benchmark for soil testing and soil P fertility management on farms.

4.2 Materials and methods

4.2.1 Experiment sites

Four sites were established near Yass (34^0 56'54.02'' S and 148^0 55'48.02'' E), Burrinjuck (34^0 52'11.80'' S and 148^0 40' 21.45'' S), Belfrayden (35^0 06'58.06'' S and 146^0 59'34.51'' E) and Beckom (34^0 12'10.56'' S and 147^0 01'59.22'' E) in southern NSW. The Yass site was sown in 2012 and re-sown in 2013 and 2014. The Burrinjuck site was sown in 2013 and re-sown in 2014 while the Belfrayden and Beckom sites were only sown in 2014. The treatments at each site included twelve pasture cultivars and six P rates with three replicates providing a total of 216 plots per site.

The sites were chosen to represent permanent pasture-based livestock systems (Yass and Burrinjuck) and pasture systems that are both permanent (non-arable landscapes) or grown

in sequence with crops (Belfrayden and Beckom). The site characteristics are shown in Table 4.1.

Table 4.1 Sites and years of experimentation are shown with average annual rainfall (italics) and rainfall received in each year of the experiments (mm), soil pH ($CaCl_2$), native Colwell P (mg/kg) and Phosphorus Buffering Index (PBI).

	Yass 2012	Yass 2013	Yass 2014	Burrinjuck 2013	Burrinjuck 2014	Belfrayden 2014	Beckom 2014
AAR	*681.3*			*801.6*		*471.1*	*498.2*
Rainfall	781.8	583.5	704.5	625.1	876.0	369.5	415.9
Soil pH	5.4	5.3	5.1	6.0	5.9	5.8	4.9
Colwell P	12	11	8	13	11	14	5
PBI	52	42	nd	65	nd	82	40

AAR = average annual rainfall
nd – not determined in this growing season

4.2.2 Fertiliser application

Basal nutrients (potassium sulphate 100 kg/ha, magnesium sulphate 60 kg/ha, molybdenum trioxide 0.07 kg/ha, boric acid 1.75 kg/ha, copper sulphate 1.75 kg/ha and zinc sulphate 3.5 kg/ha) were applied at each site through a boom spray at 100 L/ha of water carrier. P was applied by hand from two pre-weighed bags per plot to establish 6 evenly spread P fertility levels. Rates included 0, 15, 30, 45, 60 and 80 kg P/ha at Yass (2012) and Burrinjuck (2013) with slightly different rates of 0, 15, 30, 50, 65 and 85 kg P/ha applied at Belfrayden and Beckom (2014). Maintenance P rates were applied at 0, 1, 3, 8, 15, 20 kg P/ha to Yass (2013 and 2014) and Burrinjuck (2014). P was applied as triple-superphosphate (P=20.7%, S=1.5%). Perennial grasses received a total of 80 kg/ha/yr of nitrogen in four equal applications during May, July, August, and October.

4.2.3 Plant materials and management

Sowing occurred on May 7 (Yass 2012), May 20 (Yass and Burrinjuck 2013), February 25 (Yass and Burrinjuck 2014) and April 2 (Belfrayden and Beckom 2014). All annual legumes were re-sown each year at 30 kg/ha to avoid possible differences in seed reserves and establishment density. Perennial legumes and perennial grass species were sown at 10 kg/ha and not re-sown annually unless establishment was considered inadequate. The germination of all cultivars was tested and sowing rates were adjusted accordingly. Legumes were inoculated with appropriate rhizobia prior to sowing (4.2). The cultivars sown (s) and harvested (h) at peak spring production are shown in Table 4.3.

Table 4.2 Scientific name, common name, inoculum group, life form (annual or perennial) and primary use in farming systems (pasture = regenerating pasture and forage = one or two year stand).

Species	Common name	Cultivar	Inoculum group	Life form	Primary use
Trifolium glanduliferum	gland clover	Prima	C	Annual	Pasture
T. hirtum	rose clover	Hykon	C	Annual	Pasture
T. incarnatum	crimson clover	Dixie	C	Annual	Forage
T. michelianum	balansa clover	Bolta	C	Annual	Pasture
T. purpureum	purple clover	Electra	C	Annual	Forage
T. spumosum	bladder clover	Bartolo	C	Annual	Pasture
T. subterraneum	subterranean clover	Leura, Narrikup, Izmir	C	Annual	Pasture
T. vesiculosum	arrowleaf clover	Zulu II	C	Annual	Forage
Ornithopus compressus	yellow serradella	Santorini, Avila	S	Annual	Pasture
O. sativus	French serradella	Margurita	S	Annual	Pasture
Biserrula pelecinus	biserrula	Casbah, Mauro	WSM1497	Annual	Pasture
Lupinus albus	white lupin	Luxor	G	Annual	Crop
Medicago truncatula	barrel medic	Sultan-SU	AM	Annual	Pasture
M. sativa	lucerne	SARDI 10	AL	Perennial	Pasture
T. ambiguum	kura clover	Kuratas	CC283b	Perennial	Pasture
T. tumens	talish clover	Permatas	B	Perennial	Pasture
Lotus corniculatus	Bird’s-foot trefoil	LC07AUYF	SU343	Perennial	Pasture
Bituminaria bituminosa	tedera	Tedera	WSM4083	Perennial	Pasture
Dactylis glomerata	cocksfoot	Porto, Uplands	n/a	Perennial	Pasture
Phalaris aquatica	phalaris	Advanced AT	n/a	Perennial	Pasture

Yass 2012 and 2013

The perennial grasses failed to establish adequately at the Yass site in 2012 because of grass weeds (mostly *Vulpia* spp.) that invaded these treatments and could not be controlled. In the legume plots grass weeds were controlled with 500 mL/ha of the grass selective herbicide Select ® (240g/L clethodim). The perennial legumes *Trifolium tumens* (cv. Permatas) and *T. ambiguum* (cv. Kuratas) established at low densities and subsequently their plant numbers were reduced further over the 2012/13 summer; consequently these treatments were not harvested and were replaced in autumn 2013. An experimental line of *Bituminaria bituminosa var. albomarginata* (tedera27) was sown in 2012 but winter frosts killed most seedlings. It was subsequently sown in spring (late September) 2012, however, it did not survive frosts in the 2013 winter in adequate numbers to be assessed for peak spring growth in 2013. The annual legumes *Biserrula pelecinus* (cv. Mauro) and *T. spumosum* (cv. Bartolo) failed to produce adequate forage over the winter and spring of 2012 and were not assessed (Table 4.3). Rutherglen bug (*Nysius vinitor*) was found on *T. spumosum* during spring and was controlled by spraying with Matador ® (250g/L lambda-cyhalothrin) at 36 mL/ha.

Yass 2014

At Yass in 2014, the annual legumes *B. pelecinus* (cv. Casbah), *T. spumosum* (cv. Bartolo) and *T. hirtum* (cv. Hykon) failed to produce adequate spring growth. Rutherglen bug (*Nysius vinitor*) was observed in large numbers on *T. spumosum* in early September and Matador ® (250g/L lambda-cyhalothrin) was applied at 36 mL/ha to provide control. By 2014, *M. sativa* (cv. SARDI 10) plant populations had reduced considerably over the 2013/14 summer and consequently it was not assessed in spring 2014 (Table 4.3). *Sclerotinia sclerotiorum* was identified on *Ornithopus sativus* (cv. Margurita) and was controlled with Prosaro® 420 SC (210 g/L prothioconazole and 210 g/L tebuconazole) at 300 mL/ha.

Burrinjuck 2013 and 2014

At Burrinjuck in 2013, the sown perennial grass failed for the same reasons outlined previously for the Yass site in 2012, and the remaining plots received 500 mL/ha of the grass selective herbicide Select ® (240g/L clethodim) to control grass weeds. The legumes *T. spumosum* (cv. Bartolo), *T. hirtum* (cv. Hykon), *B. pelecinus* (cv. Mauro) and *L. corniculatus* (cv. LC07AUYF) failed to establish in adequate numbers and were not assessed. The likely reason for this is that these species were not competitive against toad rush (*Juncus bufonius*) that had invaded these treatments over winter. In 2014, *T. spumosum* (cv. Bartolo), *T. hirtum* (cv. Hykon), *B. pelecinus* (cv. Casbah), *T. glanduliferum* (cv. Prima), *O. compressus* (cv. Santorini) and *T. subterraneum* (cv. Narrikup) failed to establish successfully again due to toad rush invasion despite an early season application of Spinnaker® (700 g/kg imazethapyr) at 70 g/ha (Table 4.3). As with the Yass site, *Sclerotinia sclerotiorum* was identified on *O. sativus* (cv. Margurita) and was controlled with Prosaro® 420 SC (210 g/L prothioconazole and 210 g/L tebuconazole) at 300 mL/ha.

Beckom and Belfrayden 2014

All species sown at these sites produced sufficient herbage in spring to enable them to be harvested (Table 4.3). However, the spring was relatively dry with only about half of the average rain falling at Belfrayden and two thirds of the average rainfall at Beckom in September and October (Table 4.4).

Table 4.3 Pasture cultivar sown (s) at each site and year, along with cultivars harvested at peak spring (h) and sown but not harvested (nh).

Cultivar	Yass			Burrinjuck		Belfrayden	Beckom
	2012	2013	2014	2013	2014	2014	2014
Prima	-	-	-	-	s, nh	s, h	s, h
Hykon	s, h	s, h	s, nh	s, nh	s, nh	s, h	s, h
Dixie	-	-	-	s, h	s, h	s, h	s, h
Bolta	-	-	-	-	s, nh	-	-
Electra	-	s, h	s, h	s, h	-	-	-
Bartolo	s, nh	s, h	s, nh	s, nh	s, nh	s, h	s, h
Leura	s, h	s, h	s, h	s, h	s, h	-	-
Narrikup	-	-	-	-	s, nh	s, h	s, h
Izmir	-	-	-	-	-	s, h	s, h
Zulu II	-	-	-	-	s, h	s, h	s, h
Avila	-	-	s, h	-	-	-	-
Santorini	s, h	s, h	s, h	s, h	s, nh	s, h	s, h
Margurita	-	-	s, h	s, h	s, h	s, h	s, h
Casbah	-	-	s, nh	-	s, nh	s, h	s, h
Mauro	s	s, h	-	s, nh	-	-	-
Sultan-SU	-	-	-	-	-	s, h	s, h
SARDI 10	s, h	h	nh	s, h	h	s, h	s, h
Kuratas	s, nh	h	-	-	-	-	-
Permatas	s, nh	-	-	-	-	-	-
LC07AUYF	s, h	h	-	s, nh	-	-	-
Tedera27	s, nh	s, nh	-	-	-	-	-
Porto	-	s, h	h	s, nh	-	-	-
Uplands	s, nh	-	-	-	-	-	-
Advanced AT	s, nh	s, h	h	s, nh	-	-	-
Luxor and Leura mix	-	-	s,h	-	-	-	-

Table 4.4 Monthly rainfall (mm) for Yass (2012, 2013 and 2014), Burrinjuck (2013 and 2014), Belfrayden and Beckom (2014) as well as long term average (1889 to 2015) rainfall for each site.

Yass	J	F	M	A	M	J	J	A	S	O	N	D
2012	51.1	120.0	204.7	29.3	42.3	51.7	44.5	51.5	44.3	41.7	46.8	53.9
2013	61.8	55.1	38.3	11.7	14.6	95.4	69.6	46.4	68.0	22.1	77.7	22.8
2014	14.5	54.0	103.3	88.7	30.9	78.6	41.2	53.6	47.4	43.8	29.4	119.1
Average	*54.8*	*45.0*	*50.6*	*48.5*	*52.7*	*62.7*	*63.5*	*61.6*	*61.8*	*66.6*	*59.5*	*53.9*
Burrinjuck	J	F	M	A	M	J	J	A	S	O	N	D
2013	18.7	52.2	42.4	14.0	26.9	124.8	77.8	70.9	70.5	28.3	74.5	24.1
2014	19.3	79.9	131.5	110.6	45.9	131.9	76.9	56.6	49.4	40.7	31.1	102.2
Average	*57.5*	*47.3*	*54.3*	*58.4*	*66.2*	*81.5*	*85.2*	*78.5*	*76.8*	*76.6*	*62.2*	*57.0*
Belfrayden	J	F	M	A	M	J	J	A	S	O	N	D
2014	27.0	22.0	72.6	45.7	27.3	66.2	23.7	18.6	30.9	11.1	18.2	52.6
Average	*45.0*	*36.5*	*39.4*	*37.7*	*39.8*	*47.2*	*44.8*	*43.6*	*40.0*	*45.7*	*39.4*	*39.1*
Beckom	J	F	M	A	M	J	J	A	S	O	N	D
2014	13.7	26.7	45.8	58.3	39.2	61.5	17.6	8.3	30.0	17.2	35.9	15.3
Average	*35.5*	*32.6*	*34.8*	*36.2*	*41.7*	*47.8*	*44.3*	*42.6*	*40.8*	*45.9*	*34.8*	*34.1*

4.2.4 Rainfall

The rainfall received over the May to October period at all sites over the 2012 to 2014 seasons was below average. For example at Yass the May to October rainfall was 93 mm, 52.9 mm and 73.5 mm below the long term (1889 to 2015) average for 2012, 2013 and 2014 respectively (Table 4.4). Similarly Burrinjuck received 65.7 and 63.5 mm below the long term average rainfall for May to October during 2013 and 2014, while Belfrayden received 89.3 mm below average and Beckom 83.3 mm below average for 2014.

While drier than average conditions were experienced over the May to October period, there was also waterlogging present at the Yass and Burrinjuck sites for 2013 and 2014 which is evident in the higher than average June rainfall figures for these years. Waterlogging was more evident at Burrinjuck and particularly in 2014 (Table 4.4).

4.2.5 Measurements

4.2.5.1 Herbage dry matter

At peak spring growth, shoots were cut from three quadrats (200 mm x 500 mm) in each plot. Shoots were dried at 70°C for 72 hours and weighed to provide an estimate of the dry matter production for each plot. Peak spring production was assessed on; November 6, October 2 and 9 at Yass for the 2012, 2013 and 2014 years respectively; October 23 and 24 at Burrinjuck for 2013 and 2014 respectively; October 1 at Belfrayden (2014) and September 18 at Beckom (2014).

4.2.5.2 Soil sampling

Soil P levels were determined at the time of the peak spring harvest by sampling 8 cores (0-10 cm depth) from each of the three areas cut for dry matter assessment (total = 24 soil cores per plot). Soils were then homogenised and dried at 40^0C. Once dry the soil samples were again homogenised and subsampled to determine the Colwell P extractable-P concentration (Colwell 1963).

4.2.5.3 Mycorrhizal colonisation of roots

Colonisation of roots by arbuscular mycorrhizal fungi (AMF) was measured on topsoil roots (0-10 cm depth) of *O. sativus*, *O. compressus* and *T. subterraneum* growth in the unfertilised treatment at all four field sites in spring 2014 at the time of the peak spring harvest. The methods by which roots were prepared and AMF colonisation was assessed are as reported in Chapter 10.

4.2.5.4 Experiment design and statistical analysis

Experimental designs were generated using DiGGer® (Coombes, 2009) to produce a design that avoids row, column and diagonal treatment duplicates with three replicates, six P rates and 12 cultivars. The analysis was performed using R version 3.3.1.1. Dry matter and soil test data were first analysed using asreml and the fixed model applied as cultivar plus P rate with testing for linear column and row effects which were checked for significance using the Wald test. The random model included row, column, rep and rep.plot effects with testing for auto-regressive correlations using loglik differences greater than 3.84. This analysis showed that effects of P rate and cultivar were significant (P>0.05) but not P rate x cultivar effects. Estimates of LSD values at the 5% level were calculated for Colwell P and dry matter measurements. Fitted data from asreml at the plot level was then used to fit Mitscherlich

equation [$y = a - b*(e^{-cx})$] where y is the shoot dry matter (kg/ha) and x is the soil Colwell P (mg/kg) which provide an estimate of critical Colwell P (ie. the Colwell P concentrations coinciding with 90% and 95% of maximum dry matter). Plot values were excluded from herbage yield analyses if: (i) herbage growth across the plots had been recorded in field notes collected during the growing season as highly variable (at all sites some plots were recoded as variable due to factors including: grazing by wild vertebrate pests, large sub-surface rocks, sclerotinia damage, root rot, water-logged hollow and/or establishment failure) and these observations coincided with (ii) a plot residual produced after LMM analysis that was greater than 3.

4.3 Results

Subterranean clover (cvs. Leura, Narrikup, Izmir) was used as a benchmark species as it is the most commonly grown legume in permanent pastures and mixed crop-pasture systems in southern Australia. It is known to have a high critical external requirement for P and this is the basis of the soil test P (STP) benchmarks that are used for pasture management in southern Australia (*Gourley et al. 2007*; Moody 2007).

4.3.1 Critical soil test P concentrations

Mitscherlich curves were fitted for yield in response to Colwell P concentration for each species grown at each site in each year (Figs. 4.1, 4.2, 4.3 and 4.4). The critical Colwell P requirement was determined as the Colwell P concentration required to achieve 90 or 95% of maximum yield (Table 4.5). The critical Olsen P concentration was derived from the relationship between Colwell P and Olsen P, which was determined for each site in each year (Table 4.6). The comparison of Colwell and Olsen P requirements, and the 90% and 95% yield thresholds provide some of the first benchmarks ever determined for the critical P requirements among the species studied. Henceforth, results in this Chapter refer to a single benchmark based on a critical Colwell P concentration that corresponds with a 90% yield threshold. Further discussion of the implications and interpretation of these benchmarks for pasture management can be found in the Conclusions (Chapter 14).

Yass

In 2012, the critical external Colwell P requirement of *T. subterraneum* (cv. Leura) was 42 mg P/kg (Fig. 4.1; Table 4.7). *Ornithopus compressus* (cv. Santorini) and *L. corniculatus* (cv. LC07AUYF) had critical Colwell P requirements and yields approximately half that of *T. subterraneum* (cv. Leura, Table 4.7; Fig. 4.1a). *T. hirtum* (cv. Hykon) had an intermediate critical P requirement but yielded as well as *T. subterraneum*. The critical P requirement of *M. sativa* (cv. SARDI 10) could not be determined as a lack of P treatments greater than 80 kg P/ha meant that a reliable maximum yield could not be estimated using the Mitscherlich function.

In 2013, *T. subterraneum* had a critical Colwell P requirement of 31 mg P/kg and, with the exception of *M. sativa* (cv. SARDI 10), yielded better than all other species. *Ornithopus compressus* (cv. Santorini), *T. purpureum* (cv. Electra) and *L. corniculatus* (cv. C07AUYF) had critical Colwell P requirements approximately half to two-thirds that of *T. subterraneum* and yields up to 70% of *T. subterraneum*. The critical Colwell P requirement of *T. hirtum* (cv. Hykon), *B. pelecinus* (cv. Mauro), *T. spumosum* (cv. Bartolo), *T. ambiguum* (cv. Kuratas), *B. bituminosa* (line 27), *D. glomerata* (cv. Porto) and *P. aquatica* (cv. Advanced AT) did not significantly differ to that of *T. subterraneum*. As in 2012, the critical P requirement of *M. sativa* could not be reliably determined.

In 2014, the critical Colwell P requirement of *T. subterraneum* was 31 mg P/kg. O. compressus (cvs. Avila, Santorini) and O. sativus (cv. Margurita) had critical Colwell P requirements approximately 60% that of *T. subterraneum* and yielded as well as *T. subterraneum* (with the exception of cv. Avila). The grasses and the mixture of *L. albus* and *T. subterraneum* had lower critical Colwell P requirements but also a lower yield than the *T. subterraneum*.

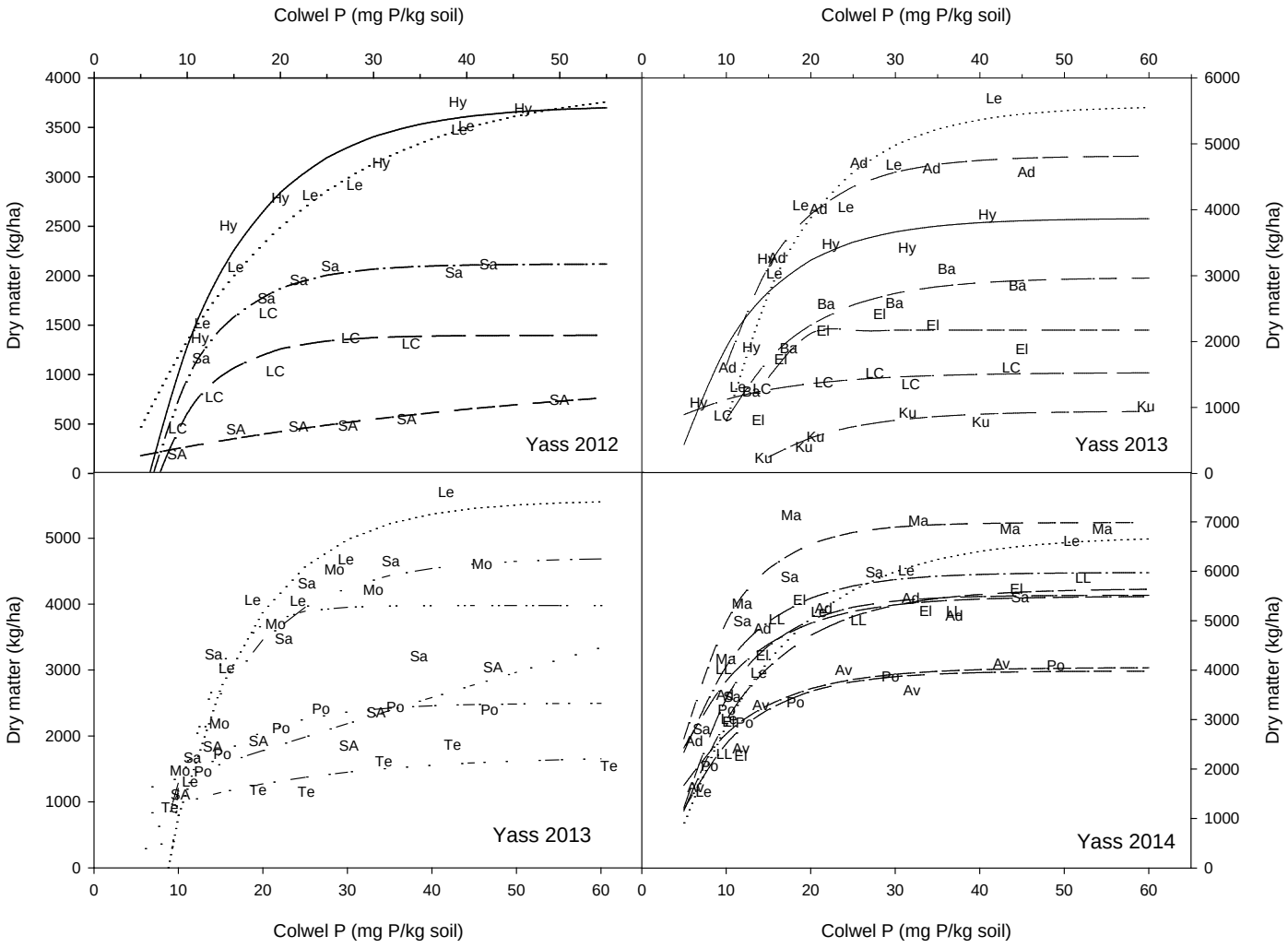


Figure 4.1 Peak spring dry matter yield (kg/ha) at Yass in response to increasing levels of soil P measured as Colwell P (mg P/kg soil). Lines are the predicted response using the Mitscherlich equation and letters are the measured mean dry matter response. Hykon symbol = Hy, line = solid; Leura symbol = Le, line = dotted; Santorini symbol = Sa, line = dash dot; LC07AUYF symbol = LC, line equals long dash, SARDI 10 symbol = SA, line = short dash, Advanced AT symbol = Ad, line = medium dash; Bartolo symbol = Ba, line = long short dash; Electra symbol = El, line = long short short dash; Kuratas symbol = Ku, line = short long dash; Porto symbol = Po, line = short dash; Tedera symbol = Te, line = short short short dash; Margurita symbol = Ma, line = short short dash; Luxor and Leura mix symbol = LL, line = medium medium dash; Avila symbol = Av, line = short long short dash. For clarity, genotypes harvested in 2013 are shown distributed over two panels with Leura (Le) shown in both for reference.

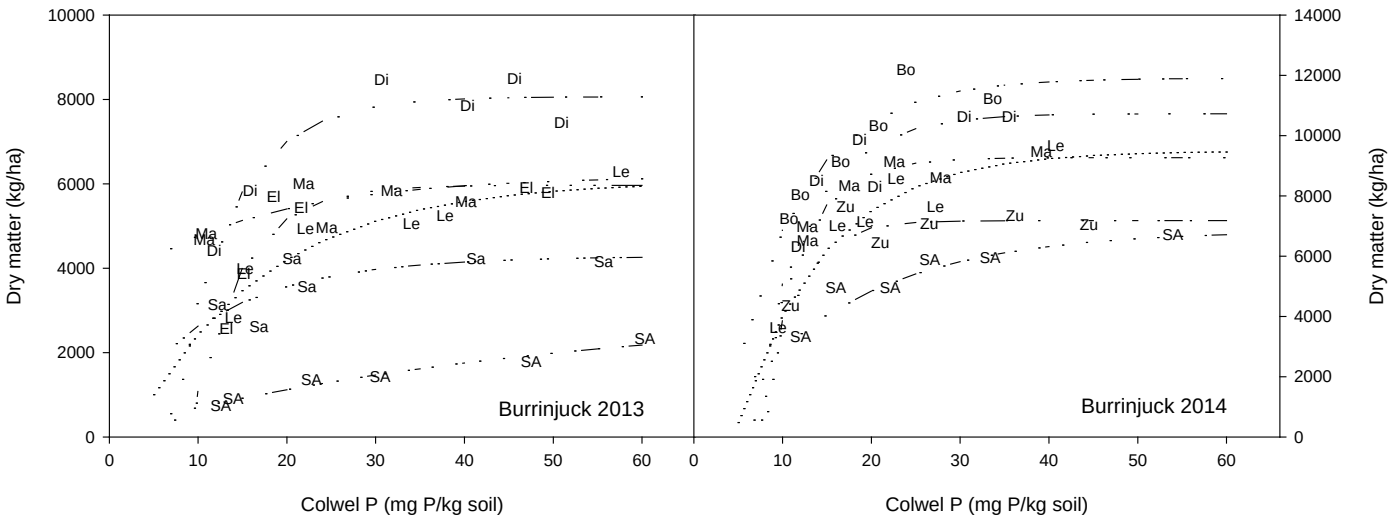


Figure 4.2 Peak spring dry matter yield (kg/ha) at Burrinjuck in response to increasing levels of soil P measured as Colwell P (mg P/kg soil). Lines are the predicted response using the Mitscherlich equation and letters are the measured mean dry matter response. Dixie symbol = Di, line = short long dash; Leura symbol = Le, line = dotted; Santorini symbol = Sa, line = dash dot; SARDI 10 symbol = SA, line = short dash, Electra symbol = El, line = long short short dash; Margurita symbol = Ma, line = short short dash; Bolta symbol = Bo, line = short short short dash; Zulu II symbol = Zu, line = medium medium dash.

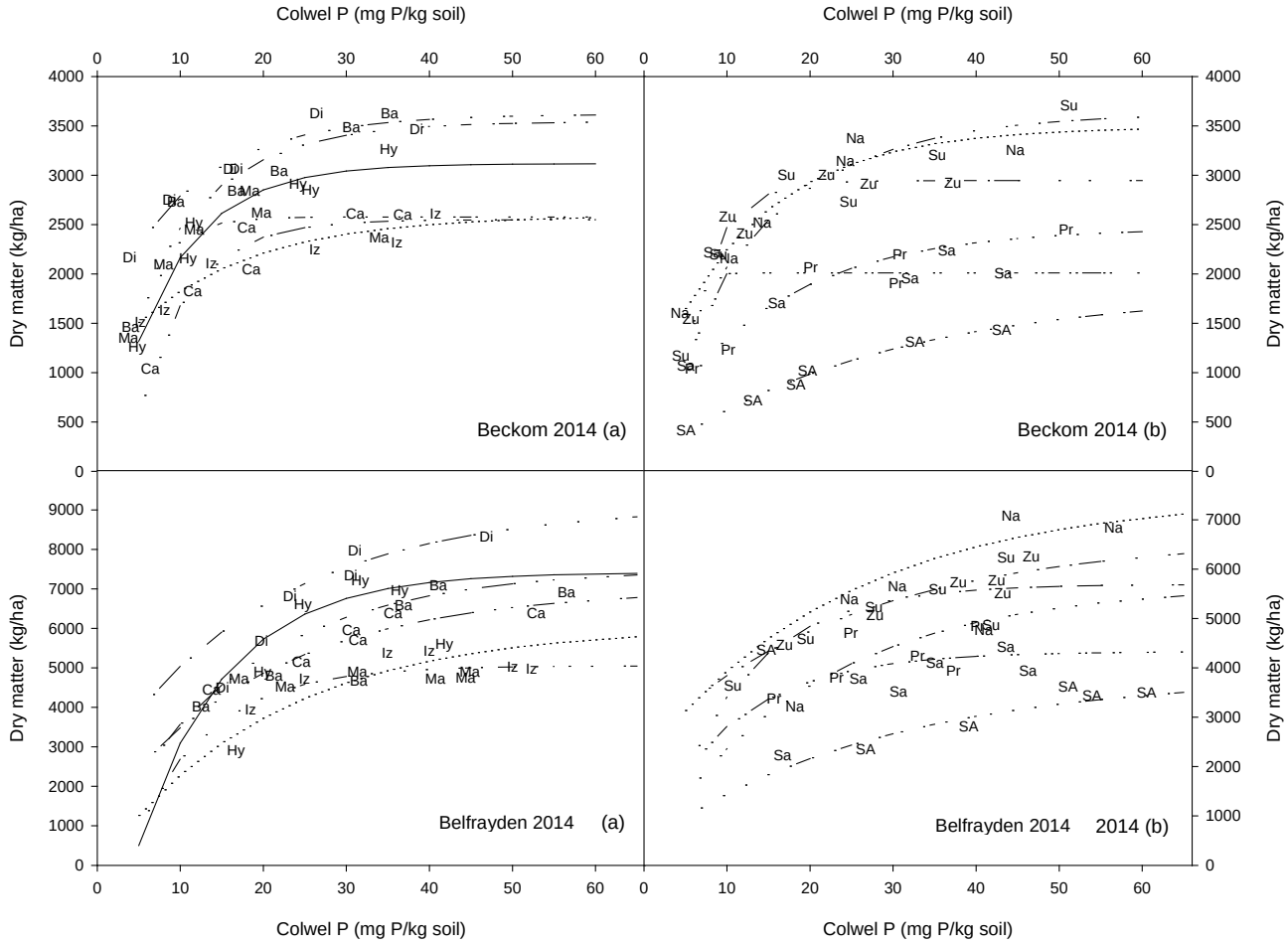


Figure 4.3 Peak spring dry matter yield (kg/ha) at Beckom and Belfrayden in response to increasing levels of soil P measured as Colwell P (mg P/kg soil). Lines are the predicted response using the Mitscherlich equation and letters are the measured mean dry matter response. Dixie symbol = Di, line = short long dash; Hykon symbol = Hy, line = solid; Bartolo symbol = Ba, line = long short dash; Izmir symbol = Iz, line = dotted; Casbah symbol = Ca, line = shor short short dash; Narrikup = symbol = Na line = dotted, Saltan SU symbol = Su, line = medium dash; Zulu II symbol = Zu, line = medium medium dash; Prima symbol = Pr, line = short dash; Santorini symbol = Sa, line = dash dot; SARDI 10 symbol = SA, line = short dash, Margurita symbol = Ma, line = short short dash. For clarity, genotypes harvested at both sites are shown distributed over two panels (a and b) with Leura (Le) shown in both for reference.

Table 4.5 Critical Colwell P requirements (mg P/kg soil) of pasture legumes and grasses determined as the soil test P concentration required to achieve either 95% or 90% of maximum herbage yield (kg dry matter/ha) for a period of spring growth. Parameters were derived by fitting a Mitscherlich response (yield = a - b*($e^{-c*Colwell\ P}$)) to data collected from the Yass, Burrinjuck, Beckom and Belfrayden sites. Maximum yield is predicted by *a*, the asymptote; responsiveness to P is reflected in *c*, the curvature parameter; and the intercept (*a*-*b*) is an extrapolation reflecting yield at a theoretical Colwell P value of zero. Equivalent critical Olsen P values (mg P/kg) are shown in parentheses and were determined using relationships between Olsen P and Colwell P measured at each site during spring (Table 4.6).

Species	**Cultivar**	**Critical STP concentration (mg/kg)**		**Parameter**			
		95%	90%	Intercept	a	b	c
Yass 2012		Colwell P (Olsen P)	Colwell P (Olsen P)				
Trifolium hirtum	Hykon	35 (13)	28 (11)	-3034	3723	-6757	0.9032
Lotus corniculatus	LC07AUYF	29 (11)	20 (8)	-3149	1397	-4546	0.8398
Trifolium subterraneum	Leura	54 (20)	42 (16)	-704	3949	-4653	0.9436
Onithopus compressus	Santorini	25 (10)	21 (9)	-3406	2117	-5523	0.8560
Medicago sativa	SARDI 10	140 (49)	106 (38)	80	1083	-1003	0.9793
Yass 2013							
Phalaris aquatica	Advanced AT	30 (11)	25 (9)	-6569	4818	-11387	0.8802
Trifolium spumosum	Bartolo	34 (12)	28 (10)	-3517	2972	-6489	0.8960
Trifolium purpureum	Electra	19 (7)	17 (6)	-1872457	2175	-1874632	0.5920
Trifolium hirtum	Hykon	31 (11)	24 (9)	-2148	3871	-6019	0.8938
Trifolium ambiguum	Kuratas	40 (14)	34 (12)	-2549	945	-3494	0.8981
Lotus corniculatus	LC07AUYF	29 (10)	21 (8)	535	1530	-995	0.9150

Table 4.5 continued

(Yass 2013)

		Critical STP concentration (mg/kg)		Parameter			
Species	Cultivar	95%	90%	Intercept	a	b	c
Trifolium subterraneum	Leura	37 (13)	31 (11)	-7969	5576	-13545	0.9011
Biserrula pelecinus	Mauro	37 (13)	30 (11)	-4671	4709	-9380	0.9042
Dactylis glomerata	Porto	31 (11)	25 (9)	-1885	2498	-4383	0.8901
Onithopus compressus	Santorini	21 (8)	18 (7)	-23929	3979	-27908	0.7939
Medicago sativa	SARDI 10	1048 (344)	801 (263)	929	16510	-15581	0.9972
Bituminaria bituminosa	Tedera27	51 (17)	38 (13)	462	1708	-1246	0.9488

Table 4.5 continued

Species	Cultivar	Critical STP concentration (mg/kg)		Parameter			
		95%	90%	Intercept	a	b	C
Yass 2014		Colwell P (Olsen P)	Colwell P (Olsen P)				
Phalaris aquatic	Advanced AT	26 (9)	20 (7)	38	5493	-5455	0.8909
Ornithopus compressus	Avila	26 (9)	20 (7)	-1305	3984	-5289	0.8801
Trifolium purpureum	Electra	32 (12)	25 (9)	-1898	5652	-7550	0.9015
Trifolium subterraneum	Leura	39 (14)	31 (11)	-2095	6715	-8810	0.9200
Onithopus sativus	Margurita	21 (8)	17 (6)	-2461	6987	-9448	0.8572
Dactylis glomerata	Porto	27 (10)	20 (7)	-169	4054	-4223	0.8918
Onithopus compressus	Santorini	24 (9)	19 (7)	-977	5976	-6953	0.8784
T. subterraneum + *Lupinus albus*	Leura + Luxor interplant	24 (9)	19 (7)	-3293	5518	-8811	0.8663
Burrinjuck 2013							
Trifolium incarnatum	Dixie	26 (10)	22 (9)	-13743	8062	-21805	0.8596
Trifolium purpureum	Electra	25 (10)	22 (9)	-24079	5964	-30043	0.8339
Trifolium subterraneum	Leura	47 (19)	37 (15)	-1010	6070	-7080	0.9354
Ornithopus sativus	Margurita	36 (14)	24 (10)	3603	6188	-2585	0.942

Table 4.5 continued

(Burrinjuck 2013)

Ornithopus compressus	Santorini	35 (14)	26 (10)	466	4281	-3815	0.9199
Medicago sativa	SARDI 10	148 (58)	113 (44)	181	3052	-2871	0.9804
Burrinjuck 2014							
Trifolium michelianum	Bolta	27 (10)	22 (8)	-5474	11903	-17377	0.8836
Trifolium incarnatum	Dixie	z	20 (7)	-17915	10726	-28641	0.8502
Trifolium subterraneum	Leura	34 (12)	27 (10)	-5472	9489	-14961	0.9037
Ornithopus sativus	Margurita	21 (8)	17 (6)	-29840	9267	-39107	0.807
Medicago sativa	SARDI 10	47 (16)	36 (13)	-679	6855	-7534	0.9361
Trifolium vesiculosum	Zulu II	18 (7)	16 (6)	-38836	7179	-46015	0.769

Table 4.5 continued

Species	Cultivar	Critical STP concentration (mg/kg)		Parameter			
		95%	90%	Intercept	a	b	c
Beckom 2014		Colwell P (Olsen P)	Colwell P (Olsen P)				
Trifolium spumosum	Bartolo	28 (13)	21 (10)	512	3542	-3030	0.902
Biserrula pelecinus	Casbah	32 (15)	18 (8)	-1764	2549	-4313	0.8524
Trifolium incarnatum	Dixie	27 (13)	19 (9)	1552	3618	-2066	0.9127
Trifolium hirtum	Hykon	24 (11)	19 (9)	-284	3117	-3401	0.8804
Trifolium subterraneum	Izmir	35 (17)	25 (12)	1052	2591	-1539	0.9323
Ornithopus sativus	Margurita	12 (5)	10 (4)	-1336	2576	-3912	0.7580
Trifolium subterraneum	Narrikup	35 (17)	26 (12)	746	3488	-2742	0.9234
Trifolium glanduliferum	Prima	43 (20)	33 (16)	296	2467	-2171	0.9356
Ornithopus compressus	Santorini	7 (3)	7 (3)	-102963	2009	-104972	0.385
Medicago sativa	SARDI 10	82 (39)	63 (30)	104	1825	-1721	0.9648
Medicago truncatula	Sultan-SU	40 (19)	30 (14)	410	3631	-3221	0.9303
Trifolium vesiculosum	Zulu II	15 (7)	12 (5)	-2838	2945	-5783	0.7792

Table 4.5 continued

Species	Cultivar	Critical STP concentration (mg/kg)		Parameter			
		95%	90%	Intercept	a	b	c
Belfrayden 2014		Colwell P (Olsen P)	Colwell P (Olsen P)				
Trifolium spumosum	Bartolo	51 (22)	39 (16)	460	7518	-7058	0.9436
Biserrula pelecinus	Casbah	56 (24)	42 (18)	1215	7012	-5797	0.9515
Trifolium incarnatum	Dixie	56 (24)	41 (17)	2518	9120	-6602	0.9531
Trifolium hirtum	Hykon	36 (15)	29 (12)	-3649	7421	-11070	0.9103
Trifolium subterraneum	Izmir	62 (27)	48 (20)	-52	6053	-6105	0.9528
Ornithopus sativus	Margurita	31 (13)	24 (9)	-1927	5054	-6981	0.8976
Trifolium subterraneum	Narrikup	65 (28)	48 (20)	2143	7489	-5346	0.9598
Trifolium glanduliferum	Prima	61 (26)	46 (19)	280	5694	-5414	0.9526
Ornithopus compressus	Santorini	31 (13)	24 (9)	534	4331	-3797	0.9125
Medicago sativa	SARDI 10	81 (35)	62 (27)	428	3847	-3419	0.9652
Medicago truncatula	Sultan-SU	65 (28)	47 (20)	2508	6642	-4134	0.9617
Trifolium vesiculosum	Zulu II	31 (13)	24 (9)	-459	5694	-6153	0.9057

Table 4.6 Relationships between Olsen P (y) and Colwell P (x) concentrations (mg P/kg soil) of topsoil (0-10 cm depth) sampled in spring at the Yass, Burrinjuck, Beckom and Belfrayden sites during the years in which P-response experiments were conducted.

Site	Year	Relationship	R^2
Yass	2012	y = 0.342x + 1.3449	R^2 = 0.93
Yass	2013	y = 0.3275x + 0.7723	R^2 = 0.95
Yass	2014	y = 0.3601x + 0.0646	R^2 = 0.96
Burrinjuck	2013	y = 0.3909x + 0.2264	R^2 = 0.94
Burrinjuck	2014	y = 0.3363x + 0.5457	R^2 = 0.97
Beckom	2014	Y = 0.4858x - 0.4622	R^2 = 0.97
Belfrayden	2014	y = 0.4572x - 1.5644	R^2 = 0.95

Table 4.7 Critical P requirement and 90% maximum yield for each cultivar grown at Yass in 2012, 2013 and 2014. Critical P requirement determined as Colwell P concentration (mg/kg) required to achieve 90% of maximum dry matter yield from a fitted Mitscherlich equation. Asymptote standard error, variance accounted and standard error are for the fitted Mitscherlich equation.

Site	Yass						
Year	2012						
Species		*T. hirtum*	*L. corniculatus*	*T. subterraneum*	*O. compressus*	*M. sativa*	
Cultivar		Hykon	LC07AUYF	Leura	Santorini	SARDI 10	
Critical P (90% max)		28	20	42	21	106	
Dry matter (90% max)		3332	1259	3543	1907	974	
Asymptote standard error		121	104	344	92	1251	
Colwell P LSD 5%	*8.0*	*Variance accounted*		*96*	*Standard error*		*205*
Dry matter LSD 5%	*530*						
Year	2013						
Species		*T. hirtum*	*L. corniculatus*	*T. subterraneum*	*O. compressus*	*M. sativa*	*T. spumosum*
Cultivar		Hykon	LCO7AUYF	Leura	Santorini	SARDI 10	Bartolo
Critical P (90%)		24	21	31	18	801	28
Dry matter (90% max)		3464	1376	5039	3541	14861	2672
Asymptote standard error		113	139	150	71	84013	119
Species		*T. purpureum*	*B. pelecinus*	*T. ambiguum*	*B. bituminosa*	*D. glomerata*	*P. aquatica*
Cultivar		Electra	Mauro	Kuratas	Tedera27	Porto	Advance AT
Critical P (90%)		17	30	34	38	25	25

Dry matter (90% max)		1922	4252	855	1539	2259	4349
Asymptote standard error		60	130	106	163	127	111
Colwell P LSD 5%	*9.0*	*Variance accounted*		*91*	*Standard error*		*403*
Dry matter LSD 5%	*608*						
Year	2014						
Species		*O. compressus*	*T. purpureum*	*T. subterraneum*	*O. compressus*	*O. sativus*	*L. albus + T. subterraneum*
Cultivar		Avila	Electra	Leura	Santorini	Margurita	Luxor+Leura
Critical P (90%)		20	25	31	19	17	19
Dry matter (90% max)		3573	5087	6051	5384	6299	4942
Asymptote standard error		203	212	214	188.5	127	137
Species		*D. glomerata*	*P. aquatica*				
Cultivars		Porto	Advanced AT				
Critical P (90%)		20	20				
Dry matter (90% max)		3626	4952				
Asymptote standard error		207	177				
Colwell P LSD 5%	*7.1*	*Variance accounted*		*87.8*	*Standard error*		*282*
Dry matter LSD 5%	*852*						

Table 4.8 Critical P requirement and 90% maximum yield for each species grown at Burrinjuck in 2013 and 2014. Critical P requirement determined as Colwell P concentration (mg/kg) required to achieve 90% of maximum dry matter yield from a fitted Mitscherlich. Asymptote standard error, variance accounted and standard error are for the fitted Mitscherlich equation.

Site	Burrinjuck						
Year	2013						
Species		*T. incarnatum*	*T. purpureum*	*T. subterraneum*	*O. sativus*	*O. compressus*	*M. sativa*
Cultivar		Dixie	Electra	Leura	Margurita	Santorini	SARDI 10
Critical P (90% max)		22	22	37	24	26	113
Dry matter (90% max)		7280	5412	5472	5572	3846	2745
Asymptote standard error		145	200	363	1160	244	2403
Colwell P LSD 5%	*8.4*	*Variance accounted*		*85.1*	*Standard error*		*811*
Dry matter LSD 5%	*1074*						
Year	2014						
Species		*T. incarnatum*	*T. michelianum*	*T. subterraneum*	*O. sativus*	*M. sativa*	*T. vesiculosum*
Cultivar		Dixie	Bolta	Leura	Margurita	SARDI 10	Zulu II
Critical P (90%)		20	22	27	17	36	16
Dry matter (90% max)		10231	10761	8517	8246	6156	6491
Asymptote standard error		173	238	276	159	324	128
Colwell P LSD 5%	*8.0*	*Variance accounted*		*87.8*	*Standard error*		*282*
Dry matter LSD 5%	*1724*						

Table 4.9 Critical P requirement and 90% maximum yield for each species grown at Belfrayden in 2014. Critical P requirement determined as Colwell P concentration (mg/kg) required to achieve 90% of maximum dry matter yield from a fitted Mitscherlich. Asymptote standard error, variance accounted and standard error are for the fitted Mitscherlich equation.

Site	Belfrayden						
Year	2014						
Species		*T. spumosum*	*B. pelecinus*	*T. incarnatum*	*T. hykon*	*T. subterraneum*	*O. sativus*
Cultivar		Bartolo	Casbah	Dixie	Hykon	Izmir	Margurita
Critical P (90%)		38	39	33	24	43	21
Dry matter (90% max)		7047	6387	8199	6668	5469	4561
Asymptote standard error		252	276	340	157	270	119
Species		*T. subterraneum*	*T. glanduliferum*	*O. compressus*	*M. sativa*	*M. truncatula*	*T. vesiculosum*
Cultivar		Narrikup	Prima	Santorini	SARDI 10	Sultan-SU	Zulu II
Critical P (90%)		44	43	24	55	40	24
Dry matter (90% max)		6796	5238	3909	3454	5801	5123
Asymptote standard error		360	685	142	401	469	164
Colwell P LSD 5%	*9.7*	*Variance accounted*		*87*	*Standard error*		*527*
Dry matter LSD 5%	*1806*						

Table 4.10 Critical P requirement and 90% maximum yield for each species grown at Beckom in 2014. Critical P requirement determined as Colwell P concentration (mg/kg) required to achieve 90% of maximum dry matter yield from a fitted Mitscherlich. Asymptote standard error, variance accounted and standard error are for the fitted Mitscherlich equation.

Site	Beckom						
Year	2014						
Species		*T. spumosum*	*B. pelecinus*	*T. incarnatum*	*T. hirtum*	*T. subterraneum*	*O. sativus*
Cultivars		Bartolo	Casbah	Dixie	Hykon	Izmir	Margurita
Critical P (90%)		21	18	19	19	25	10
Dry matter (90% max)		3195	2306	3254	2815	2976	2331
Asymptote standard error		153	139	142	109	187	81
Species		*T. subterraneum*	*T. glanduliferum*	*O. compressus*	*M. sativa*	*M. truncatula*	*T. vesiculosum*
Cultivar		Narrikup	Prima	Santorini	SARDI 10	Sultan-SU	Zulu II
Critical P (90%)		26	33	7	63	30	12
Dry matter (90% max)		3143	2226	1877	1645	3262	2942
Asymptote standard error		168	161	53	480	139	83
Colwell P LSD 5%	*8.5*	*Variance accounted*		*81*	*Standard error*		*372*
Dry matter LSD 5%	*902*						

Burrinjuck

In 2013, the critical Colwell P requirement of *T. subterraneum* (cv. Leura) was 37 mg P/kg. (Fig. 4.2; Table 4.8). *Trifolium incarnatum* (cv. Dixie), *T. purpureum* (cv. Electra), *O. sativus* (cv. Margurita) and *O. compressus* (cv. Santorini) had critical Colwell P requirements that were approximately two-thirds that of *T. subterraneum*. *Trifolium purpureum* and *O. sativus* yielded as well as *T. subterraneum*, and *T. purpureum* yielded greater than *T. subterraneum*.

In 2014, the critical Colwell P requirements of all species were lower, and dry matter yields higher, than that measured in 2013. The critical Colwell P requirement for *T. subterraneum* was 27 mg P/kg. *Ornithopus sativus* (cv. Margurita) yielded as well as *T. subterraneum* but had a critical Colwell P requirement of 17 mg P/kg. *Trifolium vesiculosum* (cv. Zulu II) also had a critical P requirement approximately half that of *T. subterraneum* but only achieved approximately 75% of the maximum yield of *T. subterraneum*. *Trifolium incarnatum* (cv. Dixie) and *T. michelianum* (cv. Bolta) yielded more than *T. subterraneum* but did not differ in critical P requirement.

Belfrayden and Beckom

At Belfrayden and Beckom *T. subterraneum* cv. Leura was not grown as it is not adapted to the climate at these locations. The better adapted cultivars Izmir and Narrikup were grown instead.

At Belfrayden, the critical Colwell P requirement of *T. subterraneum* (cvs. Izmir and Narrikup) was 43-44 mg P/kg (Fig. 4.3; Table 4.9). *Trifolium spumosum* (cv. Bartolo), *B. pelecinus* (cv. Casbah), *T. glanduliferum* (cv. Prima) and *M. truncatula* (cv. Sultan-SU) had critical P requirements that did not differ to that of *T. subterraneum*. *Trifolium incarnatum* (cv. Dixie), *T. hirtum* (cv. Hykon), *O. sativus* (cv. Margurita), *O. compressus* (cv. Santorini) and *T. vesiculosum* (cv. Zulu II) had critical P requirements as low as half that of *T. subterraneum*. Yields of the *Orntihopus* spp. were not significantly different to *T. subterraneum* cv. Izmir but were lower than that of cv. Narrikup.

At Beckom, both dry matter yields and critical P requirements were lower than that measured at Belfrayden (Fig. 4.3; Table 4.10). The critical Colwell P requirement of *T. subterraneum* (cvv. Izmir and Narrikup) was 25-26 mg P/kg. *Ornithopus sativus* (cv. Margurita), *O. compressus* and *T. vesiculosum* had critical P requirements less than half that *T. subterraneum*. The yield of *O. sativus* was not significantly lower than that of *T. subterraneum*. The remaining *Trifolium* spp., *M. truncatula* (Sultan-SU) and *B. pelecinus* (cv. Casbah) had similar critical P requirements and yields to *T. subterraneum*. At both Beckom and Belfrayden, *Medicago sativa* had a higher critical P requirement and lower yield than *T. subterraneum*.

4.3.2 Mycorrhizal colonisation

In 2014, mycorrhizal colonisation was measured on topsoil roots (0-10 cm depth) of *O. sativus*, *O. compressus* and *T. subterraneum* grown in the unfertilised treatment at all four field sites (methods as reported in Chapter 10). The roots of all three species were heavily colonised by arbuscular mycorrhizal fungi (AMF). Typically 40-55% of root length was colonised. The only exceptions being *O. sativus* and *T. subterraneum* at Burrinjuck where ~25% of root length was colonised (Fig. 4.4).

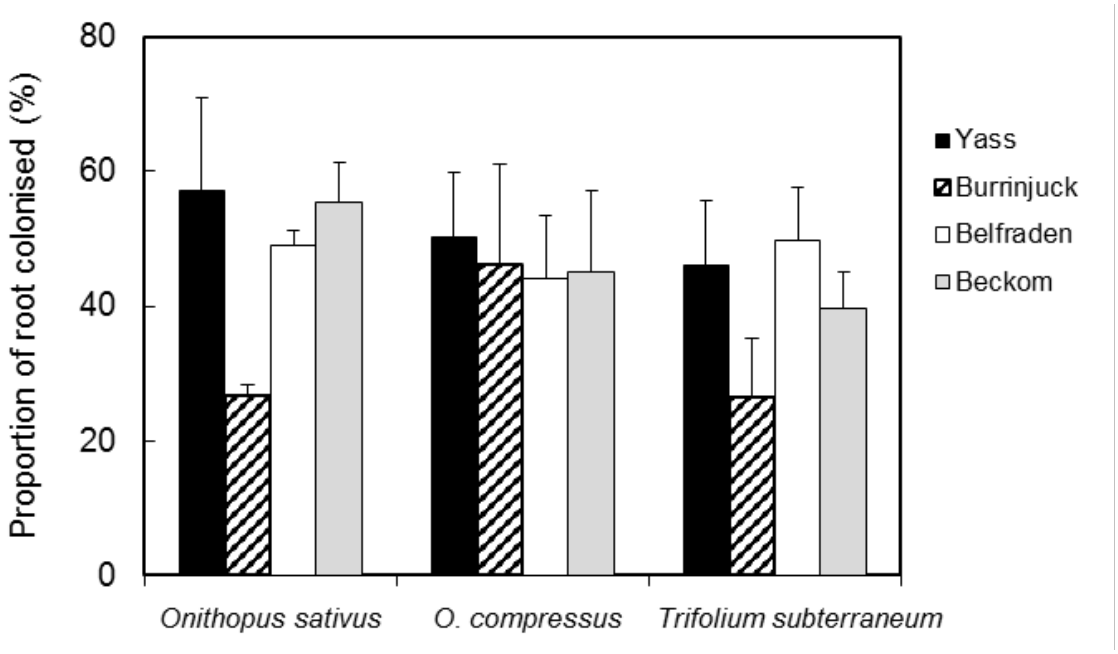


Figure 4.4 Colonisation of topsoil roots (0-10 cm depth) of *Ornithopus sativus* (cv. Margurita), *Onithopus compressus* (cv. Santorini) and *Trifolium subterraneum* (cv. Leura at Yass and Burrunjuck or cvs. Izmir/Narrikup at Beckom and Belfrayden) by arbuscular mycorrhizal fungi in the unfertilised treatments during spring 2014. Bars = 1x SE.

4.4 Discussion

4.4.1 Rankings based on critical P requirement and yield potential of the pasture legumes

Table 4.11 presents a summary of the ranges in critical Colwell P and 90% maximum yield measured for species/cultivars that were able to be grown in field experiments over at least two growing seasons or sites. Species were allocated to one of four groups based on their critical STP requirement. *Medicago sativa* was consistently found to have a higher critical P requirement than *T. subterraneum*. Its critical P requirement could not be determined because considerably higher soil P levels were required to do this than was possible in an experiment designed to benchmark the P requirements of the alternative legumes relative to that of subterranean clover. Most of the *Trifolium* spp., and *M. truncatula* and *B. pelecinus* had moderate to high P requirements, similar to that of *T. subterraneum*. The *Ornithopus* spp., *L. corniculatus* and *T. purpureum* had substantially lower critical P requirements. However, *Ornithopus sativus* (cv. Margurita) was the only pasture legume to have a very low P requirement but with dry matter yields consistently equivalent to that of *T. subterraneum*.

Within the forage legumes, *T. purpureum* (cv. Electra) had a lower external P requirement and was able to produce as much dry matter as *T. subterraneum* cv. Leura at Burrinjuck in 2013. However *T. purpureum* had a lower dry matter yield than *T. subterraneum* cv. Leura at Yass in 2013 and 2014, and did not differ in its critical P requirement relative to *T. subterraneum* cv. Leura at Yass in 2014. These results suggest adaptation differentiation for *T. purpureum* (cv. Electra) between the Yass and Burrinjuck sites and possible limited scope for its use.

A large number of the species/cultivars tested had equivalent critical P levels to that of the *T. subterraneum* (cvs. Leura, Narrikup and Izmir), and were generally able to produce as much dry matter when fertilised at their critical P concentration as well fertilised *T. subterraneum*.

The critical STP results recorded at Belfrayden in 2014 were consistently higher for all species gown at the site than recorded elsewhere. Colwell P results are known to vary with the PBI of the soil (e.g. Moody 2007) and it is possible that a high critical STP value could be due to soil with a high P buffering capacity. The measured PBI at Belfrayden was the highest recorded of the field sites (Table 4.1), but it was not high enough for more than a minor increase in the critical P concentration (*Gourley et al. 2007*; Moody *et al*. 2007). In addition critical Olsen P values are not expected to change with soil type or PBI, yet they were also high at the Belfrayden site. The reason for the high critical P requirements reported for all species at Belfrayden site is unknown. However, it is suspected that the dry spring conditions led to a very dry topsoil, which would limit diffusion of P to the roots and therefore cause critical P requirements to be artificially high. Rainfall received in September and October at Belfrayden was only half the average rainfall for that time of year (Table 4.2).

4.4.2 Interplanting with *Lupinus albus*

A mixture of *Lupinus albus* and *T. subterraneum* cv. Leura was tested in 2014 at Yass to determine if *T. subterraneum* could benefit from companion planting with a species known to mobilise “sparingly-available” P through the release of citrate from its roots (Johnson *et. al.* 1996; Neumann *et. al.* 2000). The results indicated that the combination of these species has resulted in a lower critical P compared to *T. subterraneum* cv. Leura grown as a monoculture. This was a surprisingly successful treatment because the critical STP of the mixture was as low as that achieved by the *Ornithopus* species. However, the result was only from one site and one year and would need to be further explored to verify this response.

Table 4.11 Range in critical Colwell P requirement and 90% maximum yield for 13 pasture species grown at up to four sites and across four yields.

Species	Cultivar	Critical Colwell P range (mg/kg)	Near-maximum dry matter range (kg/ha)	Number of sites or years
		Very high P requirement		
M. sativa	SARDI 10	36 - 801	974 - 14861	6
		High P requirement		
T. glanduliferum	Prima	33 - 43	2226 - 5238	2
M. truncatula	Sultan-SU	30 - 40	3262 - 5801	2
T. subterraneum	Leura	27 - 42	3543 - 8517	5
T. subterraneum	Narrikup	26 - 44	3143 - 6796	2
T. subterraneum	Izmir	25 - 43	2976 - 5469	2
T. spumosum	Bartolo	21 - 38	2672 - 7047	3
B. pelecinus	Casbah	18 - 39	2306 - 6387	2
		Moderate P requirement		
T. incarnatum	Dixie	19 - 33	3254 - 10231	4
T. hirtum	Hykon	19 - 28	2815 - 6668	4
T. vesiculosum	Zulu II	15 - 31	2945 - 7179	3
		Low P requirement		
T. purpureum	Electra	17 - 25	1922 - 5412	3
L. corniculatus	LC07AUYF	20 - 21	1259 - 1376	2
O. sativus	Margurita	10 - 24	2331 - 8246	5
O. compressus	Santorini	7 - 24	1877 - 5384	6

4.5 Conclusions

The field experiments have identified for the first time that *O. sativus* (cv. Margurita) has a significantly lower critical P requirement than *T. subterraneum* and is also capable of producing equivalent peak spring biomass. This result and that for *O. compressus* confirmed the substantial differences in critical STP requirements of *Ornithopus* spp. relative to *T. subterraneum*, and support results from glasshouse and controlled-environment experiments that are reported in subsequent chapters. These differences in external P requirement occurred whilst roots of all species were highly colonised by arbuscular mycorrizhal fungi,

despite their reputed role in P nutrition of pasture legumes. The field experiments also demonstrated that some species have specific adaptation requirements which can modify their ability thrive and their apparent P-efficiency. These constraints to widespread use of some of these alternative legumes are sometimes not adequately acknowledged.

Acknowledgements

Edward Story of "Werong" near Yass' Peter Southwell of "Fairview" near Burrinjuck; Gnadbro Pastoral Company of "Lyndoch" near Belfrayden and Peter Carmody of "Wybimbie" near Beckom are thanked for providing land for these experiments at no cost to the project. Their support for the work is gratefully acknowledged.

5 Variation in root traits associated with nutrient foraging amongst temperate pasture legumes and grasses

Zongjian Yang, Richard A. Culvenor, Rebecca E. Haling, Adam Stefanski, Megan H. Ryan, Graeme A. Sandral, Daniel Kidd, Hans Lambers, Richard J. Simpson

Published on-line in 2015: Grass and Forage Science doi: 10.1111/gfs.12199

5.1 Introduction

The most common legume genera (*Trifolium* and *Medicago* spp.) used in temperate pasture systems have high critical P requirements (i.e. the soil P concentration required for 90% of maximum shoot yield) for maximum productivity relative to grasses with which they are grown (Ozanne *et al.*, 1969; 1976). This is partly attributed to the legumes being relatively less effective at acquiring P from soil, most likely as a result of differences in root morphology and the impact of this on soil exploration (Evans, 1977). The grasses generally have relatively long, fine roots with long root hairs. In contrast, the legumes have short, thick roots with short root hairs (Hill *et al.*, 2006). High soil exploration by roots (root foraging) potentially minimises the diffusion distance for P movement to the root. It has been proposed that selecting legumes with lower critical soil P requirements could reduce the amounts of P inputs required in many pasture systems (Simpson *et al.*, 2014). To achieve this, it will be necessary to identify pasture legumes that have root foraging traits similar to those of grasses or P “mining” traits such as organic anion exudation (Richardson *et al.*, 2011).

There is little reported information on the variation in root morphological characteristics of pasture legumes and grasses. There is a particular gap in our knowledge for a range of alternative legume species that have been developed in Australia over the past 15 years (Loi *et al.*, 2005; Nichols *et al.*, 2007; 2012). These species are adapted to a variety of climate, soil and/or farming systems not adequately covered by the commonly used *Trifolium* and *Medicago* spp. Some of these species are adapted to low-P soils (e.g., *Ornithopus* spp. and *Biserrula pelecinus*; de Ruiter, 1981; Paynter, 1990; Tang *et al.*, 1998), while for others, little is known about their P requirement or root morphology.

This study assessed the variation in root morphological traits of a range of annual and perennial pasture legumes and compared them with those of the roots of two perennial grasses to determine whether any legume species have root morphological traits that could achieve similar levels of soil exploration to the grasses,. The intrinsic variation in three key root traits associated with root foraging: specific root length (root length to dry mass ratio), average root diameter and root hair length was assessed.

5.2 Materials and methods

5.2.1 Plant material

Root traits associated with P-acquisition efficiency of 13 perennial and annual legumes, and two grasses, were assessed (Table 5.1). Species were selected either as currently used key pasture legumes (*Trifolium subterraneum* L. and *Medicago sativa* L.) and grasses (*Dactylis glomerata* L. and *Phalaris aquatica* L.), or as legume species that have potential value for improving the productivity of pastures grown in nutrient- or moisture-limited environments.

Species that grow well with low external P supply were of particular interest, given the importance of root traits for maximising soil P acquisition.

5.2.2 Variation in specific root length and average root diameter

Three- and six-week old plants were screened for specific root length and average root diameter in a mixture of sand (50%), sieved loam (25%) and sieved special base (25%) consisting of recycled potting mix containing residual nutrients and added superphosphate. Pots for the harvests after three weeks (90 mm diameter; 200 mm height) and six weeks (90 mm diameter; 400 mm height) were filled with 1.299 and 2.746 kg (oven dry basis) potting mix, respectively. Pots were maintained at 15% w/w moisture (approximately 75% of field capacity) by watering with a half-strength P-free nutrient solution for three weeks followed by a full-strength P-free nutrient solution [2 mM $MgSO_4.7H_2O$, 5 mM $CaSO_4.2H_2O$, 20 mM KNO_3, 2.5 mM $(NH_4)_2SO_4$, 2.5 mM NH_4NO_3, 23 µM H_3BO_3, 46 µM $MnCl_2.4H_2O$, 15 µM $ZnSO_4.7H_2O$, 1.6 µM $CuSO_4.5H_2O$, 0.7 µM $(NH_4)_2MoO_4$, 1 µM $CoCl_2 \cdot 6H_2O$ and 50 µM Fe-EDTA] for the remainder of the experiment. Extractable P concentration of the mixed growth medium was 55 mg kg^{-1} (Colwell, 1963). Four seeds were planted per pot. Pots (except those planted with *B. bituminaria*) were inoculated the day after sowing with a slurry of peat containing an appropriate strain of rhizobium (Table 5.1). Pots were moved to the glasshouse after germination and thinned to one plant per pot. Plants were grown under natural lighting from May to July 2013 in Canberra, ACT, Australia. Temperatures were partially regulated between 20°C (max) and 15°C (min). Five replicates were grown for each species and harvest, and pots were arranged in a completely randomised design. *Trifolium tumens* was not included in this screen. Plants were harvested at three and six weeks after germination. Roots were washed from the soil, scanned in a flatbed scanner, and root length and average root diameter analysed using WinRHIZO (Regent Instruments Inc., Quebec, Canada). Roots at the harvest after three weeks were scanned immediately after they had been harvested. Due to the time requirement for processing the large root systems, roots from the harvest after six weeks were weighed to determine fresh mass and then stored in 50% ethanol at 4°C prior to analysis. Root dry mass was determined after drying at 70°C. In addition, the dry mass of roots stored in ethanol was adjusted for the loss of mass (Crush *et al.*, 2010) that occurred during storage in ethanol. Correction factors were determined for each species as the ratio of root dry mass to root fresh mass based on a sub-sample of roots that were weighed fresh and immediately dried after harvest.

Table 5.1 List of species scientific name, authority, common name, genotype, inoculants and relevance to study.

Species, authority	Common name	Cultivar	Inoculant	Uses and/or features relative to *T. subterraneum* and/or *M. sativa*	Reference
Perennial legumes					
Medicago sativa L.	lucerne	cv. SARDI 10	Group AL	Widespread use in southern Australia; possible high requirement for P (field and pot experiments)	Helyar and Anderson, 1970; Pang *et al*., 2010a; 2010b
Lotus corniculatus L.	birdsfoot trefoil	cv. LC07AUYF	Lotus special	Low external requirement for P (field observations)	Ayres *et al*., 2006
Trifolium tumens Steven ex. M. Bieb.	talish clover	cv. Permatas	Group C	Novel species for pastures; drought tolerant	Hall *et al*., 2013
Trifolium ambiguum M. Bieb.	caucasian clover	cv. Kuratas	Caucasian clover special	High yielding, persistent clover; adapted to altitude and cool climates (field experiments)	Dear and Zorin, 1985; Virgona and Dear, 1996
Bituminaria bituminosa ssp. *albomarginata* (L.) C. H. Stirt.	tedera	Breeder's line 27	Tedera special	Low external requirement for P (pot experiment)	Pang *et al*., 2010
Annual legumes					
Trifolium hirtum All.	rose clover	cv. Hykon	Group C	Low internal P concentration	Pinkerton and Randall 1994; Pinkerton *et al*., 1997
Ornithopus compressus L.	yellow serradella	cv. Santorini	Group S	Low external requirement for P (pot and field experiment)	Paynter, 1990; Bolland and Paynter, 1992; Paynter, 1992
Ornithopus sativus Brot.	French serradella	cv. Margarita	Group S	Low external requirement for P (pot experiment)	de Ruiter, 1981
Trifolium spumosum L.	bladder clover	cv. Bartolo	Group C	Novel species for pastures	Loi *et al*., 2012
Biserrula pelecinus L.	biserrula	cv. Mauro	Biserrula special	Less responsive to P (pot experiment)	Tang *et al*., 1998
Trifolium subterraneum ssp. *subterraneum* L.	subterranean clover	cv. Leura	Group C	Widespread use and adaptation in southern Australia; high requirement for P (pot experiment)	Ozanne *et al*., 1976; Paynter 1990; Hill *et al*., 2005, 2006; Nichols *et al*., 2007
Trifolium incarnatum L.	crimson clover	cv. Dixie	Group C	High-yielding clover (pot experiment)	McKel l*et al*. 1962
Trifolium purpureum Lois.	purple clover	cv. Electra	Group C	Novel species for pastures	Nichols *et al*., 2007
Perennial grasses					
Phalaris aquatica L.	phalaris	cv. Holdfast GT	n/a	Common pasture grass in southern Australia; moderate requirement for P (pot experiment)	Hill *et al*., 2005, 2006
Dactylis glomerata L. ssp. *hispanica* (Roth) Nyman	cocksfoot	cv. Uplands	n/a	Common pasture grass in southern Australia; productive on low fertility, acid soils (anecdotal)	Lolicato and Rumball, 1994

5.2.3 Variation in root hair length

Root hair length of one- and four-week old plants grown in a field soil was screened in separate experiments. A sandy loam soil (Yellow Chromosol, Isbell 1996) was collected from 2–15 cm depth at Ginninderra Experiment Station, Canberra, ACT, Australia (35°10'30''S, 149°02'33.4''E). The soil was pasteurised with steam, sieved to < 5 mm and mixed with lime (1 g lime kg^{-1} soil) to raise pH (1:5 $CaCl_2$) to 5.5. Extractable P was 7 mg kg^{-1} (Colwell, 1963).

For the one-week old plants, cylindrical plastic pots (40 mm diameter, 70 mm depth) were filled with 0.106 kg (oven dry basis) of soil, which was watered to 18% w/w. Each pot was sown with 9 to 12 seeds of each species and watered with 2 mL of the nutrient solution described previously at one-quarter strength. Pots were situated in 8°C to break any seed dormancy and after three days moved to a growth cabinet set at 22°C during the 14 hour light period (photon flux density 600 µmol m^{-2} s^{-1}) and 15°C during the 10 hour dark period. Plants were harvested four to seven days later in an order determined by appearance of root tips at the base of the pot. Four replicates were grown for each species and pots were arranged in a completely randomised design. *Trifolium incarnatum*, *O. sativus* and *T. purpureum* were not included in the one week screen.

For the four-week old plants, nutrients were pre-mixed into the soil at rates of 197.7 mg kg^{-1} dry soil KH_2PO_4, 41.1 mg kg^{-1} $MgSO_4.7H_2O$, 43.0 mg kg^{-1} $CaSO_4.2H_2O$, 169 mg kg^{-1} KNO_3, 27.5 mg kg^{-1} $(NH_4)_2SO_4$, 16.7 mg kg^{-1} NH_4NO_3, 119 µg kg^{-1} H_3BO_3, 759 µg kg^{-1} $MnCl_2.4H_2O$, 359 µg kg^{-1} $ZnSO_4.7H_2O$, 33.3 µg kg^{-1} $CuSO_4.5H_2O$, 72.1 µg kg^{-1} $(NH_4)_2MoO_4$, 19.8 µg kg^{-1} $CoCl_2 \cdot 6H_2O$ and 1530 µg kg^{-1} Fe-EDTA. The extractable P concentration of the soil was 33 mg kg^{-1} (Colwell, 1963). Cylindrical PVC pots (90 mm diameter; 200 mm depth) were filled with 1.333 kg (oven dry basis) of soil wet to 11% w/w. Five seeds were planted per pot. Pots were moved to a growth cabinet after germination and thinned to two plants per pot. The photon flux density in the growth cabinet was approximately 600 µmol m^{-2} s^{-1} with 12 hours of light per 24 hour period. Temperatures were 20°C during the light period and 15°C during the dark period. Pots were inoculated with a slurry of peat containing an appropriate strain of rhizobium two weeks after sowing. All pots were maintained at a water content of approximately 75-80% of field capacity by daily weighing and watering to replace water loss from the pots. Four replicates were grown for each species and pots were arranged in a completely randomised design. Plants were harvested four weeks after seedling emergence.

At harvest, roots were washed from the soil and stored in 50% (v/v) ethanol at 4°C. Images of fully elongated root hairs were taken using a Leica MZFLIII Fluorescence microscope fitted with a Zeiss AxioCam camera. Ten images were captured per plant and root hair length of ten root hairs per image was measured using ImageJ software (Rasband, 1997-2014).

5.2.4 Statistical analyses

The effect of harvest time and species on specific root length (calculated as root length divided by root dry mass) and average root diameter were analysed using a two-way analysis of variance. The effect of species on root hair length for the one-week and four-week experiments were analysed using a one-way analysis of variance. Data were analysed in the R statistical package (R Core Team, 2013).

The mean specific root length (SRL), average root diameter (D) and average root hair length (RHL) were used to calculate an estimate of the specific root hair cylinder volume of each species, i.e. the volume of the cylinder of soil that encompasses the root and root hair zone per unit root mass, using the equation:

Specific root hair cylinder volume ($cm^3\ g^{-1}$) = SRL × π $[(0.5 \times D)+RHL]^2$

The standard error of the specific root hair cylinder volume was estimated using the delta method for propagation of error (Rice, 2007).

5.3 Results

The pasture legumes examined in this study varied considerably in the root morphological traits that were assessed. There was up to a 3.6-fold range in specific root length (79–281 m g^{-1}; three-week old plants), a 1.6-fold range in average root diameter (0.28–0.43 mm; three-week old plants) and a 6.1-fold range in root hair length (0.12–0.75 mm; four-week old plants) of the pasture legumes (Figs. 5.1 and 5.2). *Trifolium subterraneum* had a relatively low specific root length (159–171 m g^{-1}) and short root hairs (0.23 mm; four-week old plants). *Medicago sativa* had values intermediate for the legumes, but values were not consistently greater than those measured for *T. subterraneum*. *Ornithopus compressus*, *O. sativus* and *B. pelecinus* had long root hairs (0.56–0.75 mm; four-week old plants) compared with the other legumes species, and the specific root length of these three species (299–320 m g^{-1}) was also at the upper end of the range measured for the legume species when measured on six-week old plants. In comparison with the legumes, the grasses had relatively high specific root length (371–603 m g^{-1}; six-week old plants), low average root diameter (0.17–0.25 mm; six-week old plants) and long root hairs (0.86–1.0 mm; four-week old plants). Average root diameter was negatively correlated with specific root length on both three-week (R=0.89) and six-week (R=0.92) old plants (Fig. 5.3).

The variation in root morphological traits across species was generally consistent over different harvesting times (Fig. 5.4). The specific root length and average root diameter of most legume species did not significantly differ between the three- and six-week old plants (Fig. 5.1), and root hair lengths were highly correlated between the one- and four-week experiments (Fig. 5.4). However, specific root length of the grasses doubled between the three- and six-week harvests, and also increased markedly with time for *B. pelecinus* and *O. compressus*. The root hair lengths of *L. corniculatus*, *M. sativa* and *P. aquatica* appeared to be longer on 4-week old plants compared with those of one-week old plants, but shorter (by 50%) on *T. subterraneum* (Fig. 5.2).

The potential for spatial exploration of the soil by the root system was estimated by calculating the specific root hair cylinder volume (Table 5.2). The variation in root morphological traits of the legume species translated into a 24-fold range in the estimated specific root hair cylinder volume. Species with high specific root length tended to have longer root hairs (R=0.87; Fig. 5.5), which translated into similar rankings amongst the species for specific root length, average root diameter, root hair length and consequently the estimate of specific root hair cylinder volume. Hence *B. bituminosa*, which had the lowest specific root length, thickest roots and shortest root hairs of all of the species, had the smallest specific root hair cylinder volumes (28.3–31.0 $cm^3\ g^{-1}$). *Trifolium subterraneum* also had a very low specific root hair cylinder volume (82.6–77.8 $cm^3\ g^{-1}$). The specific root hair

cylinder of *D. glomerata* (1152.8–2313.6 cm^3 g^{-1}) was up to 30-fold greater than that of *T. subterraneum*, but only up to three-fold greater that of the *Ornithopus* species.

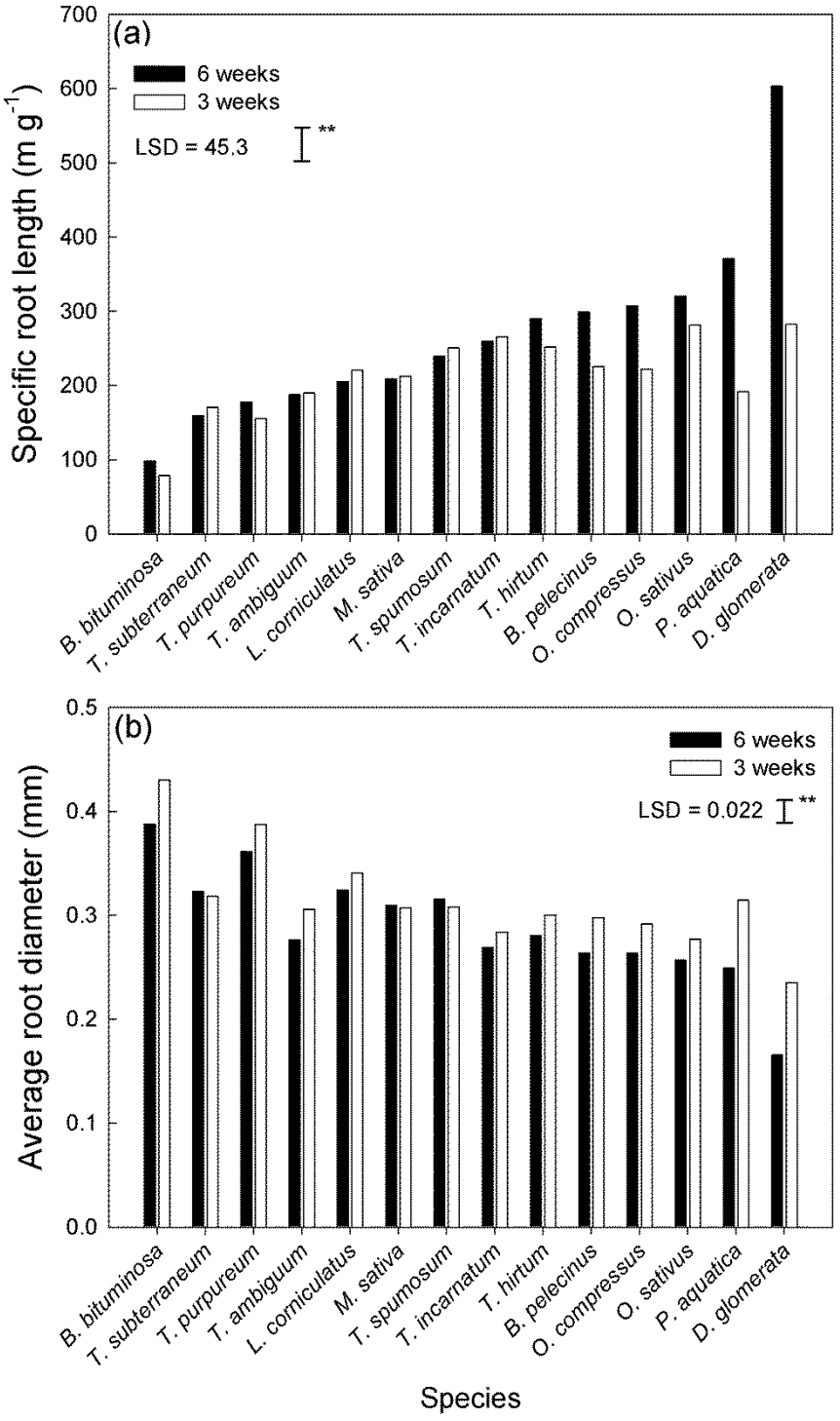


Figure 5.1 (a) Specific root length and (b) average root diameter of pasture legumes and grasses grown for three and six weeks. Bar shows LSD for two-way interaction between species and harvest (P<0.05; n=5).

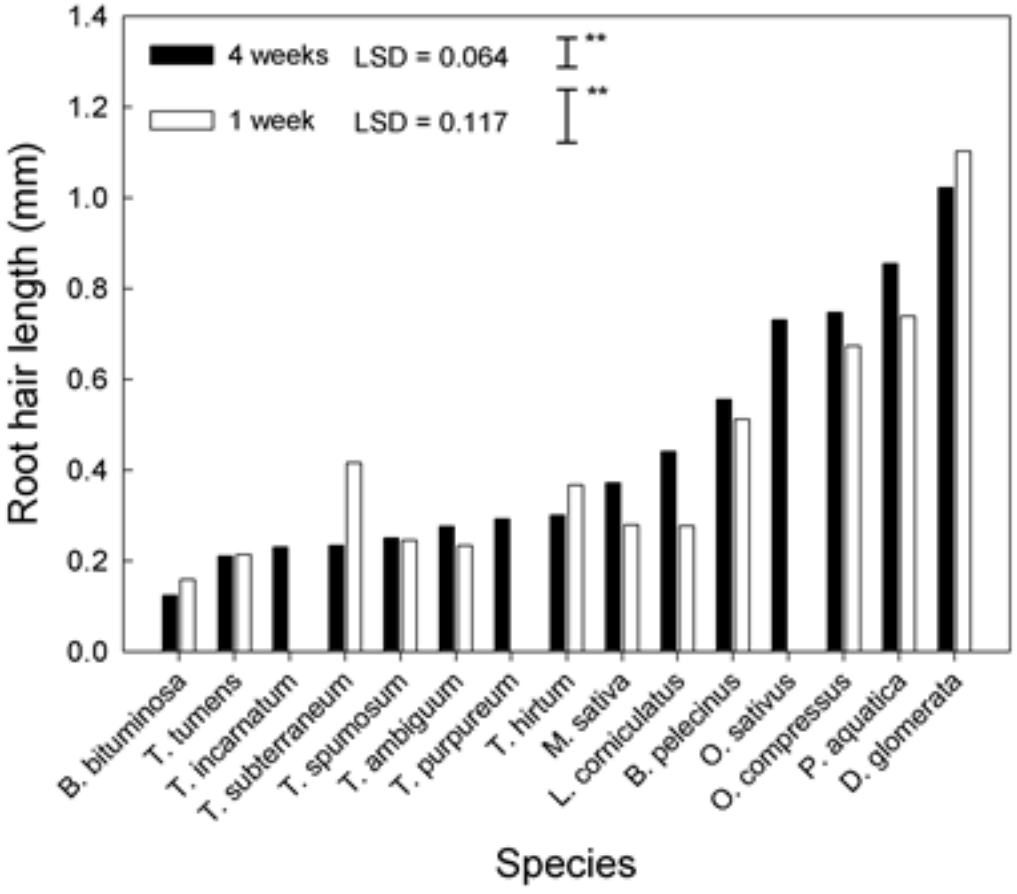


Figure 5.2 Root hair length of pasture legumes and grasses grown in separate experiments for one week and four weeks. Bar shows LSD for species effect in each experiment (P<0.05; n=4).

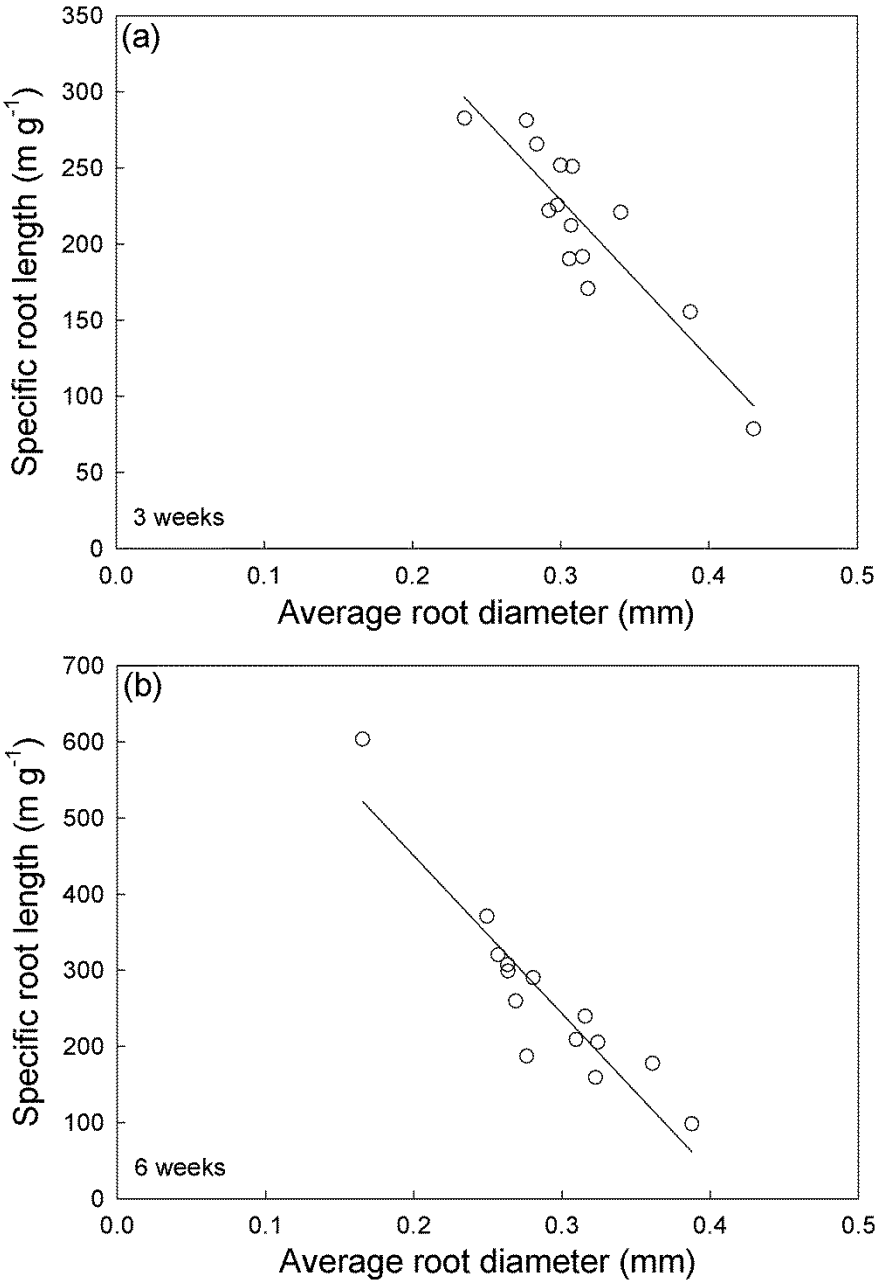


Figure 5.3 Correlation between specific root length and average root diameter of pasture legumes and grasses grown for (a) three weeks (R = 0.89) and (b) six weeks (R = 0.92).

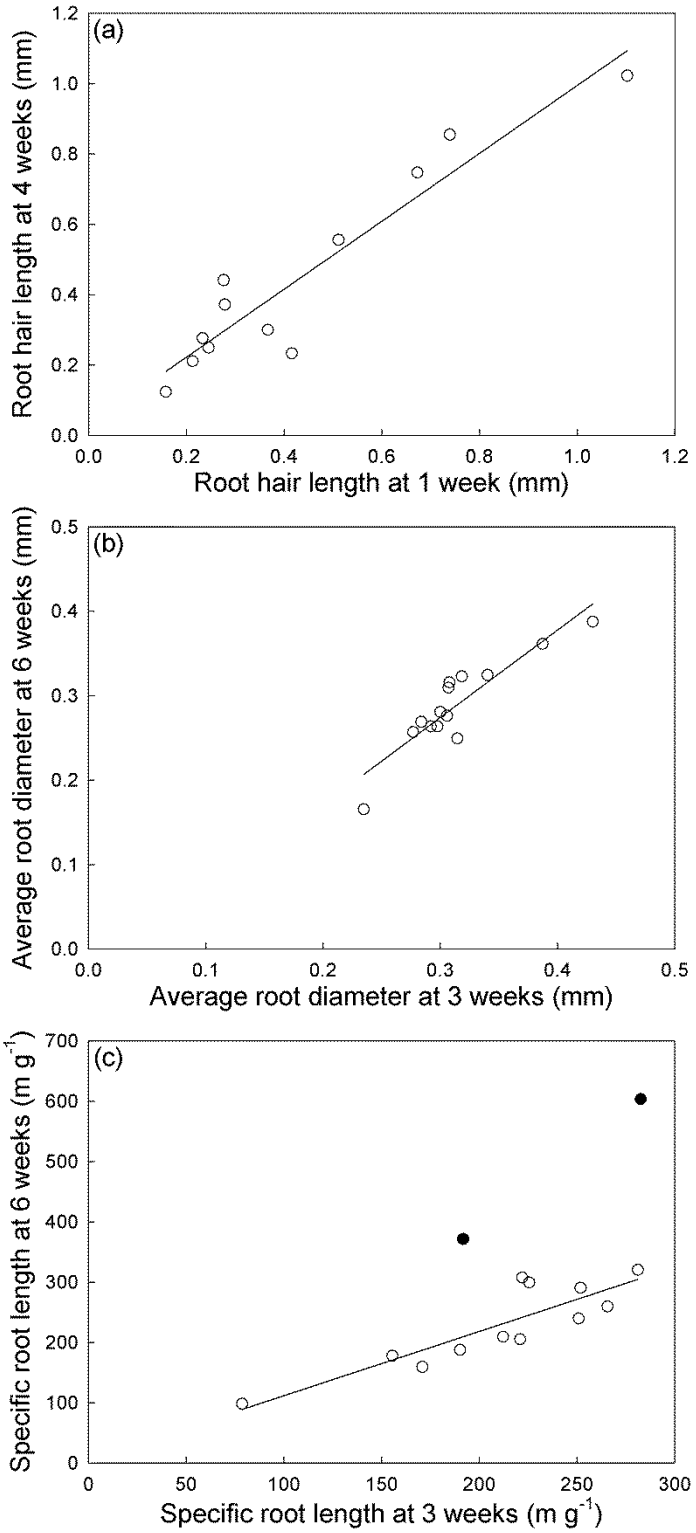


Figure 5.4 Correlation of root morphology traits between different harvest times: (a) root hair length (y = 0.97 x + 0.03; R = 0.94) (b) average root diameter (y = 1.04 x - 0.04; R = 0.90) and (c) specific root length (y = 1.07 x + 5.16; R = 0.87). For (c) grasses, shown as solid symbols, were not included in correlation.

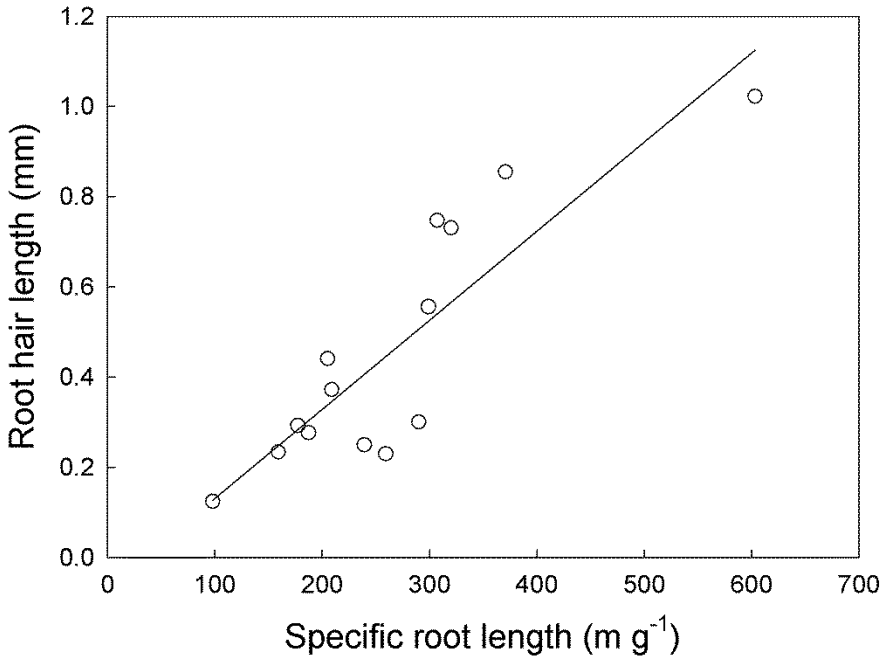


Figure 5.5 Correlation (R = 0.87) between specific root length (six week plants) and root hair length (four week plants).

Table 5.2 Estimated specific root hair cylinder volume based on specific root length (SRL) of three- and six-week old plants, and root hair length of four-week old plants.

	Specific root hair cylinder volume ($cm^3\ g^{-1}$)	
Species	three-week SRL	six-week SRL
Bituminaria bituminosa	28 ± 2	31 ± 3
Trifolium. subterraneum	83 ± 6	78 ± 3
Trifolium ambiguum	110 ± 10	101 ± 11
Trifolium purpureum	115 ± 7	108 ± 8
Trifolium incarnatum	115 ± 14	125 ± 7
Trifolium spumosum	128 ± 8	125 ± 8
Trifolium hirtum	161 ± 5	177 ± 8
Medicago sativa	184 ± 12	182 ± 14
Lotus corniculatus	259 ± 34	234 ± 19
Biserrula pelecinus	352 ± 20	444 ± 30
Ornithopus compressus	556 ± 51	746 ± 72
Phalaris aquatica	617 ± 74	1118 ± 126
Ornithopus sativus	667 ± 50	742 ± 75
Dactylis glomerata	1153 ± 148	2314 ± 299

5.4 Discussion

We assessed the variation in root morphological traits of a range of annual and perennial pasture legume species. The work aimed to identify pasture legumes that have different intrinsic abilities to forage for poorly mobile nutrients such as P. The factors that determine specific root hair cylinder volume (i.e. specific root length, average root diameter and root hair length) were examined.

Legumes with a major role in the temperate pastures of southern Australia, *T. subterraneum* and *M. sativa*, had relatively low specific root length and short root hairs in comparison with the common “companion” grasses. These root features are considered likely to contribute to the relatively high P requirement of legumes (Ozanne *et al.*, 1969; 1976) and this determines the soil P fertility management targets for the soils on which they are grown (Simpson *et al.*, 2014). However, potentially valuable differences in root traits associated with P-acquisition efficiency were observed amongst the range of “alternative” legumes examined in the present study. Most notable were the two *Ornithopus* species and *B. pelecinus*. These species, particularly the *Ornithopus* species, had root hair lengths and specific root lengths that were similar to those of the grasses. These features are likely to be a factor contributing to the relatively low requirement for P that has been observed for these legume species (de Ruiter, 1981; Paynter, 1990; Tang *et al.*, 1998). Interestingly, the specific root length and root hair length of *B. bituminaria* were low, despite this species being identified as having a relatively low requirement for P (Pang *et al.*, 2010a; 2010b).

The specific root hair cylinder volume was calculated to provide an estimate of how specific root length and root hair length affected soil exploration. The short root hairs and low specific root hair length of *T. subterraneum* translated into an effective volume of soil explored per dry mass of root that was a fraction of that achieved by the grasses. Even species such as *T. hirtum*, which had a relatively high specific root length but short root hairs, explored substantially less soil per unit root dry mass than the legumes and grasses that had both high specific root length and long root hairs. Evans (1977) found similar contrasts in root hair lengths and specific root lengths among several pasture grasses and three *Trifolium* species, and also noted the absence of root hairs on 30 to 40% of the root lengths of the clovers. This translated into six- to 30-fold differences in the specific root hair cylinder volumes among the legumes and grasses (Evans 1977). Such differences are likely to result in substantial differences in the ability of the plants to acquire P, given that the zone of root hair exploration closely mirrors the zone of depletion of P in the soil by the plant (Gahoonia *et al.*, 1997a; 1997b). Evans (1977) also calculated that the low root length density achieved by legumes in comparison with that of the grasses would mean that most of the legume roots would be competing with grass roots for P, but this would only represent a small proportion of the grass roots. This could further hinder the performance of legumes grown in sub-optimal P conditions, and highlights the importance of calculating parameters that estimate the effective exploration of soil, rather than measuring root dry mass alone.

The root hair lengths of many of the legume species, including the *Trifolium* spp., are likely within the range where mycorrhizal colonization benefits P uptake under conditions of low P availability. Schweiger *et al.* (1995) demonstrated an inverse relationship between root hair length and mycorrhizal benefit of growth with increasing growth benefit below root hair lengths of approximately 0.4 mm. *Ornithopus compressus* (root hair length 0.48 mm) and *Lolium rigidum* (Gaud.; 1.12 mm) showed no significant benefit from inoculation with a

Glomus sp. in contrast with *T. subterraneum* (0.22 mm). Schweiger *et al.* (1995) did not find a significant correlation between mycorrhizal benefit and root diameter. However, in our work, we found that species that had longer root hairs tended to also have higher specific root length. Specific root length was highly correlated with average root diameter which in itself suggests that for the species that were compared the intrinsic variation in specific root length was not caused by large differences in root tissue density or presence of root aerenchyma. Aerenchyma is, for some species (e.g., maize), an adaptation to P deficiency (Fan *et al.*, 2003), so a role in P stress adjustment for the species examined in this study is not precluded. In the current work, we assessed the intrinsic variation in root traits. While it is expected that adaptation of these traits in response to P stress would occur, we do not anticipate that it would change the relative ranking of the species. Hill *et al.* (2006) compared the root morphological traits of a range of grasses and a legume in response to P stress, and found that intrinsic root characteristics were a better indicator of inter-specific differences in critical P requirements than adaptation to P stress.

Absolute measures of root hair length and specific root length were mostly consistent across plant ages. The marked increase in specific root length of the grasses, *Ornithopus* spp. and *B. pelecinus* as the plants grew, was likely to have reflected an increase in lateral root length over time and hence lower relative contribution of thicker seminal roots (grasses) or taproots (legumes) to the total root length. The much larger difference in specific root length for *P. aquatica* compared with *D. glomerata* is likely to be a result of the slower establishment of this species relative to *D. glomerata*. The long root hairs measured on *T. subterraneum* plants at one week is not consistent with the root hair lengths measured either on other *Trifolium* spp. within this study or other studies of *T. subterraneum* (e.g., 0.22 mm Evans, 1977; 0.22 mm, Schweiger *et al.*, 1995; 0.27 mm, Hill *et al.*, 2010) and the lengths measured at this time did not persist. Further work is required to confirm this result as it may have implications for methodologies for screening for root hair length, and our understanding of root hair growth and function.

This study demonstrated that significant differences in root foraging traits exist outside the commonly-used temperate legume species such as *T. subterraneum* and *M. sativa*. A few legume species (e.g., *Ornithopus* spp.) had traits that approached those of grasses, and this is expected to be a major factor in their relatively low critical P requirements compared with other legumes. Other legumes (e.g., *B. pelecinus* and *L. corniculatus*) had intermediate root foraging traits. *Ornithopus* species are noted for their ability to tolerate low-fertility sites (Paynter, 1992), so work to quantify the critical soil P requirement of these species is important, as they may have a role in development of pastures that can be managed at a lower extractable soil P concentration than is presently possible with species such as *T. subterraneum* and *M. sativa*.

6 Growth and root dry matter allocation by pasture legumes and a grass with contrasting external critical phosphorus requirements

Rebecca E. Haling, Zongjian Yang, Natalie Shadwell, Richard A. Culvenor, Adam Stefanski, Megan H. Ryan, Graeme A. Sandral, Daniel R. Kidd, Hans Lambers, Richard J. Simpson

Published in Plant and Soil (2016) 407: 670-679.

6.1 Introduction

It is known that the grasses and the legume component (commonly *Trifolium* or *Medicago spp*.) of temperate mixed pastures have very different critical phosphorus (P) requirements (here defined as the concentration of extractable-P in soil, or the amount of P applied for 90% of maximum growth rate) (Mengel *et al*. 2001). The grass species achieve near maximum growth at much lower extractable-P concentrations in the soil than the associated legume (Ozanne *et al*. 1969; 1976; Hill *et al*. 2006). This is attributed to grasses having roots that can explore soil more effectively (nutrient foraging). In particular, temperate grasses have long, fine roots and long root hairs that enable development of relatively large specific root hair cylinder volumes (i.e. the soil volume defined by the root diameter and root hair length per unit root dry mass; Evans 1977; Horst *et al*. 1993; Gahoonia and Nielsen 1997a; 2004; Hill *et al*. 2006). The grasses also have lower critical internal P concentrations (i.e. the concentration of P in herbage at near-maximum growth rate) (Pinkerton and Randall 1994).

Grass-legume pastures in Australia rely on legume nitrogen (N) fixation as the sole source of N input. The disparity in the P requirement of the grasses and legumes means that the high P requirement of the legume determines the P fertiliser application rate for high pasture production. However, the P use efficiency of the pasture systems is low because P accumulates in Australian soils as a result of their high propensity for P sorption (Simpson *et al*. 2014; 2015). Pastures used for sheep and beef production, for example, require 5–9 units of P to be applied as fertiliser for an output of 1 unit of P exported in animal products (Weaver and Wong 2011). However, long-term monitoring of the P balance of grazed pasture systems has shown that lower P inputs will be achieved if pastures can be managed at lower concentrations of extractable P in the soil without reducing production (Simpson *et al*. 2014; 2015). Legumes that are as productive as current mainstream pasture legume species (e.g. *T. subterraneum; T. repens*) at lower soil test P concentrations are needed to achieve this.

There have been many studies of the P requirement of *Trifolium* spp., most of which have demonstrated only marginal differences amongst genotypes; where differences are reported, interpretation has sometimes been confounded by differences in yield and ontogeny (McKell *et al*. 1962; Jones *et al*. 1970). Attempts to select for improved P acquisition efficiency and/ or productivity under low P conditions have been relatively unsuccessful. For example, while significant genotypic variation in root morphology traits, and/ or response to P, has been identified within *T. repens* (Caradus 1981; Crush *et al*. 2008; Jahufer *et al*. 2008), the value of this for improving the productivity of *T. repens* under low P conditions in the field has not been fully realised (e.g. Caradus 1994; Caradus and Dunn 2000).

Temperate pastures in southern Australia rely heavily on the use of three main groups of pasture legumes: *T. subterraneum* (acid soils, temperate southern Australia), *T. repens* (high rainfall, temperate zone) or annual *Medicago* spp. (low rainfall, neutral-alkaline soils). However, over several decades Australia has invested in the development of "novel" legumes, through breeding and ecotype selection, to fill soil or environmental niches not already covered by the main pasture legumes and, in some instances, as potential alternatives to the mainstream species (Nichols *et al*. 2007). The P requirements of the alternative legumes are largely unknown although it is reported that *Ornithopus compressus* can yield as well as *T. subterraneum* in the sandy soils of Western Australia with about half the amount of applied P (Paynter 1990). The features of grass root systems and of other species that are likely to enable plants to achieve high yield at low critical P levels include high specific root length and long root hairs (Lynch 2007; Evans 1977). Yang *et al*. (2015) have reported that some of the novel pasture legumes have high specific root lengths, long root hairs and root hair cylinder volumes approaching that of some grasses (Chapter 5). This may confer improved potential for P acquisition in low P soil and lower critical external requirements for P than is achieved by the mainstream legume species.

Here we report the first study of the response to P fertiliser by five alternative pasture legumes and a companion grass species and assess their root dry matter allocation, internal P distribution and P acquisition efficiency in response to P application.

6.2 Materials and Methods

6.2.1 Plant material

Five annual pasture legumes and one perennial grass were selected on the basis of differences in their root morphology traits (Chapter 5) to represent a range in potential for nutrient foraging capability. Two of the species are commonly grown as companions in temperate pastures. *Trifolium subterraneum* L. (subterranean clover cv. Leura), is a cultivar of the most widely-used legume in the pastures of southern Australia. It has relatively low specific root length (171 m g^{-1}) and short root hairs (0.23 mm). *Dactylis glomerata* L. (cocksfoot cv. Porto) is often grown in pastures with *T. subterraneum.* It is reputed to grow well in infertile soils (Lolicato and Rumball 1994) and has a high specific root length (603 m g^{-1}) and long root hairs (1.10 mm). The other legumes used in this experiment are presently considered as potential alternatives to *T. subterraneum* for use in temperate pastures (Nichols *et al*. 2007). *Biserrula pelecinus* L. (biserrula cv. Mauro) has high specific root length (299 m g^{-1}) and intermediate root hair length (0.56 mm); *Ornithopus sativus* Brot. (French serradella cv. Margurita) has a high specific root length (320 m g^{-1}) and long root hairs (0.73 mm); *Ornithopus compressus* L. (yellow serradella cv. Santorini) has high specific root length (307 m g^{-1}) and long root hairs (0.75 mm) and *Trifolium hirtum* All. (rose clover cv. Hykon) has high specific root length (290 m g^{-1}) and short root hairs (0.37 mm) (Yang *et al*. 2015).

6.2.2 Soil and nutrient treatments

A sandy loam soil (Yellow Chromosol; Isbell 1996) with a low concentration of extractable P (8.3 mg kg^{-1} P; Colwell, 1963) was collected from Ginninderra Experiment Station, Canberra, ACT, Australia (35°10'30"S, 149°02'33.4"E). The soil was steam pasteurised (60-65°C) to reduce levels of disease inoculum, sieved to < 5 mm and mixed with lime (1.06 g $CaCO_3$ kg^{-}

[1]) to raise pH (1:5 w/v; 0.01M $CaCl_2$) to 5.5 and lower the concentrations of Al^{3+} to negligible levels. Nutrients were then mixed into the soil at rates of 41.1 mg kg^{-1} soil $MgSO_4.7H_2O$, 43.0 mg kg^{-1} $CaSO_4.2H_2O$, 169 mg kg^{-1} KNO_3, 27.5 mg kg^{-1} $(NH_4)_2SO_4$, 16.7 mg kg^{-1} NH_4NO_3, 119 µg kg^{-1} H_3BO_3, 759 µg kg^{-1} $MnCl_2.4H_2O$, 359 µg kg^{-1} $ZnSO_4.7H_2O$, 33.3 µg kg^{-1} $CuSO_4.5H_2O$, 72.1 µg kg^{-1} $(NH_4)_2MoO_4$, 19.8 µg kg^{-1} $CoCl_2 \cdot 6H_2O$ and 1530 µg kg^{-1} Fe-EDTA. These nutrient additions ensured that all nutrients except P were in adequate supply. Six P-fertilised soil treatments were established by mixing KH_2PO_4 with sub-samples of the amended soil at rates of 0, 15, 30, 70, 135 and 250 mg P kg^{-1} soil. This resulted in extractable P concentrations of 8.3, 19.2, 30.9, 58.4, 111 and 203 mg kg^{-1} (Colwell 1963). Pots (cylindrical PVC; 87 mm internal diameter, 190 mm soil height) were filled with a bottom layer of the soil (1.00 kg; 11% moisture) that was not fertilised with P (the subsoil) and then with a topsoil layer of P-fertilised soil (0.333 kg; 11% moisture). The resultant P application rates were 0, 4.5, 9.0, 21.0, 40.5 and 75.0 mg P pot^{-1}. The mixing of phosphate throughout the topsoil layer of the pots mimicked the stratified concentration of P in fields that results from application of P fertiliser to the soil surface of grazing lands. The boundary of the fertilised topsoil and unfertilised subsoil layers was marked by placing small alkathene beads around the interior edge of the pot. To ensure that the grass had an adequate supply of N for growth, an additional 21 mg N pot^{-1} (supplied as NH_4NO_3) was applied to the *D. glomerata* plants at 32 days after sowing. Further N was not applied to the legumes in order to promote N_2-fixation.

6.2.3 Plant growth conditions and experimental design

Seed (50 mg viable seed pot^{-1}) of each species was spread evenly across the pot surface area and covered with the topsoil to achieve a 5 mm sowing depth. Five replicate pots of each species at each rate of applied P were prepared. Plants were grown in a controlled-environment growth cabinet with 12 hours of light (720 µmol $quanta^{-1}$ m^2 s^{-1}) and 12 hours dark, at 25/15°C, respectively. Pots were arranged in a randomised complete block design and rotated within blocks every 3 to 7 days to minimise the effect of light gradients within the cabinet. When plant growth exceeded the rim of the pot, sleeves with a reflective inner surface were fitted to the outside of the pots and raised daily to equal plant height. This was used to reproduce light conditions in a pasture sward. Soil moisture was maintained at approximately 75 to 80% of field capacity by daily weighing and watering of pots. Seven days after sowing, the legumes were inoculated with rhizobium; Group C for *Trifolium* spp., Group S for *Ornithopus* spp. and Biserrula special for *B. pelecinus*.

6.2.4 Harvest and measurements

Plants were harvested six weeks after sowing when still in the vegetative growth stage (40 days after sowing for *T. hirtum*; 41 days for *B. pelecinus*, *O. sativus* and *T. subterraneum*; 42 days for *D. glomerata* and *O. compressus*). Shoots were cut at the surface of the soil and dried at 70°C for dry mass determination. Soil was removed from the pots as an intact core and cut at the interface of the fertilised topsoil (0-45 mm depth) and the subsoil (45-190 mm depth) as identified by the alkathene beads. Roots from each layer were washed from the soil. A sub-sample of each was weighed fresh and stored in 50% ethanol at 4°C for subsequent measurements of root morphology (not reported here). An additional sub-sample was weighed fresh and immediately dried to facilitate calculation of a correction factor to account for loss of root mass during storage in the ethanol solution (Crush *et al*. 2010). The total dry mass of roots in each soil layer was determined after drying all samples

at 70^{o}C. The mass of the sub-sample of roots stored in 50% ethanol was corrected to allow for loss of mass (Root DM = $DM_{[after\ alcohol\ storage]}$ * correction factor) using the following correction factors determined for each species and, where necessary, for an effect of P application: (*O. sativus* 1.06; *O. compressus* 1.13; *D. glomerata* 1.13; *T. subterraneum* 1.15; *T. hirtum* 1.32; *B. pelecinus* 1.64 at 0 mg P kg^{-1} and 1.27 for 15–250 mg P kg^{-1}).

Shoots and root samples that had not been stored in ethanol were milled to a fine powder and 25 to 50 mg samples ashed in a muffle furnace for 4 hours at 550^{o}C. The ashed material was dissolved in 2M HCl and P concentration determined colorimetrically using malachite green (Irving and McLaughlin 1990).

6.2.5 Statistical analysis

For each species, the yield of shoot dry matter growth in response to P application was analysed by fitting a Mitscherlich non-linear curve (Equation 1) in R (R Core Team, 2013).

$$y = a - b*(e^{-cx}) \quad [1]$$

where y is the shoot dry matter and x is the P application rate.

The critical external P requirement of each species was defined as the amount of P applied to achieve 90% of maximum yield. Estimates and confidence intervals for critical P and maximum yield (a) were determined by least squares and assume that the model is approximately linear around the estimate. Estimates and confidence intervals for other parameters, including the shoot yield at no P addition to the soil, were obtained by re-parameterising the model to the value of the key parameter using R and GenStat 16th Edition (VSN International, UK). Differences between the critical P concentrations, the maximum yield (or asymptote) and the zero P yields were tested by considering the estimates and approximate standard errors for each measure simultaneously, and testing for significant pairwise differences. Significance was determined by calculating a standardised difference that weighted the two contributing standard errors. Values greater than two standard errors were considered significantly different (P = 0.05). No adjustment was made for multiple comparisons.

For each species, critical internal P concentration was determined as the herbage P concentration corresponding to the critical external P application rate based on curvilinear responses between shoot P concentration (%) and rate of P addition (Hill *et al*. 2005). Internal P-use efficiency (PUE; g DM/ g shoot P) was calculated using two alternative approaches. “Physiological PUE” was determined as: (shoot yield at 90% maximum yield - shoot yield at 0 mg P pot^{-1}) divided by (total shoot P at 90% maximum shoot yield - total shoot P at 0 mg P pot^{-1}) (Baligar *et al*. 2001). Based on the Rose *et al*. (2016) definition of PUE, the reciprocal of shoot P concentration of each species was also determined at a common shoot P content (“PUE at 6.2 mg shoot P”). Linear relationships between ln(shoot P) and PUE (calculated as above) were fitted in GenStat 16th Edition (VSN International, UK) and PUE was predicted at ln(6.2 mg shoot P per pot). This was the lowest shoot P content that allowed all species to be compared at a common shoot P content in the P application range below the critical P requirement of all species. Propagation of errors for critical Shoot P and PUE were performed using the Delta method (Agresti 2002).

Root mass fractions were calculated separately as the mass of roots in the topsoil, subsoil or total root system divided by the total plant mass. P uptake per unit topsoil root mass was calculated as the total plant P (i.e. P in topsoil roots, subsoil roots and shoots) divided by the mass of roots in the topsoil. Relative shoot yield was calculated as: shoot yield at the given level of P pot^{-1} divided by the maximum potential shoot yield determined from the asymptote of Equation 1.

The effect of P addition on shoot dry matter, root mass fraction and P uptake per unit root mass in the topsoil layer of the six species was analysed using general analysis of variance in GenStat 16th Edition (VSN International, UK). A split-plot analysis of variance with Species and P addition as whole-plots and Soil Depth as split-plots was used to analyse root dry matter. Likewise, a split-plot analysis of variance with Plant Part as the sub-plot was used to analyse the P% of the roots in the topsoil and subsoil, and the shoots.

Simple linear regression with groups in GenStat 16th Edition (VSN International, UK) was used to fit regressions for the range over which the response between relative shoot yield (considered an indicator of P sufficiency) and topsoil root mass fraction was linear, and to assess differences in intercept and gradient of the regressions between species.

6.3 Results

6.3.1 Shoot dry matter response and critical external P requirement

Shoot dry matter of all species increased in response to addition of P, however, the initial slope of the P response function (c in Equation 1) and the maximum shoot yield (a in Equation 1) varied among species (Fig. 6.1 and Table 6.1). *Dactylis glomerata* had the highest maximum shoot yield (3.69 g pot^{-1}), almost double that of the lowest yielding species (*B. pelecinus* and *T. hirtum*; Fig. 6.1, Table 6.1). *Ornithopus compressus*, *O. sativus* and *T. subterraneum* had maximum yields that were intermediate to those of *D. glomerata* and *B. pelecinus* or *T. hirtum*.

Dactylis glomerata had the lowest critical external P requirement at 6.6 mg P pot^{-1} and *T. subterraneum* had the highest critical external P requirement at 26.7 mg P pot^{-1}; the latter was significantly higher than all other species (Table 6.1). *Ornithopus compressus* and *O. sativus* had relatively low critical external P requirements more comparable to that of the grass (7.6 and 11.3 mg P pot^{-1}, respectively). The critical external P requirement of *O. compressus* did not differ significantly from that of *D. glomerata*. The critical external P requirements of *B. pelecinus* and *T. hirtum* were between that of *T. subterraneum* and the *Ornithopus* spp.

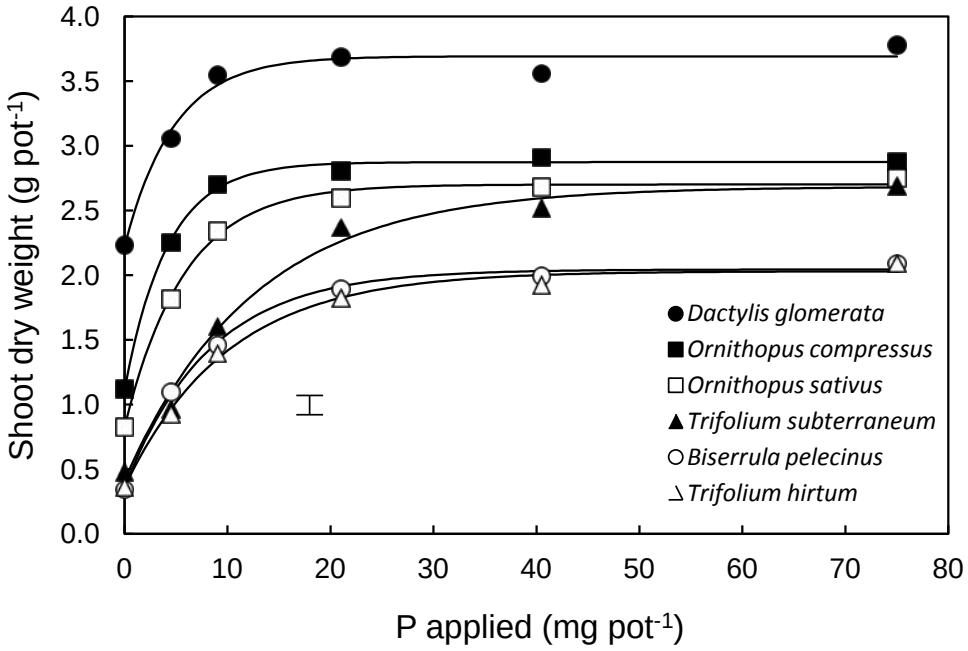


Figure 6.1 Shoot dry weight of five legume and one grass species in response to phosphorus (P) applied in the topsoil of a pot (n=5). Lines show fitted Mitscherlich curves for each species. Bar shows LSD for the Species x P applied interaction (*P*<0.05).

Table 6.1 Critical external phosphorus (P) requirement (mg P pot^{-1}) for five legume and one grass species determined as the rate of P required to achieve 90% of maximum shoot yield from a fitted Mitscherlich response ($y = a - b*(e^{-cx})$). Values ± standard error. Different letters denote significant differences within each column.

Species	Critical external P (mg P pot^{-1})	Parameter			
		Intercept	a	b	c
D. glomerata	6.6 ± 0.6a	2.22 ± 0.07a	3.69 ± 0.03a	1.47 ± 0.06	0.811 ± 0.017
O. compressus	7.6 ± 0.5a	1.12 ± 0.05b	2.87 ± 0.03b	1.76 ± 0.06	0.788 ± 0.016
O. sativus	11.3 ± 0.5b	0.83 ± 0.04c	2.70 ± 0.03c	1.87 ± 0.06	0.841 ± 0.011
T. subterraneum	26.7 ± 1.3c	0.41 ± 0.05d	2.68 ± 0.05c	2.27 ± 0.06	0.923 ± 0.005
B. pelecinus	17.3 ± 1.0d	0.36 ± 0.05d	2.04 ± 0.04e	1.69 ± 0.06	0.885 ± 0.010
T. hirtum	21.1 ± 1.5e	0.36 ± 0.06d	2.03 ± 0.04e	1.67 ± 0.06	0.905 ± 0.008

6.3.2 Root dry matter

The legumes allocated a similar mass of roots to the fertilised topsoil layer (0-45 mm depth) and the unfertilised subsoil layer (45-190 mm depth) at the higher rates of P application (Fig. 6.2). Some of the legumes made adjustments to root dry matter allocation in response to low P supply. Where this occurred (i.e. at or below the critical P application rate), more root dry matter was allocated to the fertilised topsoil than the unfertilised subsoil. In contrast to the

legumes, *D. glomerata* allocated more dry matter to the subsoil layer at all P application rates.

Trifolium subterraneum, *B. pelecinus* and *T. hirtum* produced 1.3–1.6 fold more root dry matter in the P-fertilised topsoil when grown at soil P fertility levels immediately below their critical P level (9–21 mg kg^{-1}; Fig. 6.2). However, these species were not able to achieve this dry mass allocation at P application rates <4.5 mg P pot^{-1} and their root dry mass in the unfertilised soil was significantly less than that at all other rates of P addition.

Ornithopus sativus only marginally adjusted root dry mass in the topsoil in response to P supply below its critical P level; root dry matter of *O. sativus* peaked at P addition of 9 mg pot^{-1} (Fig. 6.2). *Ornithopus compressus* did not adjust root dry mass at P levels below its critical P level. In contrast to the legumes, root dry mass of the grass was decreased in response to lower supply of P; root dry mass of *D. glomerata* grown at 0 mg P pot^{-1} was 75% of the maximum root dry mass achieved at ≥ 21 mg P pot^{-1}.

In the subsoil, the root dry mass of all species, except *O. compressus*, was lowest in the unfertilised treatment. *Ornithopus compressus* did not adjust its root mass and the reductions in root mass by *O. sativus* and *D. glomerata* were relatively small. The species with higher critical P requirements, *T. subterraneum*, *B. pelecinus* and *T. hirtum*, achieved subsoil root dry masses that were only about 50% of their maximum root mass in the fertilised topsoil. For these species, lower root dry mass occurred when P supply rates were less than the critical P level for the species.

6.3.3 Root mass fraction

All species increased the proportional allocation of biomass to their roots (i.e. total root mass fraction) in response to lower soil P fertility (Fig. 6.3a). Adjustments in root mass fraction occurred regardless of whether or not the species had increased root dry matter in the fertilised topsoil layer in response to lower soil P fertility (Fig. 6.2). For example, *D. glomerata* allocated less dry matter to roots at the lowest level of P fertility (Fig. 6.2f) but root mass fraction was nevertheless increased at this level of P fertility.

Generally, species that had lower critical external P requirements (Table 6.1), had lower overall relative allocation of biomass to roots compared to the species with higher critical external P requirements. For example, relative allocation of biomass to roots ranged from 15 to 34% (over the tested range of P addition) for *O. compressus* (low external P requirement), compared to 21 to 46% for *T. subterraneum* (high external P requirement). Despite these differences, all legumes more than doubled their relative allocation of biomass to roots in response to P stress while that of the grass increased by only a third.

The root mass fraction of roots in the topsoil differed considerably from that in the subsoil (Fig. 6.3b and 6.3c, respectively). The overall adjustment the species made in total root mass fraction (Fig. 6.3a) was largely the result of adjustments to biomass allocation in the fertilised topsoil. Adjustment to root mass fraction in the subsoil only occurred when no P fertiliser had been applied to the topsoil resulting in a soil profile with a uniformly low extractable P concentration at all depths. Under this circumstance, adjustment to root mass fraction in the subsoil was similar to that occurring in the topsoil. Regressing root mass fraction against relative shoot yield (an index of P sufficiency) demonstrated that all of the species had adjusted root mass fraction in response to P stress (Fig. 6.4). The total root

mass allocation response to P stress (i.e. the gradient of the total root mass fraction-relative shoot yield relationship) was similar for most of the species, but significantly lower allocation responses (i.e. the gradients) were observed for *T. hirtum* and *D. glomerata* (Fig. 6.4a). By contrast, the root mass allocation response to P stress in the fertilised topsoil layer differed substantially among the species with *T. subterraneum* > *T. hirtum* = *B. pelecinus* > *Onithopus species* > *D. glomerata* (Fig. 6.4b). The root mass fraction allocated to the topsoil layer was expected to represent the roots that were most involved in nutrient foraging.

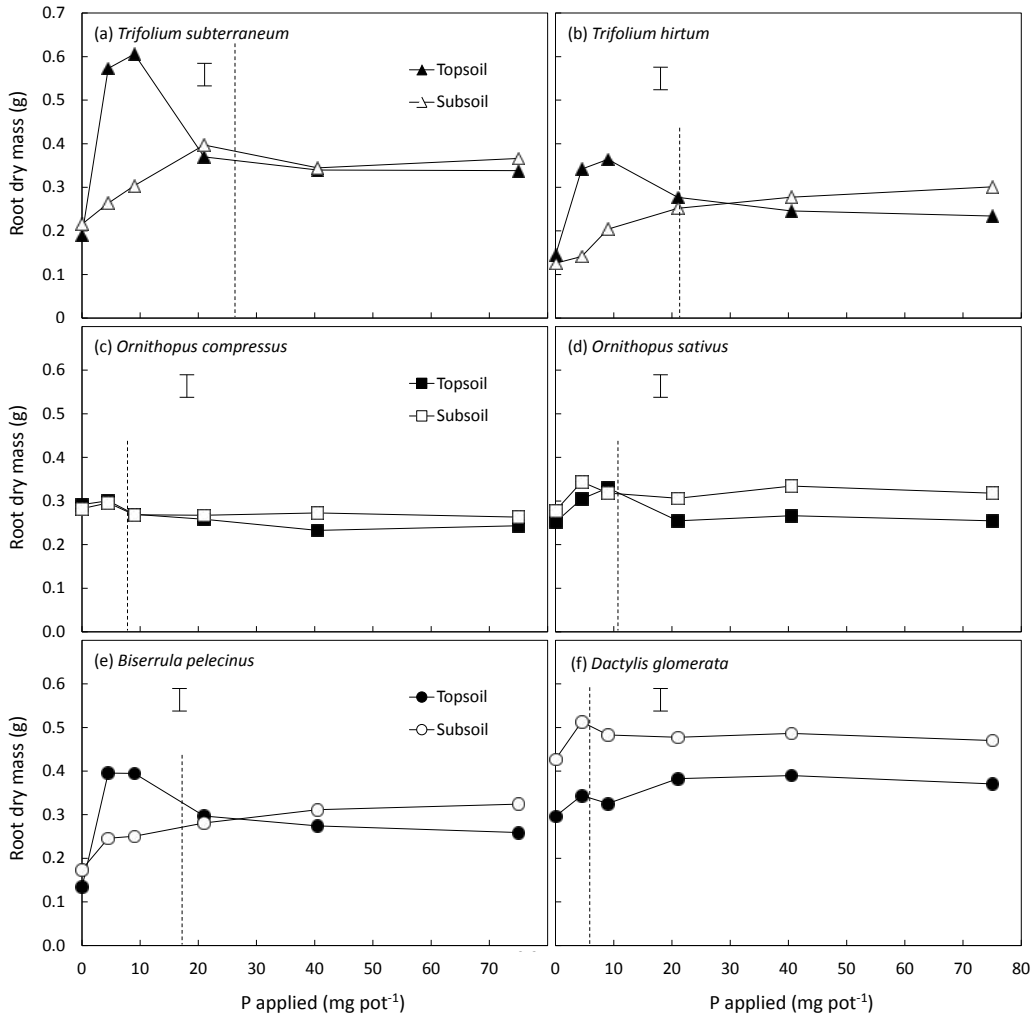


Figure 6.2 Root dry mass in topsoil and subsoil in response to phosphorus (P) applied in the topsoil of a pot for (a) *Trifolium subterraneum* (b) *Trifolium hirtum* (c) *Ornithopus compressus* (d) *Ornithopus sativus* (e) *Biserrula pelecinus* and (f) *Dactylis glomerata* (n=5). Bar shows LSD for the Species x P applied x Soil interaction (P<0.05). Dashed line shows critical external P requirement for each species.

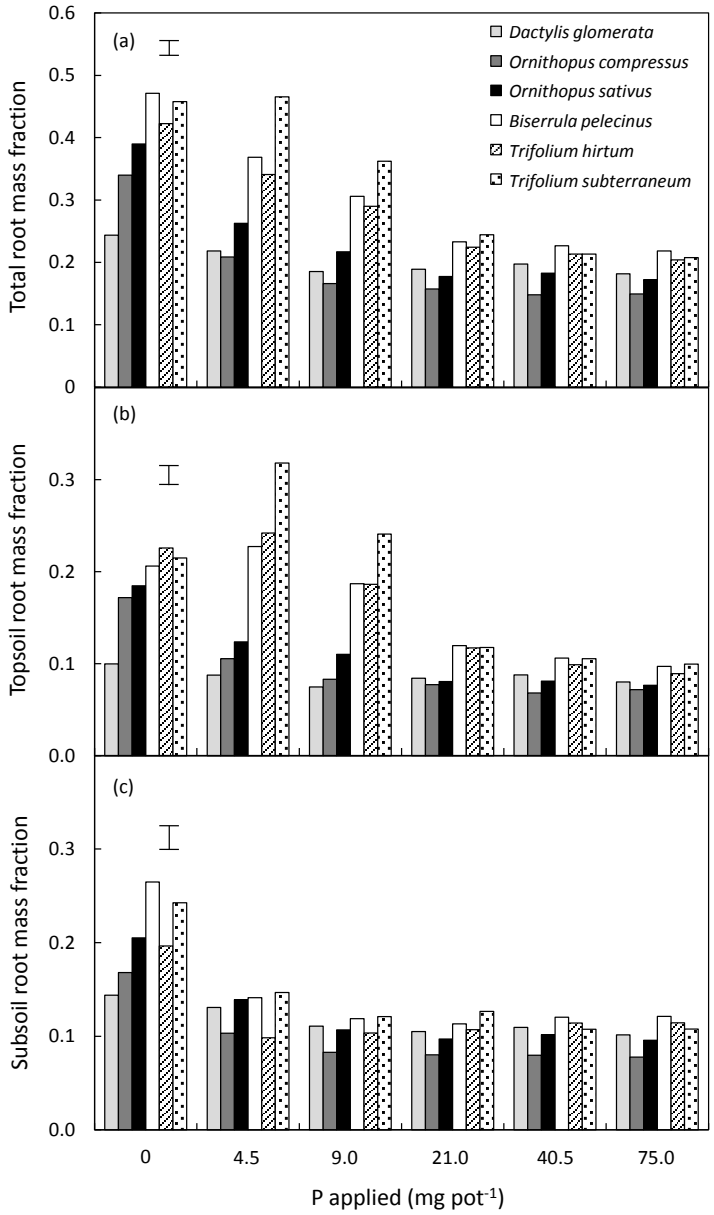


Figure 6.3 Root mass fractions for (a) total root mass (b) topsoil root mass and (c) subsoil root mass of five legume and one grass species in response to phosphorus (P) applied in the topsoil of a pot (n=5). Bars show LSD for the Species x P applied interaction (*P*<0.05).

6.3.4 Tissue P concentration, critical internal P requirement and internal P use efficiency

Shoot P concentration, topsoil root P concentration and subsoil root P concentration of all species increased with higher rates of P application (Fig. 6.5). The concentration of P in topsoil roots, subsoil roots and shoots was generally comparable for each legume grown in soil at rates of P application below its critical external P requirement. Above the critical external P requirement of each legume species, the P concentration of topsoil roots increased to a greater extent than that of the shoot or subsoil root P concentration. For example, above 9 mg P pot^{-1}, the shoot P concentration of *O. compressus* increased from 0.40% to 0.71% of dry matter, compared to 0.50% to 0.90% for topsoil roots and 0.26% to 0.38% for subsoil roots. *Dactylis glomerata* differed from the legumes in that the P concentration of the shoot was greater than that of topsoil and subsoil roots. In contrast to the legumes, the P concentration of the roots in each layer was similar.

Insets to Fig. 6.5 and associated regressions (Table 6.2) demonstrate that a disproportionate increase in the P concentration of topsoil roots of *T. subterraneum* and *T. hirtum* occurred when P application exceeded the critical P requirement of these species. In contrast, the shoot P concentration of all other species increased linearly and was correlated with the increase observed for root P concentration. In contrast to the legumes, the P concentrations of *D. glomerata* roots in each layer were similar, even at rates above the critical external P requirement.

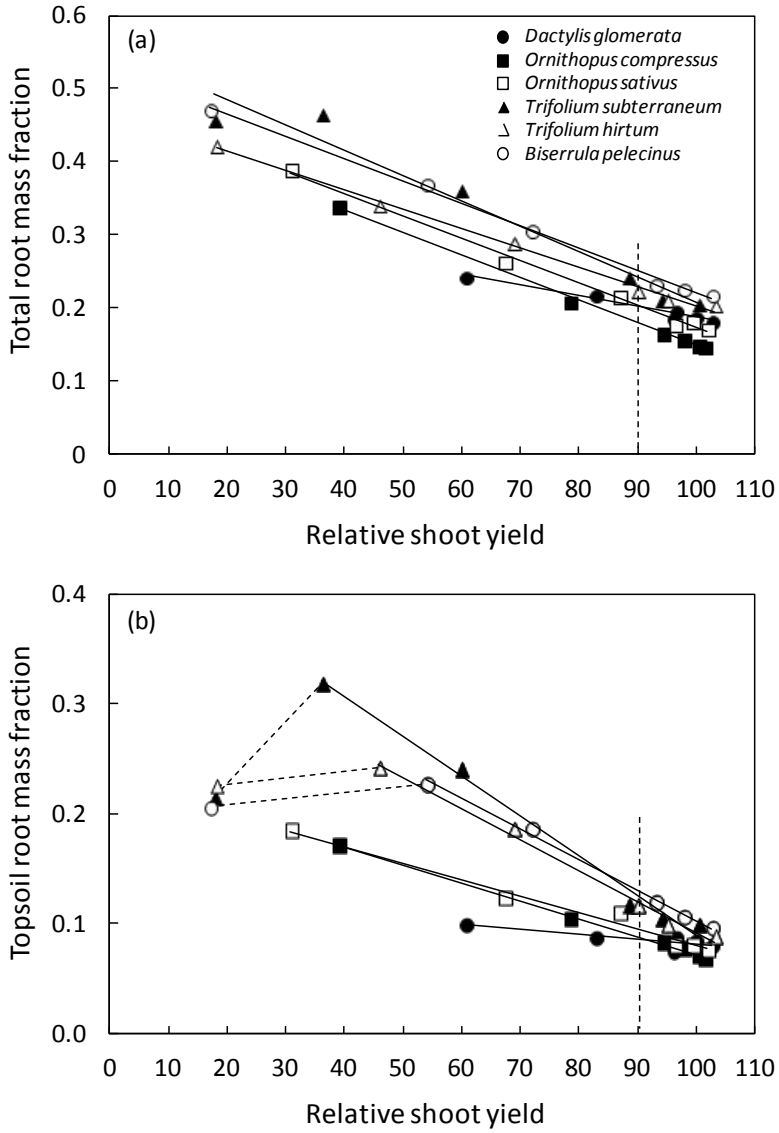


Figure 6.4 (a) Regressions fitted for relationship between relative shoot yield [an indicator of phosphorus (P) sufficiency] and total root mass fraction for five legumes and one grass species. Gradient *Dactylis glomerata* > *Trifolium hirtum* > *Ornithopus sativus* = *Biserrula pelecinus* = *O. compressus* ≥ *T. subterraneum*. Intercept *T. subterraneum* > *B. pelecinus* > *O. sativus* = *T. hirtum* = *O. compressus* > *D. glomerata* (*P*<0.05). (b) Regressions fitted for linear range between relative shoot yield and topsoil root mass fraction. Gradient *D. glomerata* > *O. sativus* = *O. compressus* > *B. pelecinus* = *T. hirtum* > *T. subterraneum*. Intercept *T. subterraneum* > *T. hirtum* = *B. pelecinus* > *O. compressus* = *O. sativus* > *D. glomerata*) (*P*<0.05). Dashed vertical line shows critical external P requirement of 90% of maximum shoot yield.

Table 6.2 Relationship between shoot phosphorus (P) concentration (x) and root P concentration (y) for five legumes and one grass species grown at six levels of P supply (n=5).

Species	Topsoil fit	R^2	Subsoil fit	R^2
Dactylis glomerata	y = 0.66x - 0.02	0.97	y = 0.36x + 0.08	0.96
Ornithopus compressus	y = 1.5x - 0.10	0.95	y = 0.45x + 0.08	0.92
Ornithopus sativus	y = 1.40 - 0.10	0.96	y = 0.50 – 0.000	0.92
Trifolium subterraneum	$y = 4.25x^2 - 0.71x + 0.14$	0.97	y = 0.70x + 0.04	0.94
Biserrula pelecinus	y = 1.28x - 0.11	0.93	y = 0.63x + 0.04	0.93
Trifolium hirtum	$y = 2.44x^2 + 0.32x + 0.06$	0.96	y = 0.58x + 0.06	0.96

Table 6.3 Critical internal phosphorus (P) concentration (%) and internal P-use efficiency assessed by alternative approaches for five legume and one grass species based on a polynomial fit between P applied per pot (x) and shoot P concentration (%; y). Values ± standard error. Different letters denote significant differences. [1] After Baligar *et al*. (2001) [2] After Rose *et al*. (2016).

Species	Critical internal P (%)	Physiological PUE[1] (g DW g^{-1} P)	PUE at 6.2 mg shoot P[2] (g DW g^{-1} P)	Polynomial fit	R^2
D. glomerata	0.23 ± 0.01	263 ± 27	350 ± 14a	$y = -7E\text{-}05x^2 + 0.0117x + 0.1554$	0.993
O. compressus	0.24 ± 0.01	327 ±19	313 ± 11bc	$y= -6E\text{-}05x^2 + 0.0118x + 0.1535$	0.995
O. sativus	0.30 ± 0.02	267 ± 19	296 ± 10b	$y = -8E\text{-}05x^2 + 0.0140x + 0.1520$	0.995
T. subterraneum	0.33 ± 0.02	258 ± 17	327 ± 9ac	$y = -7E\text{-}05x^2 + 0.0126x + 0.0440$	0.996
B. pelecinus	0.35 ± 0.02	250 ± 18	248 ± 8d	$y = -8E\text{-}05x^2 + 0.0133x + 0.1464$	0.996
T. hirtum	0.32 ± 0.02	267 ± 20	254 ± 8d	$y = -9E\text{-}05x^2 + 0.0125x + 0.0942$	0.994

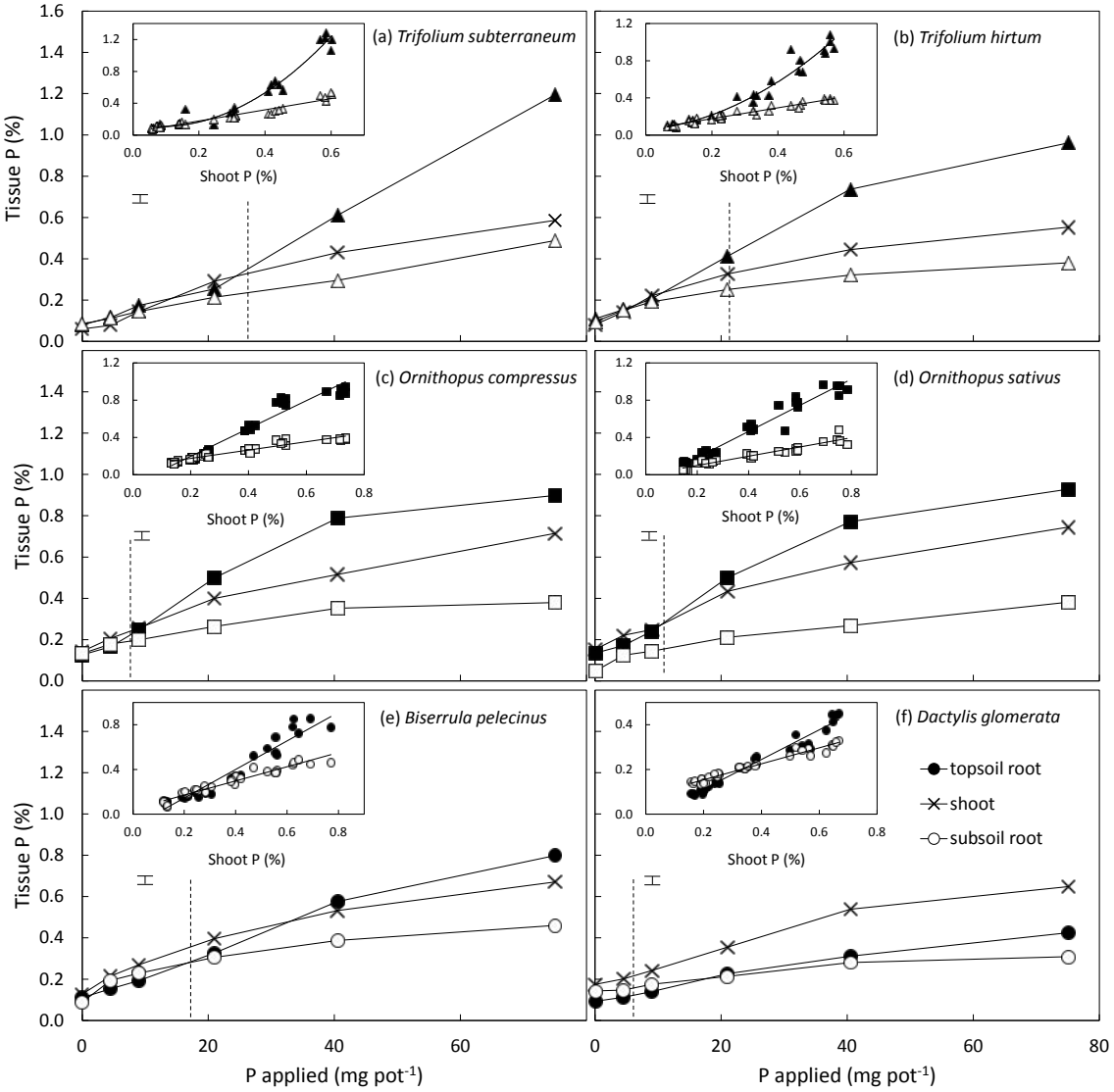


Figure 6.5 Tissue phosphorus (P) (%) of topsoil roots (closed symbols), subsoil roots (open symbols) and shoots (×) in response to P applied to the topsoil of a pot for (a) *Trifolium subterraneum* (b) *Trifolium hirtum* (c) *Ornithopus compressus* (d) *Ornithopus sativus* (e) *Biserrula pelecinus* and (f) *Dactylis glomerata* (n=5). Bar shows LSD for the Species x P applied x Soil interaction (P<0.05). Dashed line shows critical external P requirement for each species. Inset shows the relationship between shoot P (%) and P (%) of topsoil roots (closed symbols) and subsoil roots (open symbols).

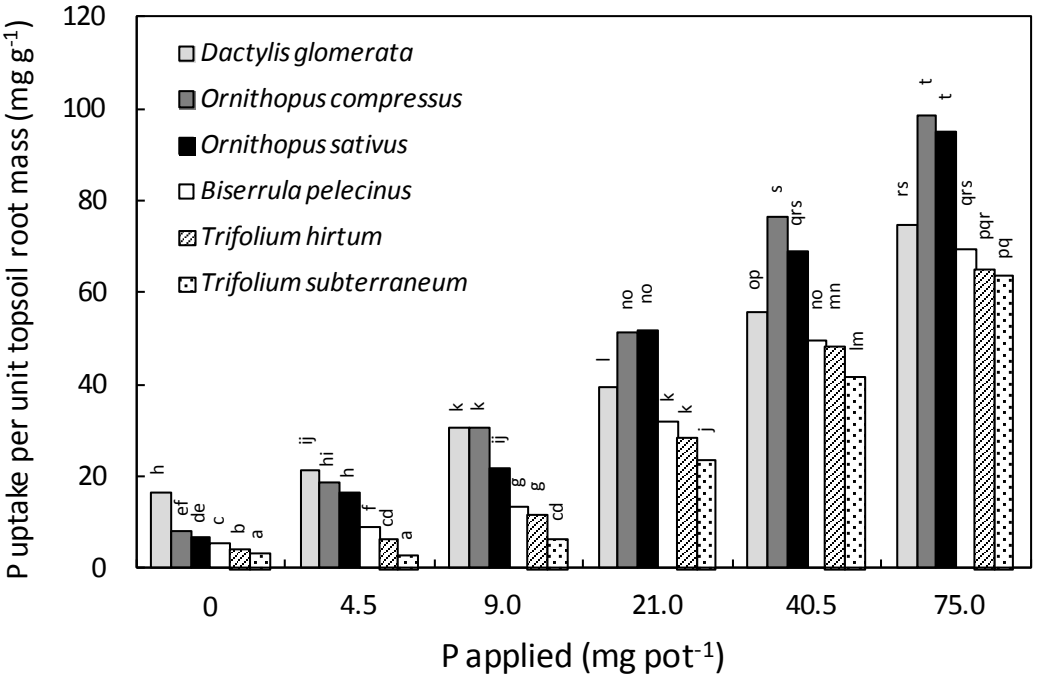


Figure 6.6 Total plant phosphorus (P) uptake per unit mass of roots in the topsoil for five legume and one grass species in response to P applied in the topsoil of a pot (n=5). Different letters indicate significant differences for Species x P applied interaction (*P*<0.05).

Curvilinear fits (Table 6.3) between rate of P addition and shoot P concentration (Fig. 6.5) were used to determine the internal P concentration of shoot dry matter for the six species that corresponded with their critical external P requirement (Table 6.1). The critical internal shoot P concentration of the species determined by this method ranged from 0.23% for *D. glomerata* to 0.35% for *B. pelecinus*. Most of the legumes had a similar critical internal P concentration (range 0.30 to 0.35) in shoot dry mass. However, the critical internal P requirement for *O. compressus* was lower and more similar to that of the grass. Physiological PUE calculated at 90% relative yield was similar among all species (250-267 g dry mass g^{-1} P), except for *O. compressus*, which was higher (327 g dry mass g^{-1} P; Table 6.3). When PUE was compared at a common amount of shoot P content (in this case, 6.2 mg shoot P pot^{-1}), *D. glomerata* had the highest PUE (350 g dry mass g^{-1} P) and *B. pelecinus* and *T. hirtum* the lowest PUE (248 and 254 g dry mass g^{-1} P, respectively).

6.3.5 P uptake per unit mass of roots in the topsoil

P uptake per unit mass of roots in the topsoil increased in response to increasing supply of P (Fig. 6.6). Species that had lower critical external P requirements had larger P uptake per unit mass of root, with differences most pronounced at the lower levels of P addition (i.e. below the critical external P requirement for each of the species). For example, at 4.5 mg P pot^{-1}, P uptake per unit root mass of *O. compressus* (18.9 mg P g^{-1} root) was 6-fold larger than that of *T. subterraneum* (3.0 P g^{-1} root). At 75 mg P pot^{-1}, P uptake per unit root mass of

both species was larger again but the difference between the two species was less pronounced (1.5-fold).

6.3.6 Relationship between topsoil root dry mass allocation, topsoil P uptake per unit root dry mass and nutrient foraging potential

Topsoil root mass allocation responses (i.e. the gradients in Fig. 6.4b) were negatively correlated with previously reported estimates (Yang *et al*. 2015) of the specific root hair cylinder volumes of the species (Fig. 6.7a). Topsoil P uptake per unit root dry mass was positively correlated with these estimates of specific root hair cylinder volumes (Fig. 6.7b).

6.4 Discussion

6.4.1 Dry matter partitioning and root morphology acclimation by plants grown in P deficient soil

Increased proportional allocation of plant dry matter to the root system is a general response to nutrient deficiency (Brouwer 1962) and an acclimation that particularly assists continued acquisition of a relatively immobile nutrient such as P (Lynch and Brown 2001; Nielsen *et al*. 2001). This response is typically characterised by proliferation of roots in relatively concentrated zones of P in an otherwise P deficient soil and is often observed in surface soils due to P-enrichment from cycling of organic material or application of fertilisers (Drew *et al*. 1975; Manske *et al*. 2000; Lynch and Brown 2001). In the present experiment, acclimation of roots to P-deficiency essentially only occurred in the P-enriched layer of the soil profile. For this reason, assessments of the allocation of root biomass in response to P stress, and P uptake per unit root dry mass could be considered using root growth in the P-enriched layer alone.

When relative shoot yield was used as an index of the level of “P sufficiency” experienced by each species it was apparent that increased allocation of biomass to roots in the fertilised soil was triggered when P supply began to reduce yield (i.e. at or near the critical P level of all of the species) and as a response to P stress. Large differences in the acclimation “effort” made by each species were apparent. The largest adjustments in root mass fraction were made by the species with relatively high critical P requirements and less by the species with low critical P requirements. This indicated that a high rate of root mass fraction adjustment alone was not a particularly effective way to achieve a low critical P requirement and may have been more a consequence of P starvation, than it being a strategic acclimation to low P soil.

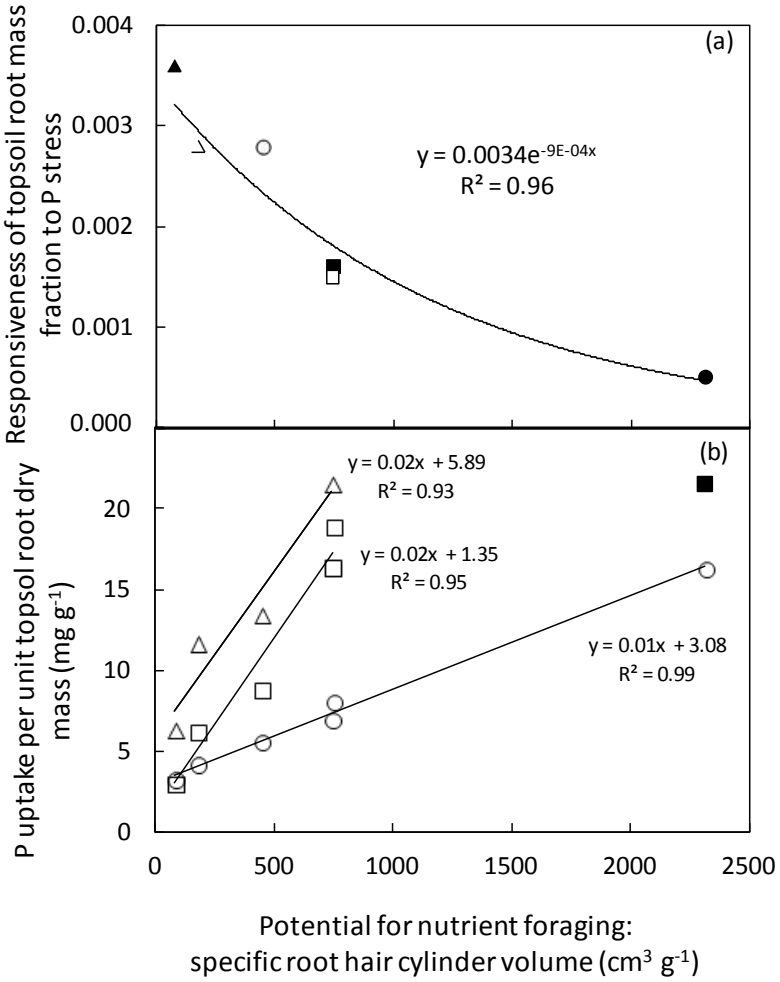


Figure 6.7 (a) Relationship between specific root hair cylinder volume (an index of the potential for nutrient foraging from Yang *et al*. 2015) (cm^3 g^{-1}) and responsiveness of topsoil root mass fraction to phosphorus (P) stress (i.e. the gradients of the relationships between root mass fraction in the topsoil layer and relative shoot yield from Fig. 6.4b) for *Dactylis glomerata* (●), *Ornithopus compressus* (■), *O. sativus* (□), *Biserrula pelecinus* (○), *Trifolium hirtum* (Δ) and *T. subterraneum* (▲) (b) Relationship between specific root hair cylinder volume (cm^3 g^{-1}) and P uptake per unit topsoil root dry mass (mg g^{-1}) at 0 mg P pot^{-1} (○), 4.5 mg P pot^{-1} (□) and 9 mg P pot^{-1} (▲). Species are only included in the regressions when grown with rates of P application below their critical external requirement. *D. glomerata* (■) at 4.5 mg P pot^{-1} was assumed to be an outlier and not included in this relationship.

6.4.2 Internal P distribution, critical internal P concentration and internal P use efficiency

The ability of plants to more efficiently distribute and utilise P internally could also contribute to a lower external P requirement. With the exception of *O. compressus*, the legumes had very similar critical internal P concentrations and physiological PUE based on the method of Baligar *et al*. (2001). However, these rankings were not consistent with those calculated using the method of Rose *et al*. (2016)) i.e. PUE at 6.2 mg shoot P). Nevertheless, PUE did not appear to be an overriding influence on the critical P requirement of the legumes. *Trifolium hirtum* has been reported to have a low critical shoot P concentration (Pinkerton *et al*. 1997) but, it was not different to *T. subterraneum* in the present study.

All legumes developed higher P concentrations in roots directly exposed to the fertilised soil. In contrast, the P concentration of roots of *D. glomerata* were similar in the fertilised topsoil and unfertilised subsoil suggesting a greater capacity for translocation of P to roots exploring low P soil. *Dactylis glomerata* also had a lower critical internal shoot P concentration than most of the legumes.

Root P concentration of most species was linearly correlated with shoot P concentration. However, both *Trifolium* spp. were exceptions in that the P concentration of roots exposed to the fertilised topsoil accumulated P at an increasing rate relative to shoot P concentration suggesting less capacity of their roots to regulate P uptake when exposed to luxury P supply. It may be relevant that *T. subterraneum* is susceptible to P toxicity (Rossiter 1952; Greenwood and Hallsworth 1960; Kim *et al*. 1985; Culvenor *et al*. 1989).

6.4.3 Efficiency of P acquisition

In the present study, significant differences in the critical external P requirement were measured amongst the species examined. Species that had a lower external critical P requirement were found to allocate less dry matter to the fertilised soil but absorbed more P per unit root mass than those with a higher critical P requirement. This was particularly evident at low levels of P addition below the critical external P requirement.

Large differences in the root morphology traits associated with nutrient foraging have been reported previously for these species (Yang *et al*. 2015). The *Ornithopus* spp. and *Dactylis glomerata* had long root hairs and high specific root length, which conferred a high specific root hair cylinder volume (746 and 2314 $cm^3\ g^{-1}$, respectively) i.e. root hair cylinder volume per unit root mass, and high potential for nutrient foraging. In contrast, *T. subterraneum* had short root hairs, low specific root length and a low specific root hair cylinder volume (78 $cm^3\ g^{-1}$). Specific root hair cylinder volumes of *T. hirtum* and *B. pelecinus* were intermediate (177 and 444 $cm^3\ g^{-1}$, respectively). Essentially all of the acclimation to P stress by the species occurred in the P-enriched topsoil layer. Differences in the rate of adjustment in topsoil root mass fraction to P stress were negatively correlated with the potential specific root hair cylinder volumes (Yang *et al*. 2015) of the species (Fig. 6.7a). The results suggest that root morphology differences among the species influenced how much root dry mass was allocated to nutrient foraging. In contrast, P acquisition efficiency (P uptake per unit root dry mass when plants are below their critical P requirement) was positively correlated with the potential specific root hair cylinder volumes. Thus, root morphology differences appear to also be a major factor determining the effectiveness (for P uptake) of the root acclimation response to P stress. Consequently, we surmise that differences in root morphology must also be a significant factor in achieving a low critical external P requirement. Direct assessment of root morphology under simular P stress conditions is needed to confirm this hypothesis and to examine whether other nutrient acquisition traits (e.g. release of P-solubilising exudates; mycorrhiza) have also contributed to the differences among these species in their capacity for P acquisition.

6.4.4 Implications for improving P efficiency of grass-legume pasture systems

O. compressus and *O. sativus* showed the greatest potential to improve P efficiency in pasture systems. Their low critical external P requirements suggest that use of these species, as alternatives to *T. subterraneum* or other similar legumes (e.g. *Trifolium repens*,

annual *Medicago* spp.), could reduce P fertiliser application rates to levels that are closer to the requirements of temperate grasses. The critical external P requirements and P uptake per unit dry mass of roots of *B. pelecinus* and *T. hirtum* were also more favourable than that of *T. subterraneum*, but interpretation of their apparent P efficiency was confounded by the fact that they had substantially lower shoot yields.

6.4.5 Conclusions

Species that had a lower external critical P requirement allocated less dry matter to the fertilised soil but absorbed more P per unit root mass than those with a higher critical P requirement. This suggested that differences in their root morphology traits were likely to be a major factor contributing to P acquisition efficiency and, consequently, differences in the critical P requirement among these species.

7 Root morphology traits that determine phosphorus acquisition efficiency and the critical external phosphorus requirement in pasture species

Rebecca E. Haling, Zongjian Yang, Natalie Shadwell, Richard A. Culvenor, Adam Stefanski, Megan H. Ryan, Graeme A. Sandral, Daniel R. Kidd, Hans Lambers, Richard J. Simpson

Published 2016: Functional Plant Biology 43, 815-826.

7.1 Introduction

Crop and pasture plants that have low critical external phosphorus (P) requirements (i.e. the ability to achieve 90% of their maximum yield at a low soil extractable-P concentration) allow P fertiliser addition to be reduced without yield penalty, and could thereby reduce the accumulation of sparingly-available P in fertilised agricultural soils and the risk of P loss due to erosion to the wider environment (Simpson *et al*. 2014; 2015). In Chapter 6, we demonstrated that annual pasture legume species vary more than three-fold in their critical external P requirement. *Trifolium subterraneum* L. (subterranean clover), a key legume used in pastures in southern Australia, had the highest critical P requirement of all the species studied (26.7 mg P pot^{-1}). Two species, promoted as alternatives to *T. subterraneum*, *Ornithopus compressus* and *O. sativus*, yielded as well as *T. subterraneum*, but had substantially lower critical P requirements (7.6 and 11.3 mg P pot^{-1}, respectively). *Biserrula pelecinus* L. and *T. hirtum* had critical external P requirements (17.3 and 21.1 mg P pot^{-1}, respectively) intermediate to those of *T. subterraneum* and the *Ornithopus* spp., but their potential shoot yields were lower (i.e. ~76% of that of *T. subterraneum*). *Dactylis glomerata* L. (cocksfoot or orchard grass), a high-yielding perennial grass often grown as a companion species with *T. subterraneum*, also had a low critical P requirement (6.6 mg P pot^{-1}). These results were in general agreement with observations that temperate grasses often have substantially lower P requirements than the temperate pasture legumes with which they are grown (Ozanne *et al*. 1969; 1976). They also confirm the field observation that *O. compressus* can yield as well as *T. subterraneum* with about half the amount of applied P-fertiliser (Bolland and Paynter 1992). Indeed, the responses of the *Ornithopus* spp. to P fertiliser demonstrate that it will be possible to find alternative pasture legumes that have critical P requirements that approach the low critical P requirements of the temperate grasses with which they are grown (Chapter 6; Haling *et al*. 2016).

The legume species used for this study were initially selected based on potentially superior root traits for nutrient foraging relative to *T. subterraneum*. Yang *et al*. (2015; Chapter 5) found *Ornithopus* spp. and *B. pelecinus* to have long root hairs and *T. hirtum*, *B. pelecinus* and the *Ornithopus* spp. to have high specific root lengths. The species were grown in soil profiles with plant-available P concentrated in the topsoil layer, as occurs in fields to which P fertiliser has been applied (Lambers and Plaxton 2015). The species responded to low-P soil by increasing their topsoil root mass fraction (topsoil root dry mass per unit total plant mass) (Chapter 6). Large differences in this typical response to P stress (Brouwer 1962) were observed among the legumes, with the largest acclimation responses recorded for the species with higher external critical P requirements. Irrespective of the large allocation of dry matter to root foraging in the fertilised soil layer, the plants that grew best in low-P soil were those that achieved higher P uptake per unit root dry mass (Chapter 6). Other work has also

led to the suggestion that the low critical P requirement of *O. compressus* is due to its greater ability to take up P (Blair and Cordero 1978; Paynter 1990).

The results presented in Chapter 6 indicate that root morphology could be a major factor determining the capacity for P acquisition and the critical P requirements among legume species. Here we report the root morphology of the species studied in Chapter 6 and its response to low P supply. We use this information to determine how some of the species achieve external critical P requirements that are low enough to underpin development of more P-efficient grazing systems.

7.2 Material and Methods

7.2.1 Plant material

Root growth, root morphology and P-uptake parameters for five annual pasture legumes and one perennial grass were examined in the second phase of an experiment that initially determined the critical P requirement (i.e. the rate of P supply needed to achieve 90% of maximum yield) and dry matter partitioning by these species in response to P supply (Chapter 6). The species were: *Trifolium subterraneum* L. (subterranean clover) cv. Leura; *Dactylis glomerata* L. (cocksfoot or orchard grass) cv. Porto; *Ornithopus compressus* L. (yellow serradella) cv. Santorini; *O. sativus* Brot. (French serradella or pink serradella) cv. Margurita; *Biserrula pelecinus* L. (biserrula) cv. Mauro; and *T. hirtum* All. (rose clover) cv. Hykon.

7.2.2 Plant growth conditions and experimental design

The experiment was conducted as described in Chapter 6. The soil, a sandy loam (Yellow Chromosol; Isbell 1996) with a low concentration of extractable P (8.3 mg kg^{-1} P; Colwell, 1963), was pasteurised to reduce levels of disease inoculum, sieved to < 5 mm and mixed with lime (1.06 g $CaCO_3$ kg^{-1}) to raise the pH (1:5 w/v, 0.01 M $CaCl_2$) to 5.5. All essential nutrients other than P were then mixed into the soil to ensure that only P and N were in low supply. Six P-fertilised soil treatments were established by mixing KH_2PO_4 with subsamples of the amended soil at rates of 0, 15, 30, 70, 135 and 250 mg P kg^{-1} soil. This resulted in extractable P concentrations of 8.3, 19.2, 30.9, 58.4, 111 and 203 mg kg^{-1} (Colwell 1963). Pots (cylindrical PVC; 87 mm internal diameter, 190 mm soil height) were filled with a bottom layer of the soil (1 kg oven dry basis) that was not fertilised with P (the subsoil) and then with a topsoil layer of P-fertilised soil (0.33 kg oven dry basis). The resultant P-application rates were 0, 4.5, 9.0, 21.0, 40.5 and 75.0 mg P pot^{-1}. The boundary of the fertilised topsoil and unfertilised subsoil layers was marked by placing small alkathene beads around the interior edge of the pot. The legumes were inoculated with appropriate strains of rhizobia. An additional 21 mg N pot^{-1} (as NH_4NO_3) was applied to the grass, 32 days after sowing.

Micro-swards of each species were established by sowing 50 mg pot^{-1} of viable seed. Five replicates of each species by P treatment were established. Plants were grown in a controlled-environment growth cabinet [12 h light (720 µmol quanta m^{-2} s^{-1})/ 12 h dark; 25/15°C]. Pots were arranged in a randomised complete block design and rotated within blocks. Reflective sleeves were fitted to the outside of the pots and raised with plant height to reproduce the light conditions in a pasture sward. The soil was maintained at 75 to 80% of field capacity by daily watering and fully wet up once per week.

7.2.3 Harvest and measurements

Plants were harvested six weeks after sowing. Shoots were cut at the soil surface, dried at 70°C and weighed. Soil was removed as an intact core and cut at the interface of the fertilised topsoil (47 mm height) and the subsoil. Roots were washed from each layer and a sub-sample stored in 50% (v/v) ethanol at 4°C. The subsample of roots was scanned using a flatbed scanner (600 dpi) and root length and average root diameter analysed using WinRHIZO (Regent Instruments Inc., Quebec, Canada). Root hairs on roots in the fertilised topsoil were imaged using a Leica MZFLIII Fluorescence microscope (Leica Microsystems, Sydney, Australia) fitted with a Zeiss AxioCam camera (Zeiss, Sydney, Australia). The length of 10 root hairs per replicate was measured using ImageJ (Rasband 1997–2014). Mycorrhizal colonisation was measured using the grid-line intersect method (Giovannetti and Mosse 1980) after clearing (10% w/v KOH for 2-4 days followed by rinsing in water and 1% v/v HCl) and staining roots (5% v/v Schaeffer blue ink/ white vinegar solution for 1 h; Vierheilig *et al*. 1998). Roots were dried at 70°C and total dry mass of roots in each soil layer calculated; this included adjusting for loss of mass from the subsamples that were stored in ethanol.

Shoot and root samples (not stored in ethanol) were milled and a 25 to 50 mg sample was ashed at 550°C, dissolved in 2 M HCl and the P concentration determined colourimetrically using malachite green (Irving and McLaughlin 1990).

The root hair cylinder volume (RHCV) of the roots in the topsoil was calculated as the volume of the cylinder enclosing the root and root hair zone.

$$RHCV = \pi * ([ARD/2] + RHL)^2 * RL \qquad \text{Equation [1]}$$

Where: ARD = average root diameter, RHL = average root hair length measured in the topsoil and RL = total root length in the soil layer.

Phosphorus content of the shoots, roots in the topsoil and roots in the subsoil were summed to calculate total plant P uptake. Total plant P uptake was divided by the total surface area of the root hair cylinder in the topsoil to determine P uptake per unit surface area of the root hair cylinder.

7.2.4 Growth and root length extension rates during the experiment

An additional experiment was undertaken to examine the patterns of shoot and root growth over the six-week period used in the original experiment. *Trifolium subterraneum* cv. Seaton Park was grown at four rates of P applied to the topsoil of the pot (0, 4.5, 12 and 75 mg P pot^{-1}) and harvested at three, four, five and six weeks after sowing. Five replicates were grown per treatment. The experimental setup and plant growth conditions were as described for the main experiment. Shoots and roots were harvested as per the main experiment, with the exception that roots were scanned immediately for analysis of root length, and were dried at 70°C. This eliminated the need to make adjustments for loss of mass during storage in ethanol.

7.2.5 Statistical analyses

As described in Chapter 6, a Mitscherlich curve was fitted to the shoot dry matter data in response to application of P in R (R Core Team, 2013). The critical external P concentration of each species was determined as the amount of P required to achieve 90% of the maximum yield.

Parameters of root growth, morphology and P uptake were analysed in GenStat 16th Edition (VSN International, UK). Root length density and specific root length (root length divided by root mass) were analysed using a split-plot analysis of variance with Species and P addition as whole-plots and Soil Depth as split-plots. Root hair length, root hair cylinder volume (log-transformed), total plant P uptake (log-transformed) and P uptake per unit surface area of root hair cylinder (log-transformed) were analysed using a general analysis of variance with Species and P addition as factors.

Mitscherlich curves were fitted to relative yield [shoot dry mass/ average shoot dry mass at 250 mg P pot^{-1}] in response to total plant P uptake for each species. Differences between the parameters of the model were tested by considering the estimates and approximate standard errors for each measure simultaneously, and testing for significant pairwise differences. To test the hypothesis that P supply to the roots was determined by diffusion of P to the surface of the root hair cylinder (according to Fick's Law; see Discussion), a simple linear regression with groups was used to analyse the relationship between soil P concentration, surface area of the root hair cylinder and total plant P uptake for each of the legume species.

7.3 Results

7.3.1 Root length density

The root length density for all species was generally higher in the fertilised topsoil layer than in the corresponding unfertilised subsoil (Fig. 7.1). *D. glomerata* achieved high root length densities in the topsoil and subsoil relative to those of most legume species.

For some species (*T. subterraneum*, *T. hirtum*, *B. pelecinus*), root length density in the subsoil was reduced when plant growth was restricted by low P supply (Fig. 7.1). However, the root length density in the subsoil of the *Ornithopus* spp., and *D. glomerata* was not altered by the rate of P supply. In contrast, all the legumes (Fig. 7.1 a-e) increased their root length density in the topsoil by up to 1.4 to 1.9-fold at P supply rates below critical P. This acclimation response to reduced P supply was typically initiated at about the critical level of P supply, except for *O. compressus*, which appeared to begin adjusting root length density at rates of P supply above critical P. *T. subterraneum* achieved a significantly higher maximum root length density (53 cm cm^{-3}) than any other legume species (28.-.42 cm cm^{-3}). However, with the exception of *O. compressus*, the legumes were unable to achieve their maximum root length density at very low levels of P supply (i.e. 0.-.5 mg P pot^{-1}). Although the root length density of *D. glomerata* was reduced in the unfertilised topsoil treatment, it otherwise made only a marginal adjustment to root length density in response to P supply.

7.3.2 Specific root length and average root diameter

Maximum specific root lengths in the topsoil ranged from 215 and 258 m g^{-1} (*B. pelecinus* and *T. subterraneum*) to 328, 365 and 383 m g^{-1} (*T. hirtum*, *O. compressus* and *O. sativus* respectively) among the legumes (Fig. 7.2 a-e) and was 486 m g^{-1} for the grass (Fig. 7.2 f). Specific root lengths most often were unchanged or declined below the critical P requirement for each species (e.g. *T. subterraneum*, *B. pelecinus* and *D. glomerata*). *O. compressus* increased its specific root length when grown at lower rates of P addition; the adjustments were unusual in that they occurred at P supply rates above the critical P requirement of the species.

For *T. subterraneum*, specific root length did not differ significantly between the topsoil and subsoil layers at each of the respective P treatments, except in the un-amended treatment where specific root length was significantly greater in the subsoil relative to the topsoil. For all other species, specific root length was often greater in the subsoil than that in the topsoil.

Average root diameter of the grass was lower than that of the legume species (Fig. 7.3). The *Ornithopus* spp. generally had lower average root diameters than those of the other legumes species. Acclimation of average root diameter to low P supply was marginal, and differences between the topsoil and subsoil root diameters were small.

7.3.3 Root hair length and colonisation by mycorrhizal fungi

Root hair length of the *Trifolium* spp. was generally at least half that of the other legume species and the grass, and did not respond to changes in P supply (Fig. 7.4). In contrast, root hair length of *D. glomerata*, the *Ornithopus* spp. and *B. pelecinus* increased between 1.2- and 1.7-fold in response to declining P supply. In the unfertilised topsoil, *D. glomerata* had the longest root hairs (0.75 mm), followed by the *Ornithopus* spp. (0.63–0.66 mm), *B. pelecinus* (0.50 mm) and the *Trifolium* spp. (0.29 mm). *Biserrula pelecinus* achieved the longest root hairs among the legumes (0.70 mm), but, unlike the *Ornithopus* spp., did not maintain this in very low P soil (< 9 mg P pot^{-1}).

Less than 5% of the root length of every species was colonised by arbuscular mycorrhizal fungi when growing in low-P soil (0 and 5 mg P pot^{-1}; data not shown).

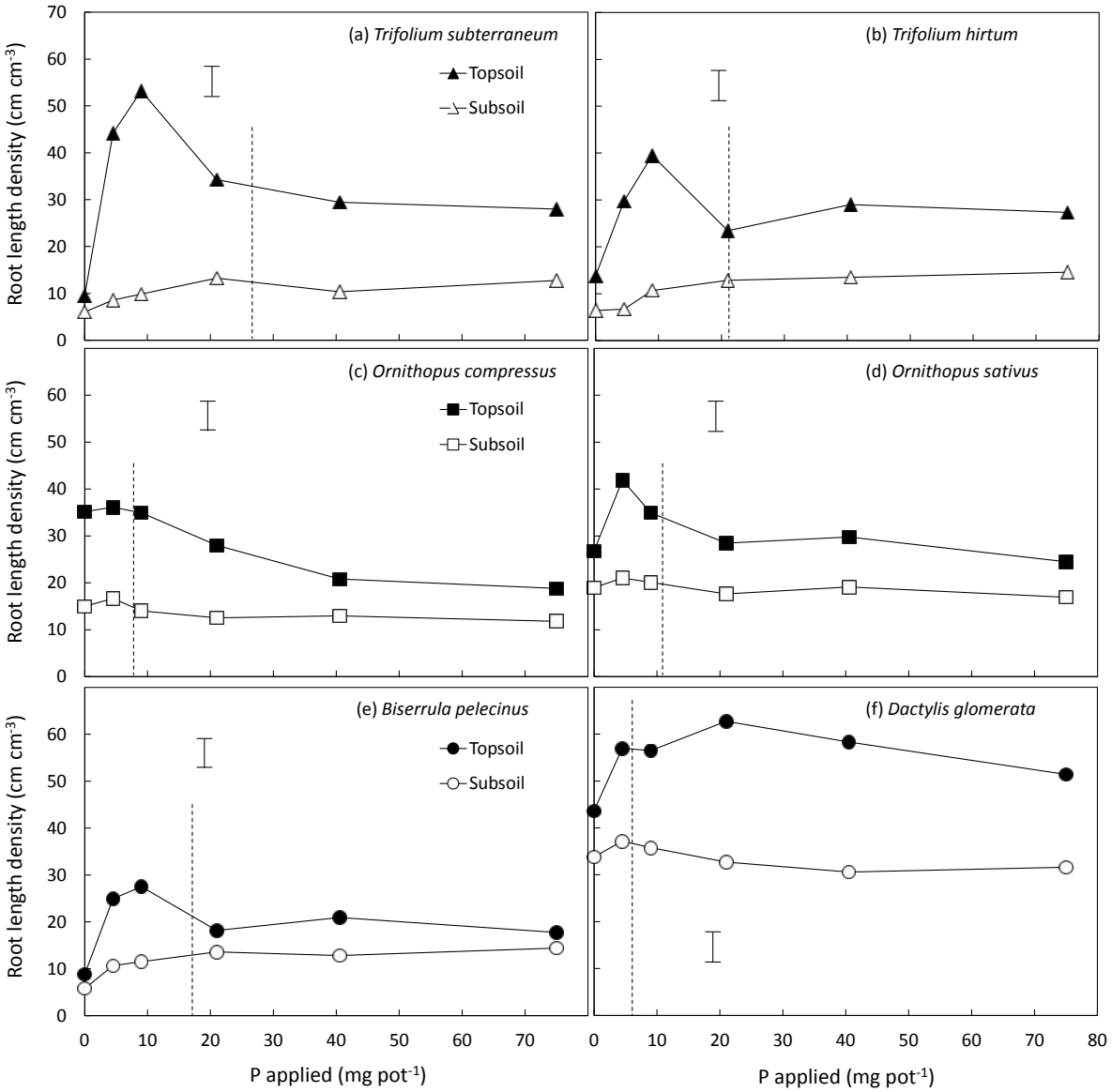


Figure 7.1 Root length density in topsoil and subsoil in response to phosphorus (P) applied in topsoil for (a) *Trifolium subterraneum* (b) *Trifolium hirtum* (c) *Ornithopus compressus* (d) *Ornithopus sativus* (e) *Biserrula pelecinus* and (f) *Dactylis glomerata*. Bar shows LSD for three-way interaction of P x species x soil depth (P<0.001; n=5). Dashed line shows critical external P requirement for each species.

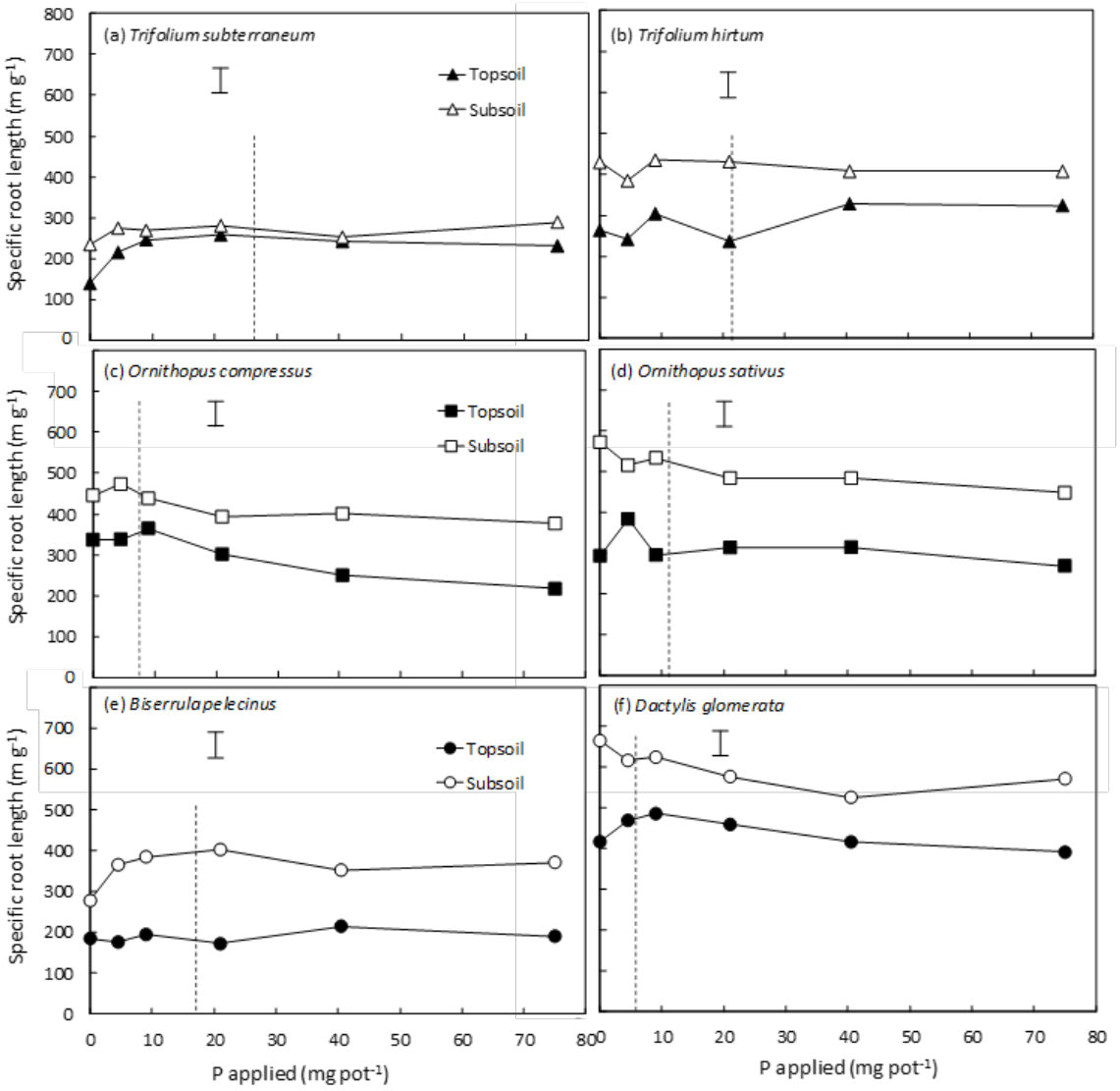


Figure 7.2 Specific root length in topsoil and subsoil in response to phosphorus (P) applied in topsoil for (a) *Trifolium subterraneum* (b) *Trifolium hirtum* (c) *Ornithopus compressus* (d) *O. sativus* (e) *Biserrula pelecinus* and (f) *Dactylis glomerata*. Bar shows LSD for three-way interaction of P x species x soil depth (P<0.05; n=5). Dashed line shows critical external P requirement for each species.

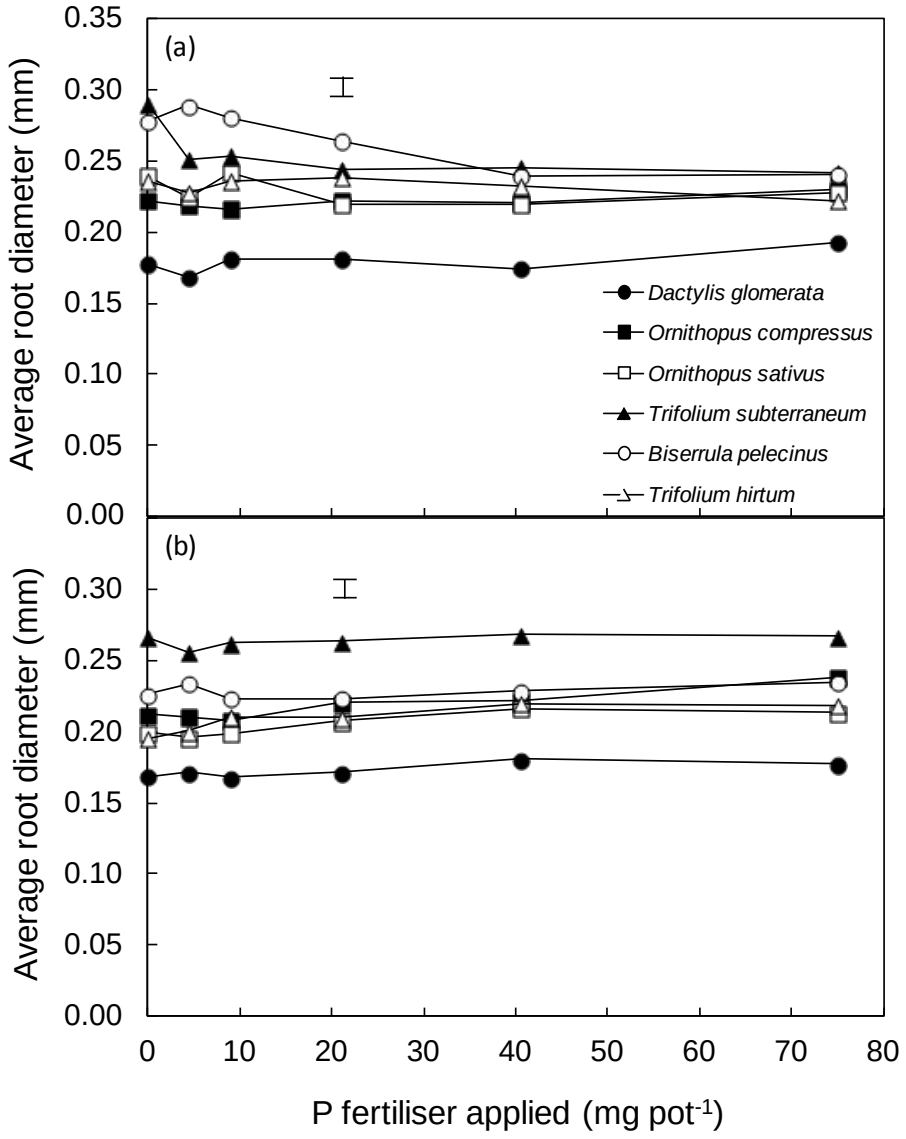


Figure 7.3 Average root diameter in (a) topsoil and (b) subsoil for one grass and five legume species in response to phosphorus (P) applied in topsoil. Bar shows LSD for three-way interaction of P x species x soil depth (P<0.05; n=5).

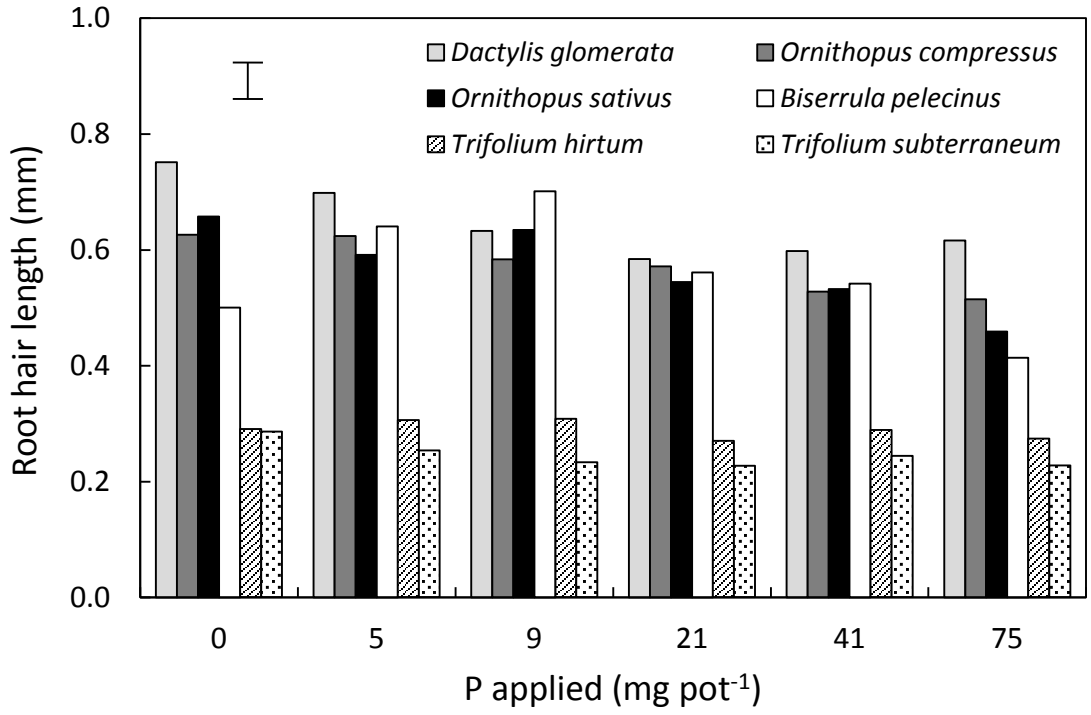


Figure 7.4 Root hair length in topsoil for five legumes and one grass in response to phosphorus (P) applied in the topsoil. Bars show LSD for two-way interaction of P x species (*P*<0.001; n=5).

7.3.4 Root hair cylinder and P uptake

Root hair cylinder volume was calculated to estimate the potential volume of topsoil explored directly by the species as a result of the length and diameter of their roots, and the length of their root hairs (Fig. 7.4a). The root hair cylinder volume of the grass approached or exceeded that of the topsoil layer and for this reason the grass was not included in calculations of P uptake per unit surface area of the root hair cylinder.

The root hair cylinder volumes of the legumes increased when the plants were grown with lower rates of P addition. At their maximum, the *Ornithopus* spp. and *B. pelecinus* had root hair cylinder volumes that were equivalent to more than half of the volume of the topsoil layer, while that of the *Trifolium* spp. was less than a quarter of the topsoil volume. Maximum root hair cylinder volumes coincided with levels of P addition at which maximum root length densities and/or root hair lengths were achieved (i.e. at 9 mg P pot^{-1} for *B. pelecinus* and the *Trifolium* spp., and 5 mg P pot^{-1} for the *Ornithopus* spp.).

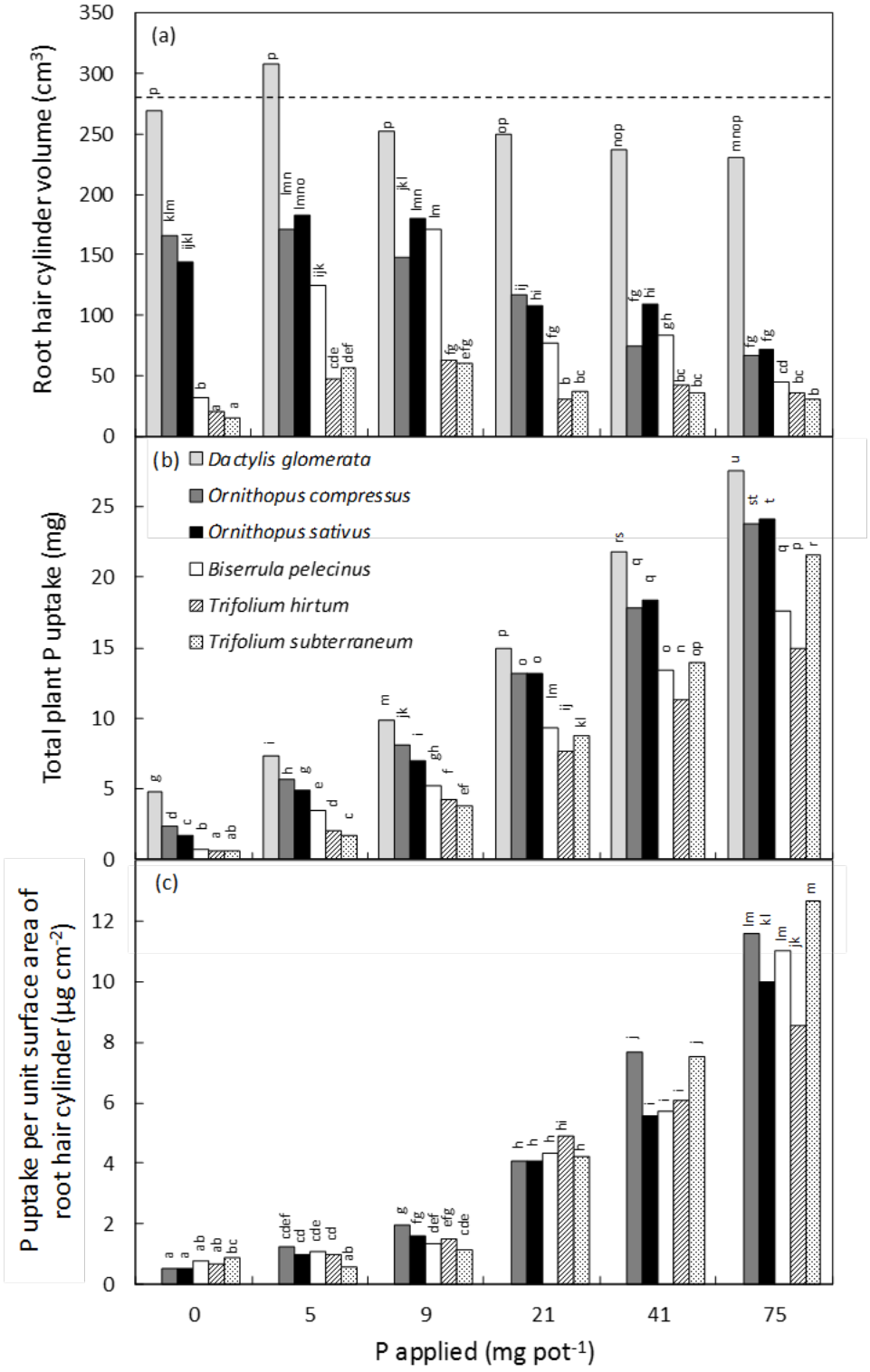


Figure 7.5 (a) Root hair cylinder volume (b) total plant phosphorus (P) uptake and (c) P uptake per unit surface area of the root hair cylinder for five legumes and one grass species in response to P applied to topsoil. Different letters denote significant differences for two-way interactions of P x species (P<0.001; n=5). Dashed line in (a) indicates volume of topsoil. P uptake per unit surface area of the root hair cylinder is not presented for *Dactylis glomerata* as the effective volume of soil explored [i.e. root hair cylinder volume presented in (a)] was equivalent to, or larger than, the volume of the topsoil.

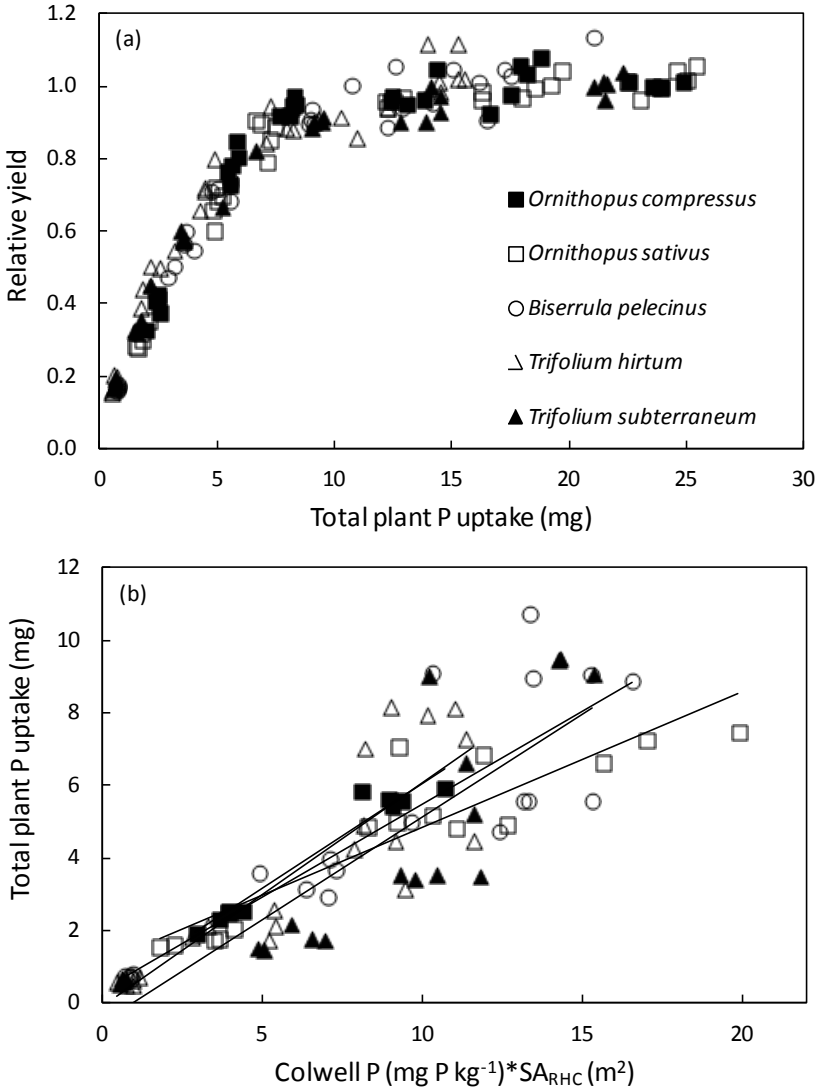


Figure 7.6 (a) Total plant phosphorus (P) uptake versus relative yield for each legume species. Critical total plant P uptake for the species in (a) are shown in Table 1. (b) Regressions fitted between product of Colwell-extractable P and surface area of the root hair cylinder (SA_{RHC}), and total plant P uptake for each legume species (see Discussion for further explanation). Data were only included for rates of P addition less than the critical external P requirement of each species. Intercepts of species did not significantly differ and there was no significant difference in gradient among species except for *Trifolium subterraneum* = *T. hirtum* > *Ornithopus sativus* (P<0.05).

Table 7.1 Critical total plant P uptake determined as total plant P uptake at 90% of relative yield from a fitted Mitscherlich response ($y = a - b*(e^{-cx})$; Fig 7.6a). Values ± standard error ($P< 0.5$; n=5). Different letters denote significant differences within each column.

Species	Critical total plant P uptake (mg P pot^{-1})	Intercept	Parameter	
			A	B
O. compressus	8.0±0.3 a	-0.30±0.08 a	1.01±0.01 a	-6.52
O. sativus	9.4±0.3 b	-0.09±0.04 b	1.00±0.02 a	-4.93
T. subterraneum	9.8±0.3 b	0.05±0.01 c	0.99±0.01 ab	-1.31
B. pelecinus	11.0±0.4 c	0.02±0.03 bc	1.05±0.03 b	-2.08
T. hirtum	9.4±0. b	0.06±0.03 c	1.06±0.04 a	-1.47

Total plant P uptake and P uptake per unit surface area of the root hair cylinder increased for all species when the plants were grown with higher rates of P application (Fig. 7.5 b, c). At a given rate of P addition, *D. glomerata* generally accumulated more P per plant, followed by *O. compressus*, *O. sativus*, *B. pelecinus* and the *Trifolium* spp. Differences were most pronounced at lower rates of P addition. However, P uptake per unit surface area of the root hair cylinder was remarkably similar among the species, particularly at the lower rates of P addition.

Parameters of Mitscherlich response curves for relative yield in response to total plant P uptake, shown in Figure. 7.5 a, were mostly similar among species (Table 7.1). Total plant P uptake was analysed in response to the product of the total surface area of the root hair cylinder and $CaCl_2$-extractable soil P concentration (for rates of applied P less than or approximately equivalent to the critical P requirement of each species; Fig. 7.6b). There was a significant linear relationship between the two fore mentioned parameters (i.e. product of the total surface area of the root hair cylinder and $CaCl_2$-extractable soil P concentration, and total plant P uptake) and the relationship for *T. subterraneum* did not differ significantly from that of most of the other legume species.

7.3.5 Patterns of growth and root length extension

Shoot and root growth of *T. subterraneum* and root length extension in the topsoil were typically exponential, but could also be described as having a lag phase (0–3 weeks from sowing), followed by an essentially linear phase of growth (3–6 weeks) (Fig. 7.7). The rate of shoot growth during the linear phase increased as the rate of P application was increased, but root dry mass increase and root length extension were highest in the moderately P-deficient soil (12 mg P pot^{-1}) reflecting the patterns of root yield and length recorded in the main experiment.

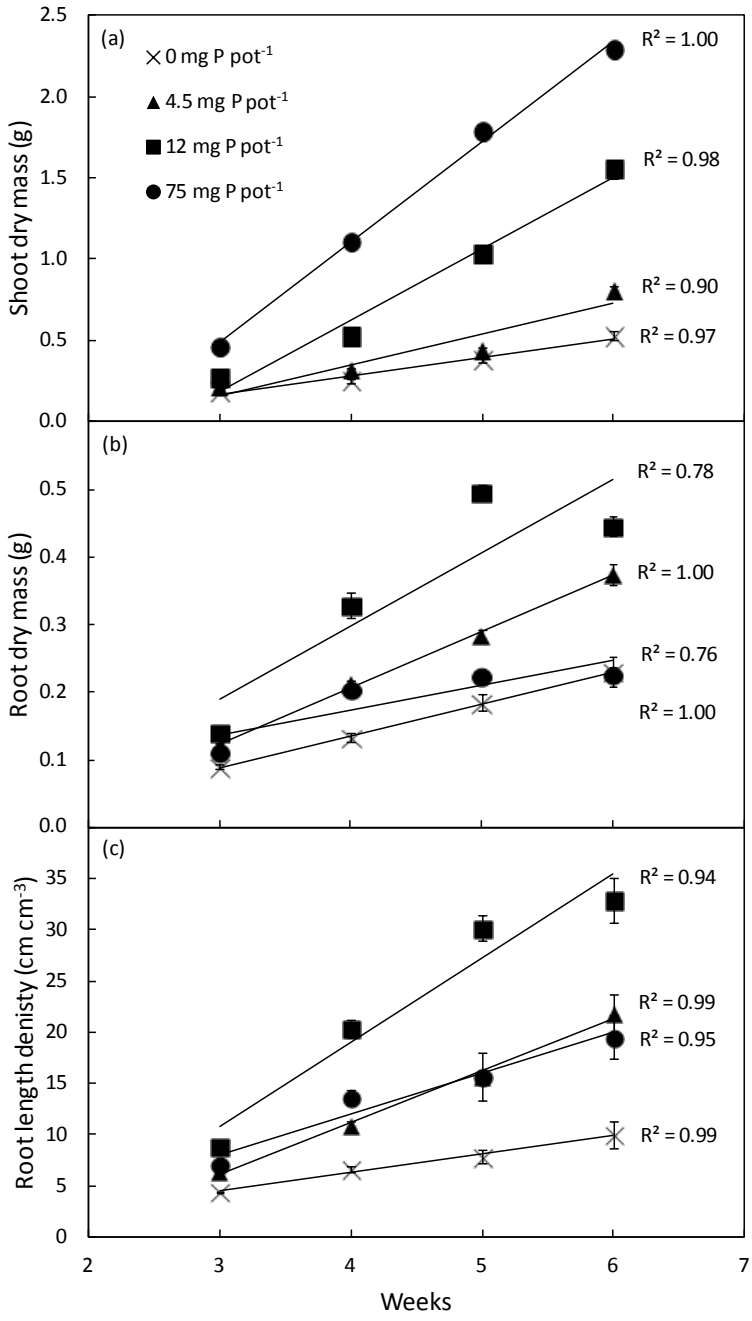


Figure 7.7 (a) Shoot dry mass (b) root dry mass in topsoil and (c) root length density in topsoil of *Trifolium subterraneum* grown at four rates of phosphorus (P) applied to the topsoil and harvested at four times after sowing. Bars show ± 1 standard error (n=5).

7.4 Discussion

7.4.1 Acclimation of root morphology to low soil P supply

Root length adjustments: High root length densities in low P soil were achieved by different strategies. All legumes adjusted root mass fraction in favour of roots when grown with moderately low P supply (Chapter 6). For the *Trifolium* spp., *O. sativus* and *B. pelecinus* this translated into a larger root mass allocation to the fertilised soil layer. Specific root length of the *Trifolium* spp. and *B. pelecinus* was either unchanged by low P supply, or was lower than that of plants grown with high P supply. This indicated that increases in root length density of these species were entirely due to increased allocations of root dry mass to the fertilised soil layer. In contrast, *O. sativus* allocated more root dry mass and/or increased specific root length at P-supply rates below its critical P supply. For *O. compressus*, all of the increase in root length density immediately above the critical P supply was associated with higher specific root length.

In contrast to the legumes, there was no obvious root length acclimation to low P by *D. glomerata*. Root length was maintained at a high density at all P supply rates, except in soil to which no P had been applied. This was associated with a high specific root length, rather than increased allocation of root dry mass to the fertilised layer. Inability to detect root acclimation in *D. glomerata* does not, however, preclude the potential for acclimation in this species. It had the lowest critical P requirement of all species we examined and there were few P supply rates employed below its critical P level. This limited our ability to detect root morphological adjustments.

Adjustments that resulted in some legumes having a lower specific root length when grown with low P supply was a response opposite to that considered most efficient for acquiring scarce P resources in soil (Lynch and Brown 2001). This was further examined in an additional experiment in which root adjustments by *T. subterraneum* were compared between single plants grown without constraint to canopy spreading and plants grown as a pasture sward (see Fig. S1, available as Supplementary Material to this paper). The specific root length of plants grown in non-sward conditions acclimated to low soil P very differently. Specific root length was increased in response to P stress (until an apparent failure-point was reached). We conclude that apparent adjustment in specific root length in response to low soil P by some species in our experiment when grown as pasture swards was the result of a complex interaction between P supply, shoot growth and shade responses. The shoots of all species showed a typical shade response (i.e. etiolation) within the high leaf area index canopies developed with high rates of applied P. This is a typical response during periods of high pasture growth in spring. Intraspecific competition for light is assumed to have also altered the allocation of biomass to roots among grass species grown at different levels of shading (Ryser and Eek 2000; Wahl *et al.* 2001)

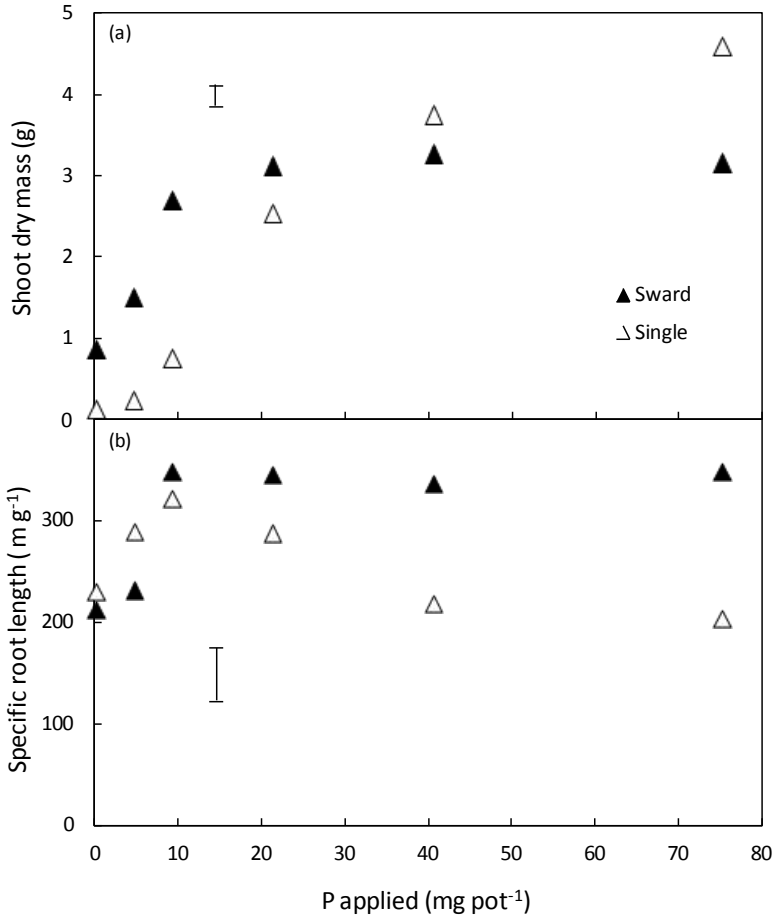


Figure 7.8 (a) Shoot dry mass and (b) specific root length in topsoil of *Trifolium subterraneum* (cv. Seaton Park) grown with phosphorus (P) applied to the topsoil either in a sward (50 mg viable seed per pot; reflective sleeves raised daily to equal plant height) or as single plants (without reflective sleeves). Experimental conditions were otherwise as described in the Materials and Methods of the main experiment. Bars show LSD for two-way interaction of P x plant density (P<0.05; n=3). Correlation R^2 between specific root length and average root diameter for plants grown in a sward or as single plants was 0.62 and 0.61, respectively.

These authors found that the grasses increased leaf area in response to shading, but maintained total root length. Although allocation of biomass to roots was lower, higher specific root length and lower construction costs per volume of root enabled root length to be maintained.

Root hair length and soil exploration: Although *D. glomerata* did not adjust root length density in response to differences in soil P supply, the length of its root hairs was increased (up to ~20%) when grown with a P supply below its critical P. In the *Ornithopus* spp. and *B. pelecinus* the length of their root hairs was also increased by a similar magnitude (*Ornithopus* spp.) or greater (*B. pelecinus*) when the P supply was reduced, but these adjustments were made even when P was supplied at rates above critical P. In contrast, the *Trifolium* spp. increased root length density substantially at moderately-low P supply but made no adjustment to the length of their short root hairs in response to P deficiency.

Differences in root hair length appeared to be a crucial factor in soil exploration potential of each of the species. This is best illustrated by comparing the estimates of potential root hair cylinder volume achieved by *D. glomerata*, *O. sativus* and *O. compressus* with that of *T. subterraneum* and the factors that contributed to their achievement of a large root hair cylinder volume. The peak root length density achieved by *T. subterraneum* under low-P soil conditions was equivalent to that of *D. glomerata* and 30-50% greater than that of the *Ornithopus* spp., yet the maximum root hair cylinder volume of *T. subterraneum* was only 20-30% of that of the grass and the *Ornithopus* spp., respectively. *O. sativus*, *O. compressus* and grasses in general, have intrinsically long root hairs in comparison with those of other pasture legumes (Evans 1977; Yang *et al.* 2015; Chapter 5). In the present experiment, the root hair lengths of the *Ornithopus* spp. were similar to those of *D. glomerata*. As a result, *D. glomerata* and the *Ornithopus* spp. effectively explored at least 3-fold more soil in the fertilised soil layer than *T. subterraneum* (as indicated by their potential root hair cylinder volumes) and could do so by investing approximately half the quantity of dry matter in topsoil roots (Chapter 6).

Previously it was argued that the species that had a low critical P requirement for shoot growth did so by achieving high P uptake per unit of root dry mass allocated to the P-enriched soil layer (Chapter 6). The present examination of root morphology has revealed that this primarily occurred when a high root length density was deployed in combination with long root hairs.

7.4.2 P uptake and critical external P requirements

Root acclimation to soil P conditions was limited to, or was most pronounced, in the P-enriched topsoil layer. This indicated that it is highly likely that P uptake and the external critical P requirement of each species may be explained predominantly by root foraging for P in the topsoil layer, along with any differences in the internal P-utilisation efficiency of each species. The species did display some differences in their shoot P-utilisation efficiencies (Chapter 6). However, 90% of maximum yield for each species was achieved at an essentially similar amount of total P uptake per sward (Fig. 7.6a, Table 7.1). This indicated that differences in shoot dry mass were associated with differences in P-utilisation efficiency, but P-utilisation efficiency did not influence the external critical P requirements of the species.

We hypothesised that under P-limiting conditions (with negligible mass flow of P), it should be possible to approximately explain P supply to roots using Fick's law of diffusion, unless there were significant rhizosphere P interactions (e.g. phosphate release by microorganisms or root exudates) that enabled the species to differentially access sparingly-available forms of P in soil:

$$J = K \times \mathrm{d}C/\mathrm{d}X, \quad \text{……Equation [2]}$$

where J is the diffusion flux (amount of P per unit area and time: moles m^{-2} s^{-1}), K is the effective diffusion coefficient for phosphate in the soil (area per unit time: m^2 s^{-1}) (this is expected to vary exponentially with temperature), C is the concentration of phosphate in soil solution of the bulk soil (moles m^{-3}) and X is the length of the diffusion path (m) ($\mathrm{d}C/\mathrm{d}X$ is the concentration gradient for phosphate between the bulk soil and the root). In turn, P uptake and the critical P requirement of a species would be explained largely by the area of the

nutrient-absorption interface of its root system with the soil. To test this hypothesis, it was necessary to make several assumptions (e.g. see Barber *et al*. 1984). The main assumptions and the evidence underpinning them were as follows.

(i) *The fertilised soil layer was the source of most of the P taken up by the legumes*. This was assumed to be the case because yield and P uptake in the unfertilised treatment was <40% of that needed for maximum growth (Figs 7.5b and 7.6a) and because the legumes only showed adjustments to root growth and length in the fertilised topsoil layer.

(ii) *The surface of the root hair cylinder in the fertilised topsoil layer was the effective interface for P uptake between the roots and the soil*. Models of P uptake by roots often estimate P influx using the total surface area of the root hairs (e.g. Föhse *et al*. 1991). However, the density of root hairs on the roots of all of the legumes was assumed to be sufficiently high for zones of P depletion of adjacent root hairs to be overlapping (Ma *et al*. 2001). Indeed, micro-sectioning of rhizosphere soil of a *Hordeum vulgare* cultivar with high root hair densities has shown that the concentration gradient for $NaHCO_3$-extractable phosphate extending out from the root is initiated close to the surface of the root hair cylinder with the phosphate concentration unchanged across the root hair zone (Gahoonia *et al*. 1997a; Gahoonia and Nielsen 2003). Furthermore, the low concentration of $NaHCO_3$-extractable phosphate at the root surface of a mutant lacking root hairs was similar to that at the surface of the root hair cylinder of the wild-type *H. vulgare* line with root hairs (Gahoonia and Nielsen 2003).

(iii) *Plant growth and root length extension into new soil was essentially linear for the duration of the experiment at each P supply level*. This will not be entirely true because growth from germination is exponential, but the additional experiment showed that shoot growth of *T. subterraneum* was linear from three weeks until the end of the experiment for each P supply level (Fig. 7.7). Eighty percent of the growth occurred during the linear phase. Root length extension was also essentially linear during this phase, although there was some suggestion that root dry matter increase in the moderately P-deficient treatment (12 mg P pot^{-1}) may have slowed during the last week of the experiment.

(iv) *The fertilised soil layer was homogeneous and K* (*the diffusion coefficient for phosphate*) *and d*X (*the distance for diffusion from the bulk soil to the surface of the root hair cylinder*) *were either constant or changed in a similar manner for all species over the duration of the experiment*. To facilitate this, soil moisture was maintained in a narrow range (75-80% of field capacity) by daily watering of pots to weight and watering pots once per week to field capacity. Furthermore, it was considered that this assumption did not hold for plants that had over-utilised the soil layer. For this reason, *D. glomerata*, whose potential root hair cylinder volume approached or exceeded that of the volume of the soil layer, was not included in the assessment.

If diffusion of phosphate and root foraging were the main determinants of P uptake, it is anticipated that at each rate of P supply (i.e. at approximately equivalent d*C*), P uptake per unit surface area of the root hair cylinder volume would be similar for all of the legume species:

$$\text{i.e.} \quad J = \text{P uptake}/SA_{RHC} = K * dC/dX \; ; \qquad \text{Equation [3]}$$

where P uptake is total P content of shoots and roots, SA_{RHC} is the surface area of the root hair cylinder and *K* and d*C*/d*X* are effectively constant (similar for each species). This was generally the case when P uptake at rates of supply below critical P were considered (Fig. 7.6b). Occasional differences in P uptake per unit surface area of the root hair cylinder volume were not consistently expressed. Factors that might alter P uptake per unit surface area of the root hair cylinder include differences in the level of colonisation by arbuscular mycorrhizal fungi (AMF), which would alter the surface area of the interface of the root system with soil, or differences between the species in their capacity to influence d*C*/d*X*: e.g. differences in I_{max}, the maximum rate of net P influx (Föhse *et al*. 1991) or production of organic anions to enhance phosphate desorption (Gerke 2015). Differences such as these would not be accounted for in the present calculation (Fig. 7.5c). Two of the species examined in the experiment (*O. compressus* and *T. subterraneum*) are reported to benefit differentially when colonised by AMF (Schweiger *et al*. 1995). In low-P soil, P uptake by *T. subterraneum* is improved more than that of *O. compressus*. The differential benefit was thought to be a consequence of the species having very short (*T. subterraneum*), as opposed to relatively long (*O. compressus*) root hairs (Schweiger *et al*. 1995). However, AMF colonisation of roots was relatively low (<5%) in the present experiment so it was unclear whether a differential P nutrition benefit could have occurred.

It was anticipated that P uptake by each legume would be directly proportional to d*C* × SA_{RHC}: i.e. P uptake = (*K*/d*X*) × (d*C* × SA_{RHC}). In this case, *K*/d*X* is effectively constant or similar for each species. We examined this by assuming that $CaCl_2$-extractable phosphate at the commencement of the experiment was a reasonable surrogate for the soil solution phosphate concentration of bulk soil in each P treatment. The relationships between P uptake and $CaCl_2$-extractable P × SA_{RHC} for each species were linear (Fig. 7.6b), indicating that nutrient foraging and phosphate diffusion were major influences on P uptake. The gradients of the relationships did not differ significantly among most of the legume species. However, the gradient for *O. compressus* was significantly greater than that of the other species. This is not readily understood, because a difference in the gradient implies that the plant has altered *K* (phosphate diffusivity) or d*X* (the distance over which diffusion occurs). However, it was only possible to use data derived from the two lowest P application levels because *O. compressus* had such a low critical P requirement and the slope of the relationship for *O. compressus* was strongly influenced by data points that were very close to its critical P requirement (Fig. 7.6b).

Root acclimation to low-P soil by most of the species was initiated when soil P supply dropped below the critical P requirement of the species, indicating that adjustments to root morphology were usually made in response to P stress. There is no doubt that the root acclimation to P stress will modify the critical P requirement of a species and will offset the yield penalty of growing in P deficient soil. However, the data also indicate that having intrinsically favourable root morphology (high root length density, long root hairs) was the primary determinant of the critical P requirement of a species. Hill *et al*. (2006) also concluded that intrinsic root morphology traits were more important in determining the critical P requirement of a range of species than acclimation to P deficiency. *O. compressus* was particularly interesting because it differed from the other species by commencing adjustments to soil P at P concentrations above its critical P requirement. Further work is required to confirm this observation because it suggests that it may be possible that some

species can also adjust root morphology in ways that substantially reduce the plant's requirement for P.

7.4.3 Conclusions

Maximising the potential for soil exploration (nutrient foraging) through a combination of relatively long, thin roots combined with long root hairs enabled greater P uptake from low-P soil among the pasture species in the present study. These species with an intrinsically high nutrient foraging potential had a relatively low critical P requirement. *T. hirtum* and *B. pelecinus* had root traits equivalent to (e.g. root hair length of *B. pelecinus*) or approaching that (e.g. specific root length of *T. hirtum*) of the *Ornithopus* spp. but only achieved external requirements for P that were between that of *T. subterraneum* and the *Ornithopus* species. However, the combinations of high root length density and long root hairs achieved by the *Ornithopus* spp. allowed them to maximise soil exploration at low levels of applied P and to achieve substantially lower critical P requirements. *T. subterraneum* was exceptional in achieving high root length density in moderately low-P soil, but failed to capitalise on this for P uptake due to short root hairs. The high root length density of this species required a large allocation of biomass due to its low specific root length. Legume species that can maximise soil exploration by having root morphological traits that maximise the surface area of the root hair cylinder are more likely to have a lower critical external P requirement.

8 A wider assessment of critical external P requirements and nutrient foraging traits among the alternative pasture legumes

Graeme A. Sandral, Rebecca E. Haling, Megan H. Ryan, Andrew Price, Wayne M. Pitt, Shane M. Hildebrand, Christopher G. Fuller, Daniel R. Kidd, Adam Stefanski, Hans Lambers, Richard J. Simpson

Submitted to Crop and Pasture Science.

8.1 Introduction

The initial controlled environment experiment reported in Chapters 6 and 7 confirmed the variation in critical P requirements of five of the legume species that had been studied in the field experiments (Chapter 4). The experiment also demonstrated the role of nutrient foraging root traits in achievement of a low critical external P requirement. Species with roots capable of developing a large root hair cylinder (i.e. long roots with long root hairs) achieved the lowest critical P requirements.

Here we report a second study that built on the initial investigations (Chapters 6 and 7) to examine the critical P requirements and root morphology of a wider sample (12) of the alternative legume species. The species used in the experiment included most species that had previously been assessed in field experiments (Chapter 4) and other species that, for logistical reasons (e.g. lack of adaptation to field sites, insufficient seed, practical limits to the size of field experiments), could not be included in the field experiments.

Over the last few decades there has been increasing interest in the use of these novel, "alternative" pasture legumes in the temperate pastures of southern Australia (Nichols *et al.* 2007). The species include *Biserrula pelecinus*, and various representatives from the *Trifolium* and *Ornithopus* genera and are often used as a unique component of crop-pasture sequences or in niche environments not suited to the mainstream pasture legumes (i.e. *Trifolium subterraneum*, *T. repens* and annual *Medicago* spp.). Legumes with lower soil test P requirements for high yield will potentially reduce inefficiencies (P losses, P accumulations in soil) associated with P fertiliser use because P loss by erosion, runoff and leaching (Melland *et al*. 2008) and P accumulation in high P-sorbing soils (Barrow 1999, Simpson *et al.* 2015) are both positively correlated with the soil test P concentration (Simpson *et al*. 2014). Species with a relatively low external P requirement may also have a potential role in low input grazing systems (Dear and Virgona 1996). Efforts to improve the P-acquisition efficiency of the current mainstream species have been relatively unsuccessful (e.g. Caradus 1994; Caradus and Dunn 2000) and development of alternative legume species may be required instead.

The experiment was conducted in a glasshouse so that the plants would be grown under higher light levels than had been possible in the controlled-environment plant growth cabinets used in the initial experiments. A soil with lower intrinsic P fertility was also used. This allowed the acclimation of *Ornithopus* spp. roots to very low soil P levels to be observed and reduced the potential for site-specific and adaptation constraints that can confound broad comparisons of species in the field. It was expected that the study would reveal

differences in the critical P requirement amongst the broader range of legume species, and confirm the hypothesis that root traits that confer high soil exploration are a major factor in achieving a low critical P requirement.

8.2 Materials and Method

8.2.1 Soil

A red Kandosol (Isbell 1996) low in extractable-P (3.74 mg kg^{-1} Colwell-extractable P; Colwell, 1963; method 9b in Rayment and Lyons 2011) was collected from 0 to 150 mm depth from near Beckom, New South Wales (34°20'S, 146°58'E). Soil was steam pasteurised at 70°C for 60 minutes to reduce inoculum levels of soil-borne pathogens, dried for 72-hours at 40°C in a dehydrator, and sieved < 5 mm. Lime was mixed through the soil (0.8 g kg^{-1} soil) to raise pH to 5.5 (0.01 M $CaCl_2$-extractable). The soil was then spread across the surface of a plastic sheet to a thickness of ≤10 mm, and a P-free basal nutrient solution was applied as a fine mist before the soil was mixed again to provide rates of 43.0 mg kg^{-1} $CaSO_4.2H_2O$, 41.1 mg kg^{-1} $MgSO_4.7H_2O$, 169 mg kg^{-1} KNO_3, 27.5 mg kg^{-1} $(NH_4)_2SO_4$, 16.7 mg kg^{-1} NH_4NO_3, 119 µg kg^{-1} H_3BO_3, 759 µg kg^{-1} $MnCl_2.4H_2O$, 359 µg kg^{-1} $ZnSO_4.7H_2O$, 33.3 µg kg^{-1} $CuSO_4.5H_2O$, 72.1 µg kg^{-1} $(NH_4)_2MoO_4$, 19.8 µg kg^{-1} $CoCl_2 \cdot 6H_2O$ and 1530 µg kg^{-1} Fe-EDTA. KH_2PO_4 was mixed through sub-samples of the soil to establish seven P treatments of 0, 15, 30, 60, 90, 140 or 250 mg of applied P kg^{-1} of soil.

PVC pots (87 mm internal diameter; 210 mm height) were filled with 1208 g of the dry soil with no applied P to create a "subsoil" layer (50-200 mm depth). Several alkathene beads were placed on top of this layer of soil around the edge of each pot to allow the interface of the topsoil and subsoil to be distinguished at harvest. P-amended soil (403 g dry soil) was then added to each pot to create the "topsoil" layer (0-50 mm depth). The topsoil and subsoil layers were used to mimic the stratification of P that occurs with surface application of fertiliser to pastures. Five replicates of each P rate (0, 15, 30, 60, 90, 140 and 250 mg kg^{-1} topsoil layer) were prepared for each pasture legume species. Plant material and growth conditions

8.2.2 Plant material and growth conditions

Twelve pasture legumes (Table 8.1) were sown at a rate of 50 mg of viable seed per pot. After seven days, pots were inoculated with a slurry of peat containing an appropriate rhizobium strain (New Edge Microbial Pty. Ltd., Albury, Australia; Table 8.1.) and placed in a temperature-controlled glasshouse (max 20°C, min 16°C) under natural light (July-August, Wagga Wagga, NSW, Australia). Pots were arranged in a column and row design to allow for analysis by nearest Linear Mixed Models (LMM). Pots were maintained at approximately 80% field capacity by watering to weight three times a week. Reflective sleeves were fitted to the outside of the pots and were raised daily to equal canopy height to mimic the light conditions that occur in a pasture sward during peak spring growth.

8.2.3 Harvest and assessment

Plants were harvested six weeks after sowing by cutting at soil level. Shoots were dried at 70°C before being weighed for dry mass. Soil was removed from the pot as an intact core and cut at the interface of the P-amended topsoil (now 0-48 mm depth due to settling of the

soil layer) and the un-amended subsoil, as marked by the alkathene beads. The topsoil was cut vertically into quarters. Roots were washed from each section of the topsoil and from the entire subsoil. Roots from one quarter of the topsoil were scanned immediately using a flatbed scanner and the image was analysed for root length and average root diameter using WinRHIZO (Regent Instruments Inc., Quebec, Canada). Two images of fully elongated root hairs on these roots were taken using a Nikon SMZ25 stereomicroscope fitted with a high resolution DS-Fi2 digital camera and digital sight DS-U3 camera controller. The scanned subsample of roots and the roots washed from all other sections of the soil were dried at 70°C before being weighed for dry mass. Specific root length (length per unit dry mass) was determined using the length and mass of roots from the scanned subsample. Total length of roots in the topsoil was then calculated based on the total dry mass measured in the topsoil and the specific root length. Root hair length was determined by measuring the length of ten root hairs (five per image) on each replicate using the software package ImageJ (Rasband 1997-2014).

Shoot P concentration (% dry matter) was determined for four of the P treatments (0, 50, 90 and 140 mg P kg^{-1} for the *Ornithopus* spp., and 0, 90, 140 and 250 mg P kg^{-1} for all other species). Shoots were milled to a fine powder and 50 mg samples were ashed in a muffle furnace for 4 h at 550°C. The ashed material was dissolved in 2M HCl and the P concentration was determined colorimetrically using malachite green (Irving and McLaughlin 1990).

8.2.4 Data analysis

Data were analysed using nearest Linear Mixed Models (LMM). Shoot yields were analysed using R version 3.1.1 by applying the fixed model ‘cultivar*P rate + linear column’ and random model as ‘replicate + replicate.plot + id column + AR1 row’. The shoot yields generated from LMM at pot level were subsequently used to fit Mitscherlich equations [$y = a – b*(e^{-cx})$] for all species.

The critical P requirement for each species was then estimated as the rate of P application in the topsoil that corresponded with 90% of maximum herbage yield. Differences between the critical P concentrations, the maximum yield (or asymptote) and the zero P yields (y-intercept) were tested by considering the estimates and approximate standard errors for each measure simultaneously, and testing for significant pairwise differences. Significance was determined by calculating a standardised difference that weighted the two contributing standard errors. Values greater than two standard errors were considered significantly different ($P = 0.05$). No adjustment was made for multiple comparisons.

The critical internal P requirement for each species was determined as the shoot P concentration corresponding with the critical external P requirement of the species. This was determined by fitting linear relationships between P applied and shoot P concentration.

Table 8.1. Scientific, cultivar, common name and rhizobium inoculant of pasture legumes species assessed in this study.

Scientific name and authority	Cultivar	Common name	Rhizobium inoculant group and strain
Biserrula pelecinus L.	Casbah	Biserrula	Special, Biserrula WSM1497
Medicago truncatula Gaertn.	Sultan	Barrel medic	AM, 'Medic' WSM1115
Ornithopus compressus L.	Santorini	Yellow serradella	S, 'Serradella' WSM471
O. sativus Brot.	Margurita	French serradella	S, 'Serradella' WSM471
Trifolium glanduliferum Boiss.	Prima	Gland clover	C, 'Sub clover' WSM1325
T. hirtum All.	Hykon	Rose clover	C, 'Sub clover' WSM1325
T. incarnatum L.	Dixie	Crimson clover	C, 'Sub clover' WSM1325
T. michelianum Savi	Bolta	Balansa clover	C, 'Sub clover' WSM1325
T. purpureum Loisel.	Electra	Purple clover	C, 'Sub clover' WSM1325
T. spumosum L.	Bartolo	Bladder clover	C, 'Sub clover' WSM1325
T. subterraneum L.	Leura	Subterranean clover	C, 'Sub clover' WSM1325
T. versiculosum Savi	Zulu II	Arrowleaf clover	C, 'Sub clover' WSM1325

The root hair cylinder was considered to represent the potential volume of soil in contact with the root and root hairs. The volume and surface area of the root hair cylinder was calculated according to the following equations:

Volume of root hair cylinder = π (average root diameter/2 + root hair length)^2 × root length

Surface area of root hair cylinder = 2 π (average root diameter/2 + root hair length) × root length

Root hair cylinder volume (RHCV, cm^3), the volume of soil in contact with the root and root hairs, was calculated according to the following equation:

RHCV = [π (root diameter/2 + root hair length)^2] × root length

The effect of pasture species on specific root length, root hair length and average root diameter were analysed using a two-way ANOVA in Genstat as LMM provided no statistical improvement in the loglik value. Data for root hair cylinder volume were log transformed and analysed using a two-way ANOVA in Genstat.

8.3 Results

8.3.1 Shoot dry matter and critical external requirement for P

Maximum shoot yields ranged 1.4-fold from 1.75 g pot^{-1} (*Biserrula pelecinus*) to 2.53 g pot^{-1} (*Medicago truncatula*). The maximum yield of *T. subterraneum*, which was used in this experiment as a benchmark species because of its widespread use in pastures of southern Australia, was 2.37 g pot^{-1}. Maximum yields of most of the species tested (*T. spumosum, T incarnatum, T. gladuliferum, T. michelianum, T. vesiculosum, O. compressus and M. truncatula*) were not significantly different to that of *T. subterraneum*. Critical external requirements for P ranged 3.6-fold from 57 (*Ornithopus compressus*) to 203 (*Trifolium subterraneum*) mg applied P kg^{-1} soil (Fig. 8.1 and Table 8.2). Only *T. spumosum* had a critical external P requirement equivalent to that of *T. subterraneum*; the critical P requirements of all other species were significantly lower. *Ornithopus* spp. had the lowest critical external P requirements of all species (57-63 mg applied P kg^{-1} soil), while that of *Biserrula pelecinus* was also low relative to many of the species (77 mg applied P kg^{-1} soil). Among the eight *Trifolium* spp. there was a 2-fold range in critical external P requirement (105 [*T. michelianum*] to 203 mg applied P kg^{-1} soil [*T. subterraneum*]). The critical internal P requirement of all of the legume species ranged from 0.31 % (*O. compressus* and *T. michelanium*) to 0.46 % (*T. spumosum*) with that of *T. subterraneum* being intermediate at 0.39% (Table 8.2). Among all species, lower critical external requirements for P were associated with a lower critical internal P concentration (R^2=0.51), but were not associated with lower maximum yields (R^2=0.17; not significant) (relationships not shown; derived from data in Table 8.2).

8.3.2 Root dry matter

Topsoil root dry matter of most species was increased when the plants were grown with lower concentrations of soil P (Fig. 8.2). However, most species reached a low-P threshold below which high root dry matter was not maintained (Fig. 8.2). The threshold rate of P application at which the peak in topsoil root dry matter occurred varied among the species,

but was usually higher for species with higher critical P requirements. *Trifolium subterraneum* and *T. vesiculosum* had the most prominent peaks in topsoil root dry matter (0.52-0.47 g), and these peaks occurred at higher rates of P application (90 mg P applied kg^{-1}) than that of the other species. Generally, less subsoil root dry matter was grown when the species were grown with lower levels of P fertility in the topsoil (Fig. 8.3).

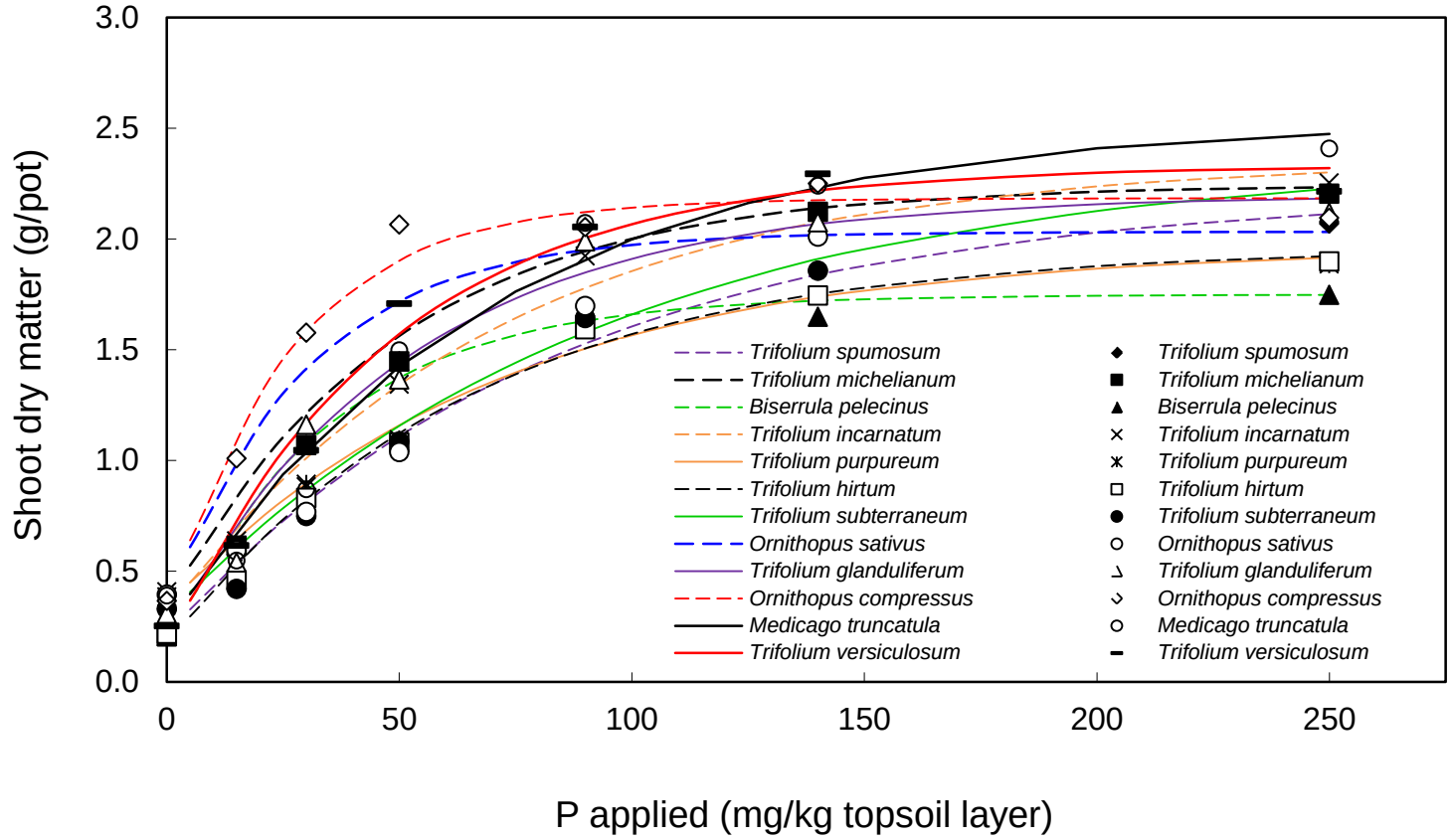


Figure 8.1 Shoot dry matter yield of 12 legume species after application of P. LSD = 0.05 for species x P applied (*P*<0.05; n=5).

Table 8.2 Critical external requirement for P (i.e. rate of applied P to achieve 90% of maximum yield), maximum shoot yield (*a*), the curvature parameter (*b*) and y-intercept for fitted Mitscherlich [$y = a + b*(e^{-cx})$]. Values followed by the same letter are not significantly different (*P* = 0.05).

Species	Critical external P requirement (mg P applied/kg topsoil)	Maximum shoot yield (*a*) (g DM/pot)	*b*	y-Intercept (g DM/pot)	Critical internal shoot P (%)
O. compressus	57±7.4 a	2.18±0.09 cd	-1.87	0.32 ± 0.13	0.31
O. sativus	63±3.4 a	2.03±0.03 bc	-1.68	0.35± 0.05	0.34
B. pelecinus	77±4.0 b	1.75±0.03 a	-1.59	0.15±0.04	0.36
T. michelianum	104±5.6 c	2.25±0.05 d	-1.91	0.34±0.05	0.31
T. versiculosum	106±5.4 c	2.33±0.06 de	-2.18	0.15±0.06	0.35
T. glanduliferum	114±7 c	2.20±0.07 d	-1.99	0.21±0.06	0.34
T. hirtum	145±6.1 d	1.96±0.05 b	-1.8	0.16±0.04	0.43
T. purpureum	151±7.8 d	1.96±0.06 b	-1.63	0.34±0.04	0.40
M. truncatula	151±8.6 d	2.53±0.09 e	-2.3	0.23±0.07	0.40
T. incarnatum	155±8.7 d	2.36±0.08 de	-2.05	0.31±0.06	0.34
T. spumosum	185±9.0 e	2.22±0.08 d	-2	0.21±0.05	0.46
T. subterraneum	203±12.7 e	2.37±0.12 de	-2.07	0.30±0.06	0.39

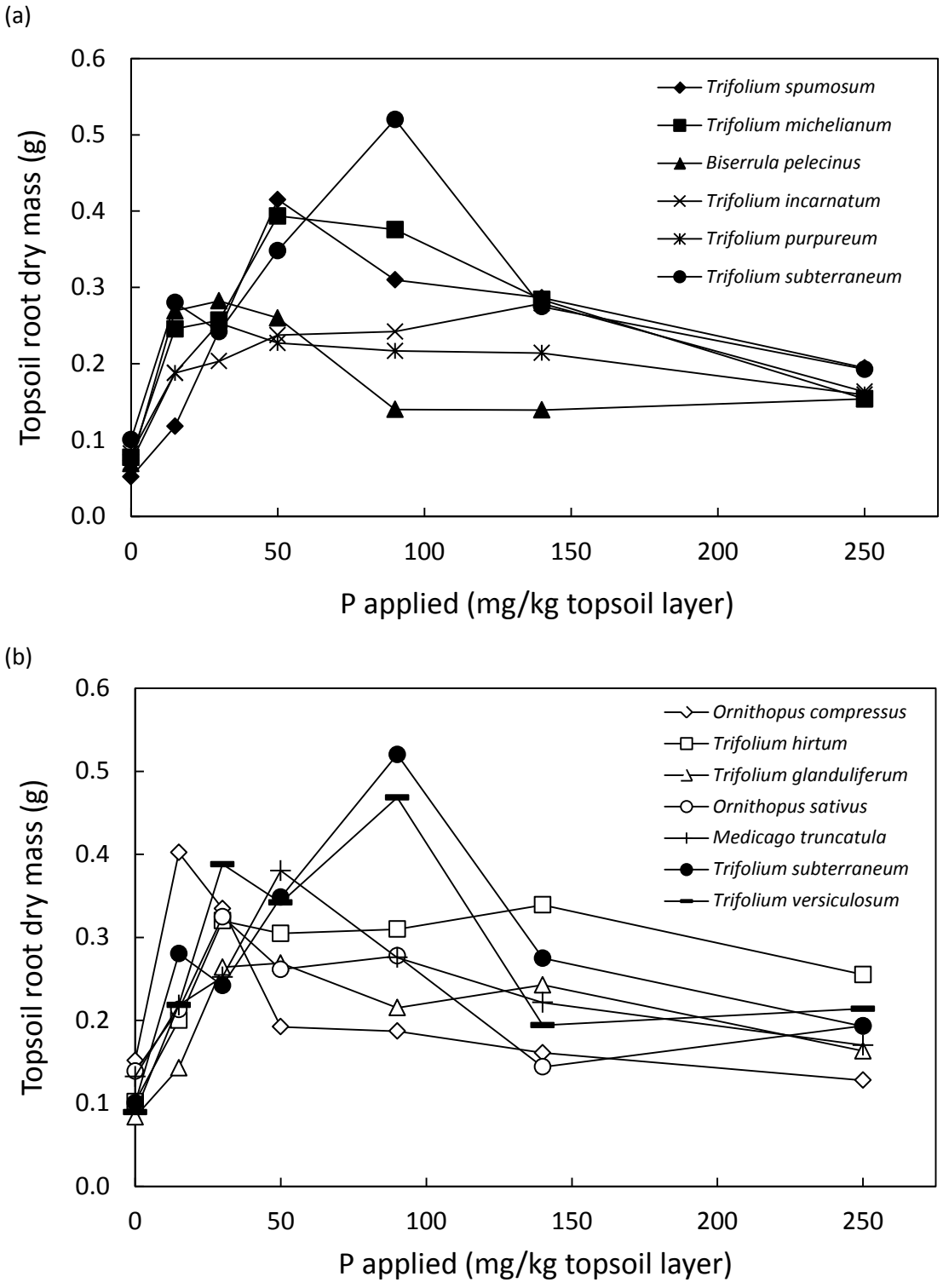


Figure 8.2 Topsoil root dry matter in response to P applied to the topsoil of a pot for 12 legume species. LSD = 0.17 for species x P applied (P<0.05; n=5).

8.3.3 Root length density

Root length density of the legumes in the topsoil layer was increased between 1.6- (*T. hirtum*) and 4-fold (*O. compressus*) and mostly demonstrated a distinct peak when the species were grown in the P supply range below their critical P requirement (Fig. 8.4). Maximum root length densities ranged from 12 (*T. purpureum*) to 39 cm cm^{-3} (*T. hirtum*). Similar to topsoil root dry matter, the level of P supply at which maximum root length density was achieved varied among the species. For example, maximum root length densities of *O. compressus* and *T. subterraneum* were achieved at 15 and 90 mg P applied kg^{-1}, respectively. Maximum root length density was mostly achieved at a P supply rate that was similar to that required for maximum topsoil root dry mass.

8.3.4 Specific root length and average root diameter in the topsoil

Some species demonstrated between a 1.2 (*T. glanduliferum*) and 1.8-fold (*T. michelianum*) increase in specific root length when grown with a low P supply but, for many species, any apparent increase in specific root length was not significant (e.g. *T. subterraneum* and *T. purpureum*; Fig. 8.5). However, threshold P supply rates were evident for specific root length and specific root length was lower for most species at the lowest rates of P supply (i.e. in the 0-15 mg P applied kg^{-1} range).

Trifolium purpureum and *T. subterraneum* consistently had amongst the lowest specific root lengths with maximum values of 156 and 176 m g^{-1}, respectively (Fig. 8.5). In contrast, *T. michelianum*, *O. sativus*, *T. hirtum* and *O. compressus* had maximum specific root lengths that exceeded 300 m g^{-1}. *Trifolium michelianum* and *O. sativus* were also notable because they were able to achieve or maintain a high specific root length at very low levels of applied P (i.e. 15 mg P applied kg^{-1}).

Despite significant changes in specific root length, the species demonstrated less adjustment in average root diameter (Fig. 8.6). *Trifolium purpureum* had consistently high average root diameters (0.38-0.43 mm) while *O. compressus* was consistently amongst the lowest for average root diameter (0.27-0.30 mm).

The root hair length of *T. hirtum*, *B. pelecinus* and *O. sativus* increased between 1.3 and 1.4-fold in response to low P supply (Fig. 8.7). No significant adjustment was detected for any other species. The *Ornithopus* spp. and *B. pelecinus* had consistently longer root hairs (up to 0.57-0.63 mm) relative to the other species, while *T. subterraneum* had the shortest root hairs (up to 0.29 mm).

Root hair cylinder volume of the species was increased between 2.0 (*T. purpureum*) and 4.8-fold (*T. michelianum*) when they were grown with low P supply (Fig. 8.8). The *Ornithopus* spp. and *B. pelecinus* achieved the largest root hair cylinder volumes (143-175 cm^3). This was equivalent to about 50% of the volume of the topsoil. These maximums occurred at very low levels of applied P (15-30 mg P applied kg^{-1}) relative to most other species. The next largest root hair cylinder volumes were achieved by *T. michelianum* which peaked at 120 cm^3 at a P application rate of 50 mg kg^{-1}. *Trifolium subterraneum* consistently had amongst the lowest root hair cylinder volumes (up to 50 cm^3); equivalent to only ~15% exploration of the topsoil.

Among the species, the specific surface area of the root hair cylinder (i.e. surface area of root hair cylinder per unit root dry mass) was negatively correlated with the critical external P requirement (Fig. 8.7). The relationship was generally strongest at lower levels of applied P. Relationships between critical external P requirement and other root traits (i.e. root length density, root hair cylinder volume, surface area of root hair cylinder volume) were not as strong and did not hold at higher levels of applied P (relationships not shown)

8.3.5 Root hair length surface area of root hair cylinder and relationship with critical external P requirement

The root hair length of *T. hirtum*, *B. pelecinus* and *O. sativus* increased between 1.3 and 1.4-fold in response to low P supply (Table 8.3). No significant adjustment was detected for any other species. The *Ornithopus* spp. and *B. pelecinus* had consistently longer root hairs (up to 0.57-0.63 mm) relative to the other species, while *T. subterraneum* had the shortest root hairs (up to 0.29 mm).

Root hair cylinder volume of the species was increased between 2.0 (*T. purpureum*) and 4.8-fold (*T. michelianum*) when they were grown with low P supply (Fig. 8.6). The *Ornithopus* spp. and *B. pelecinus* achieved the largest root hair cylinder volumes (143-175 cm^3). This was equivalent to about 50% of the volume of the topsoil. These maximums occurred at very low levels of applied P (15-30 mg P applied kg^{-1}) relative to most other species. The next largest root hair cylinder volumes were achieved by *T. michelianum* which peaked at 120 cm^3 at a P application rate of 50 mg kg^{-1}. *Trifolium subterraneum* consistently had amongst the lowest root hair cylinder volumes (up to 50 cm^3); equivalent to only ~15% exploration of the topsoil.

Among the species, the specific surface area of the root hair cylinder (i.e. surface area of root hair cylinder per unit root dry mass) was negatively correlated with the critical external P requirement (Fig. 8.9). The relationship was generally strongest at lower levels of applied P. Relationships between critical external P requirement and other root traits (i.e. root length density, root hair cylinder volume, surface area of root hair cylinder volume) were not as strong and did not hold at higher levels of applied P (relationships not shown)

8.4 Discussion

8.4.1 Critical external P requirements

This work demonstrated that there was significant variation in the critical external P requirements and nutrient foraging potential of the 12 legume species. The critical external requirements of the species were separated into five significantly different groups. The grouping from lowest to highest were *Ornithopus spp*., < *Biserrula pelecinus* < *Trifolium michelanium*, *T vesiculosum*, *T. glanduliferum* < *T. hirtum*, *T. purpureum*, *Medicago truncatula*, *T. incarnatum* < *T. spumosum* and *T. subterraneum*. This research confirmed results of Bolland and Paynter (1992) and Chapters 6 and 7, that demonstrated the *Ornithopus* spp. have the potential to yield as well as *T. subterraneum* but can achieve this yield with less than half the amount of applied P.

It is debatable whether some of the smaller differences in critical P requirements of the legumes (e.g. *T. incarnatum* cf. *T. subterraneum*) in this experiment have any practical use for farmers. This is mainly because it is highly likely that different soil types, soil chemical

conditions (e.g. soil acidity) and soil biology (e.g. root rot pathogens - see Chapter 13) and ontogenetic development of the species interact with legume root growth or root morphology, and this can modify the response of a species to P. Comparisons of the apparent critical P requirements of *B. pelecinus* among several experiments demonstrate this issue. In the present experiment it had a relatively low critical P requirement when grown in a soil sourced from Beckom, NSW (Table 8.1), in Chapter 6 (when grown in soil sourced from Hall, ACT) its P requirement was only marginally lower than subterranean clover and in all field trials (Yass, Burrinjuck, Beckom and Belfrayden), its P requirement was also only marginally lower than that of subterranean clover. Likewise, at some field sites *T. incarnatum* had a much lower apparent critical P requirement than was indicated by the present experiment. In these instances, relatively high crop growth rates (high peak spring yields) were recorded for *T. incarnatum* which is usually used as a forage species because of its high spring growth rates.

For obvious reasons recommendations about the P requirements and management of these legumes must be based on field performance. In the field experiments only three broad grouping of the legumes based on their P requirements seemed sensible (Chapter 4).

An encouraging observation is that the serradella species were always ranked with the lowest critical P requirements and this was considerably lower than the requirement of subterranean clover.

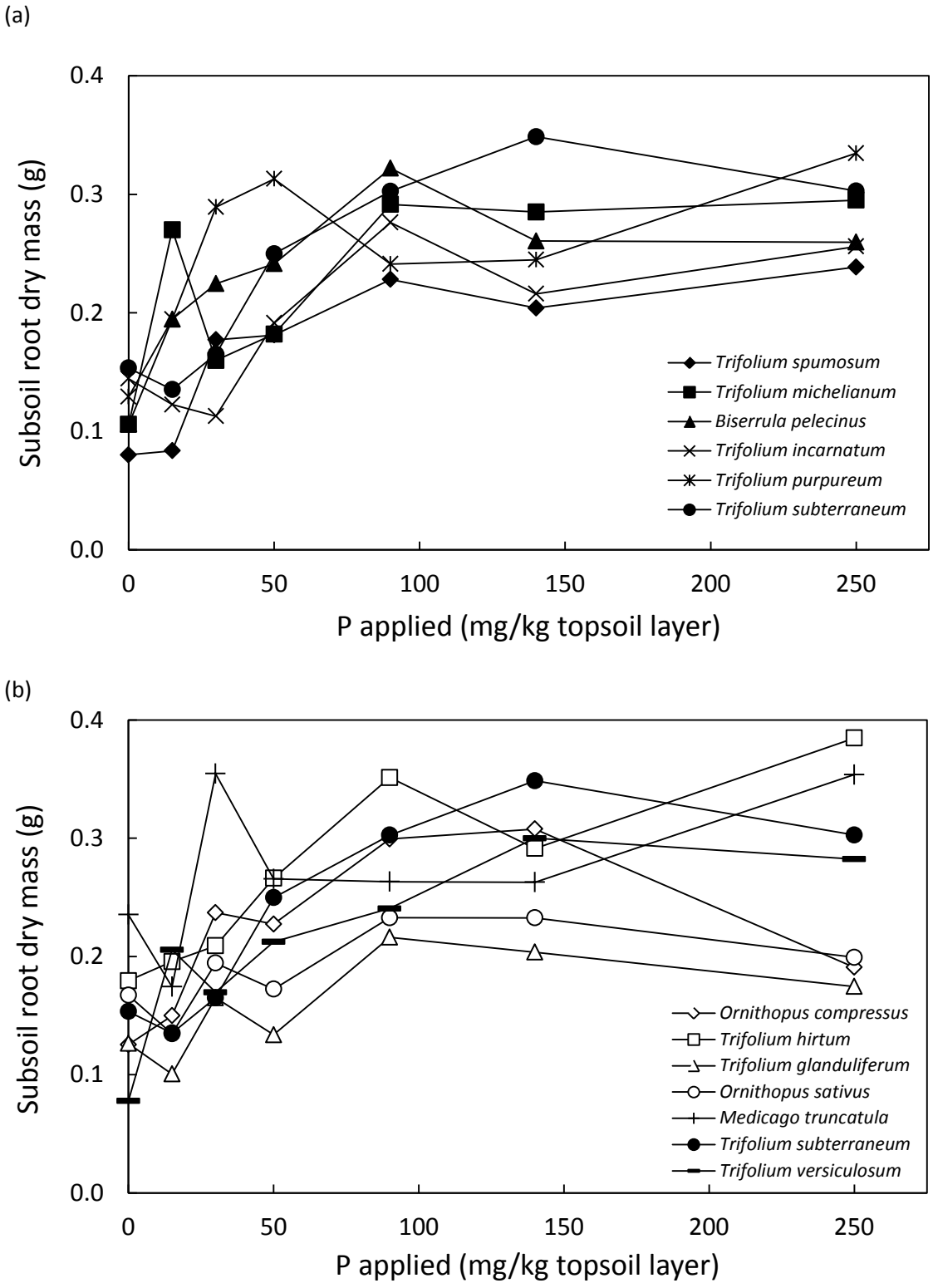
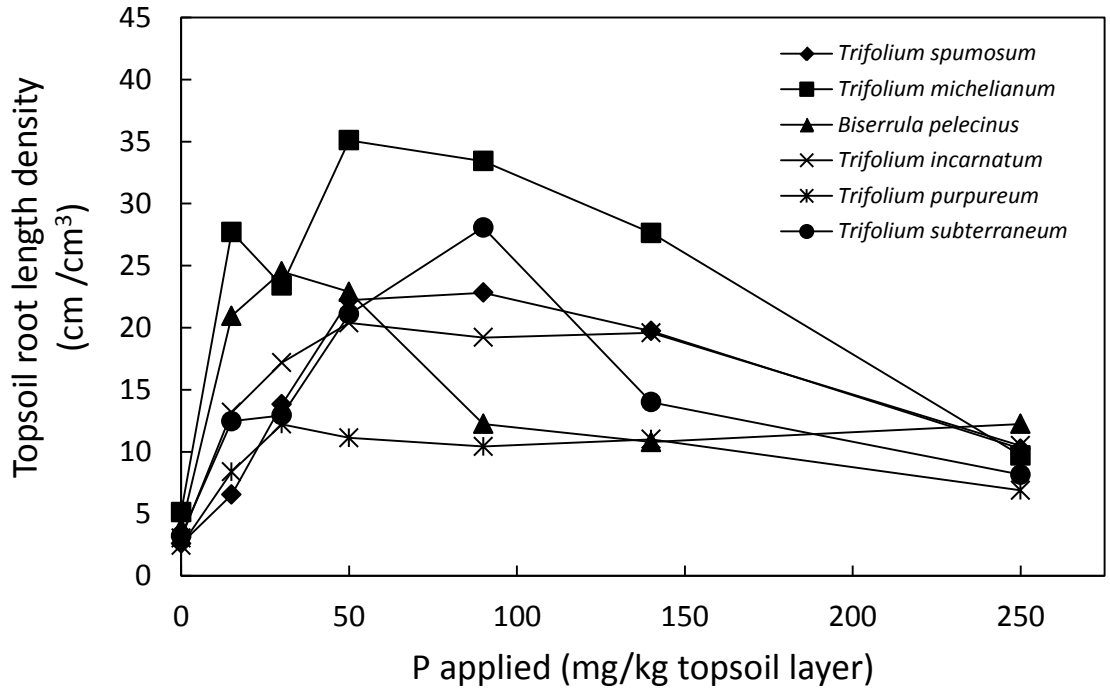


Figure 8.3 Subsoil root dry matter in response to P applied to the topsoil of a pot for 12 legume species. LSD = 0.14 for species x P applied (P<0.05; n=5).

(a)



(b)

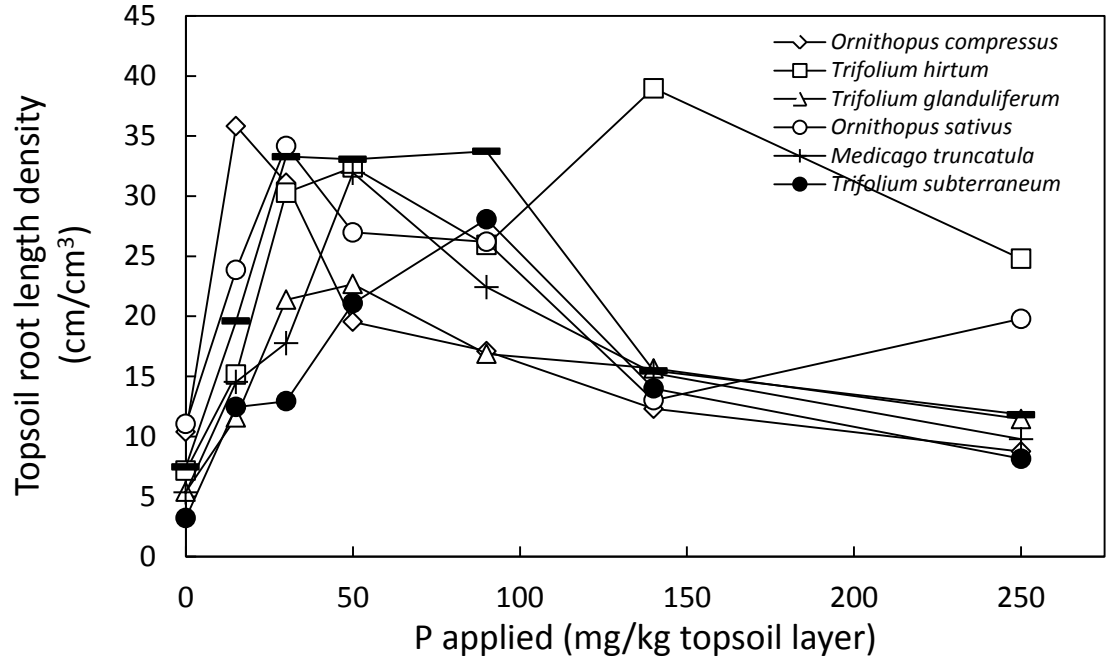


Figure 8.4 Topsoil root length density to P applied to the topsoil of a pot for 12 legume species. LSD = 9.7 for species x P applied (P<0.05; n=5).

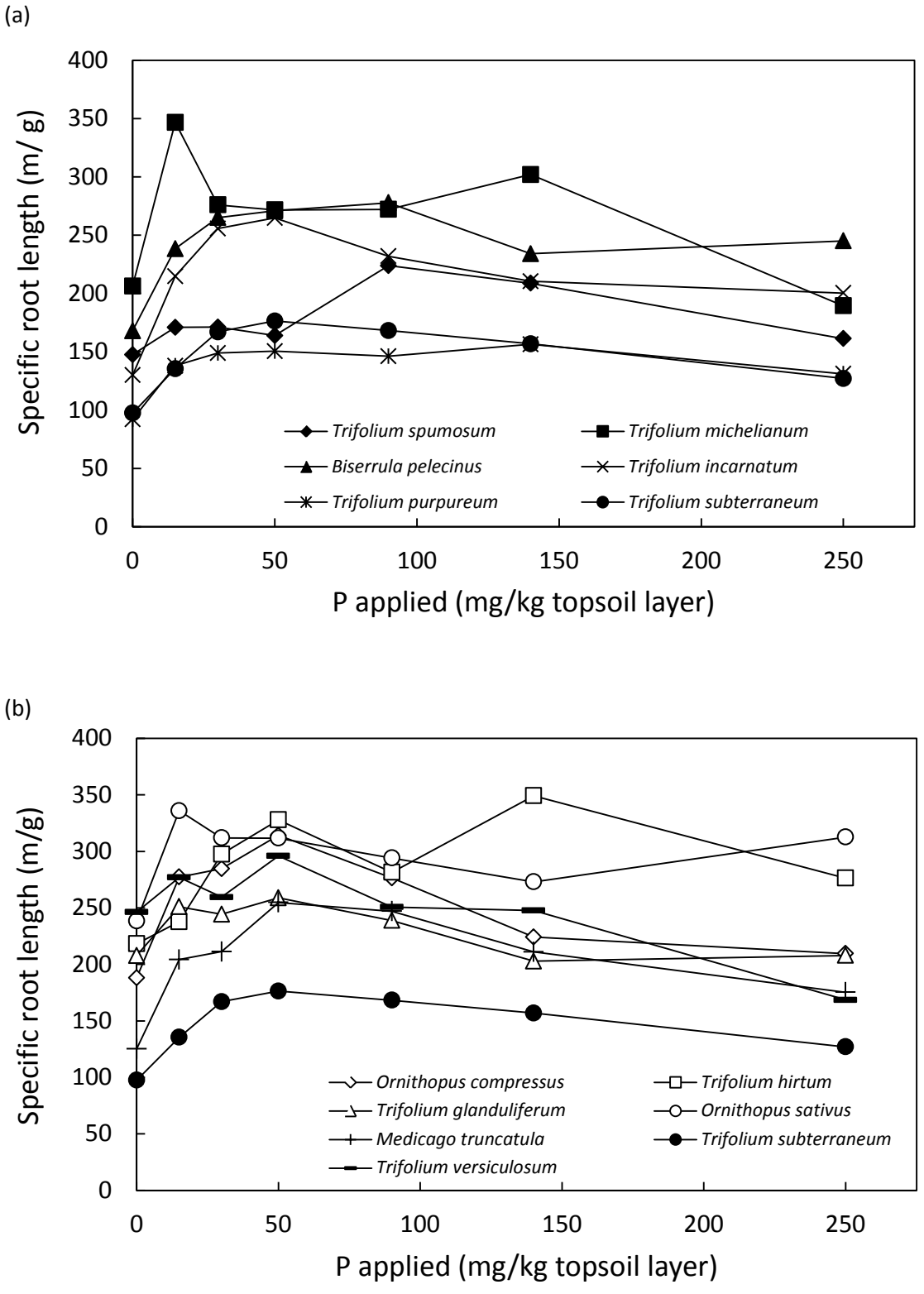
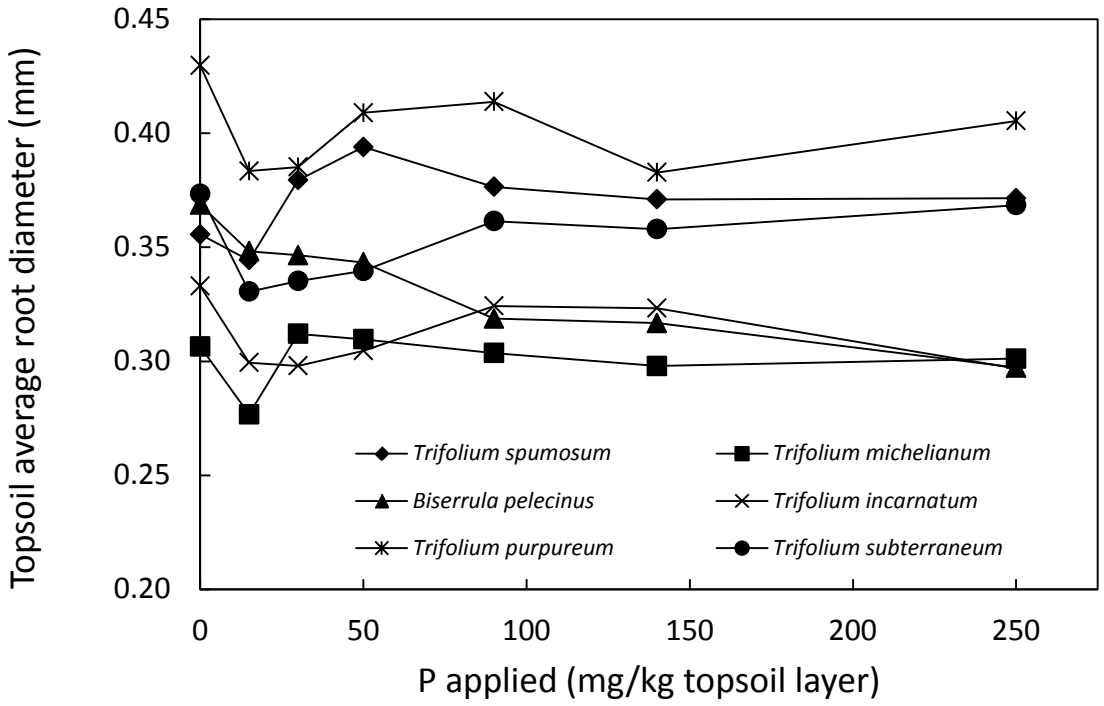


Figure 8.5 Specific root length in response to P applied to the topsoil of a pot for 12 legume species. LSD = 51.6 for species x P applied (P<0.05; n=5).

(a)



(b)

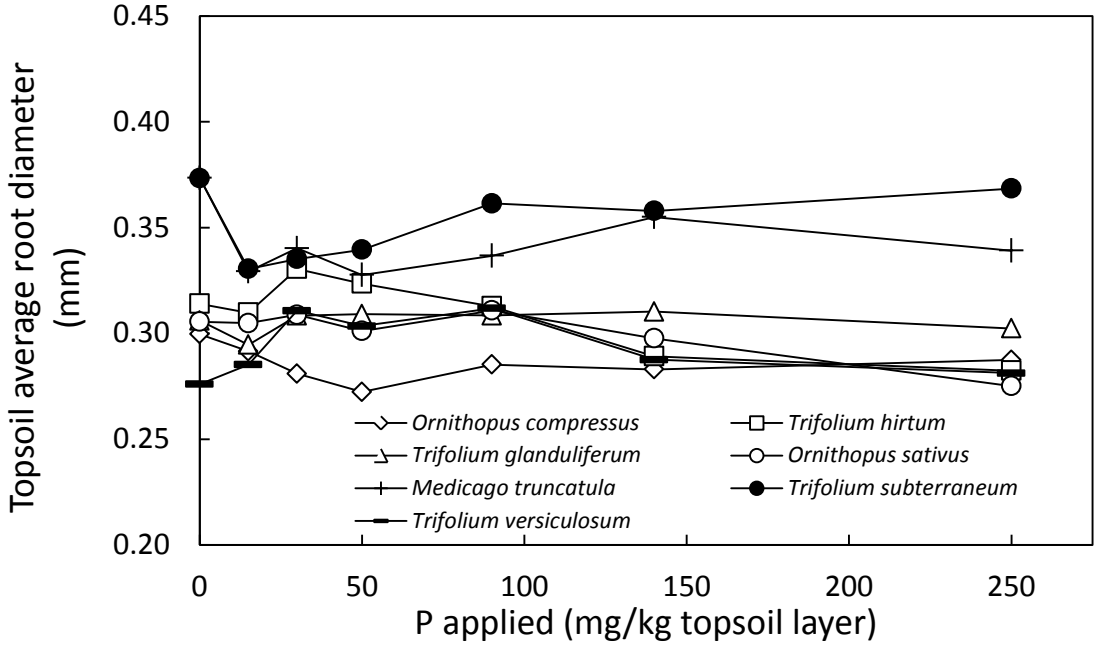


Figure 8.6 Average root diameter in response to P applied to the topsoil of a pot for 12 legume species. LSD = 0.023 for species x P applied (P<0.05; n=5).

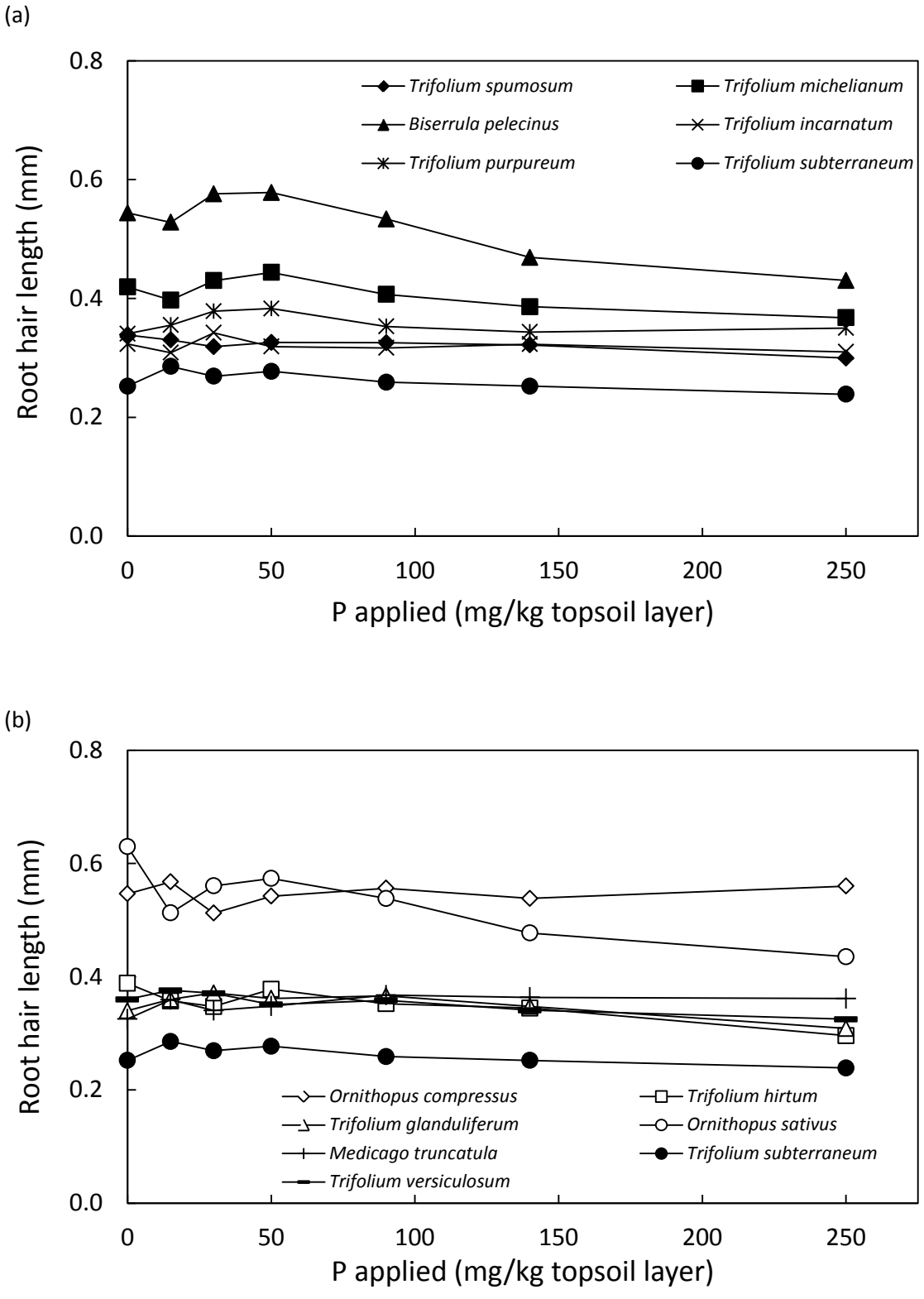


Figure 8.7 Root hair length in response to P applied to the topsoil of a pot for 12 legume species. LSD = 0.080 for species x P applied (P<0.05; n=5).

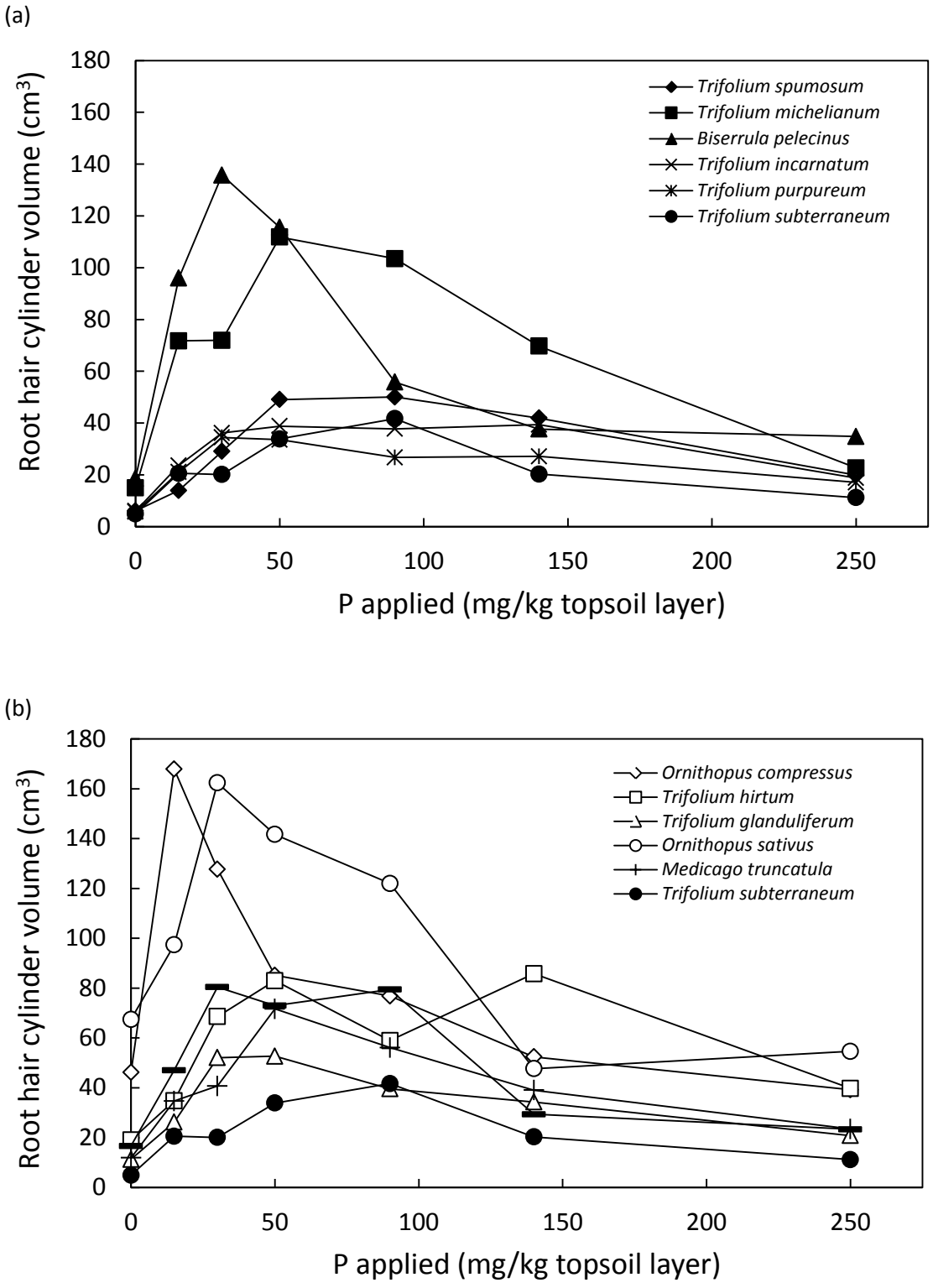


Figure 8.8 Root hair cylinder volume in response to P applied to the topsoil of a pot for 12 legume species. LSD for log(1+x) transformed data was 0.225 for species x P applied (P<0.05; n=5).

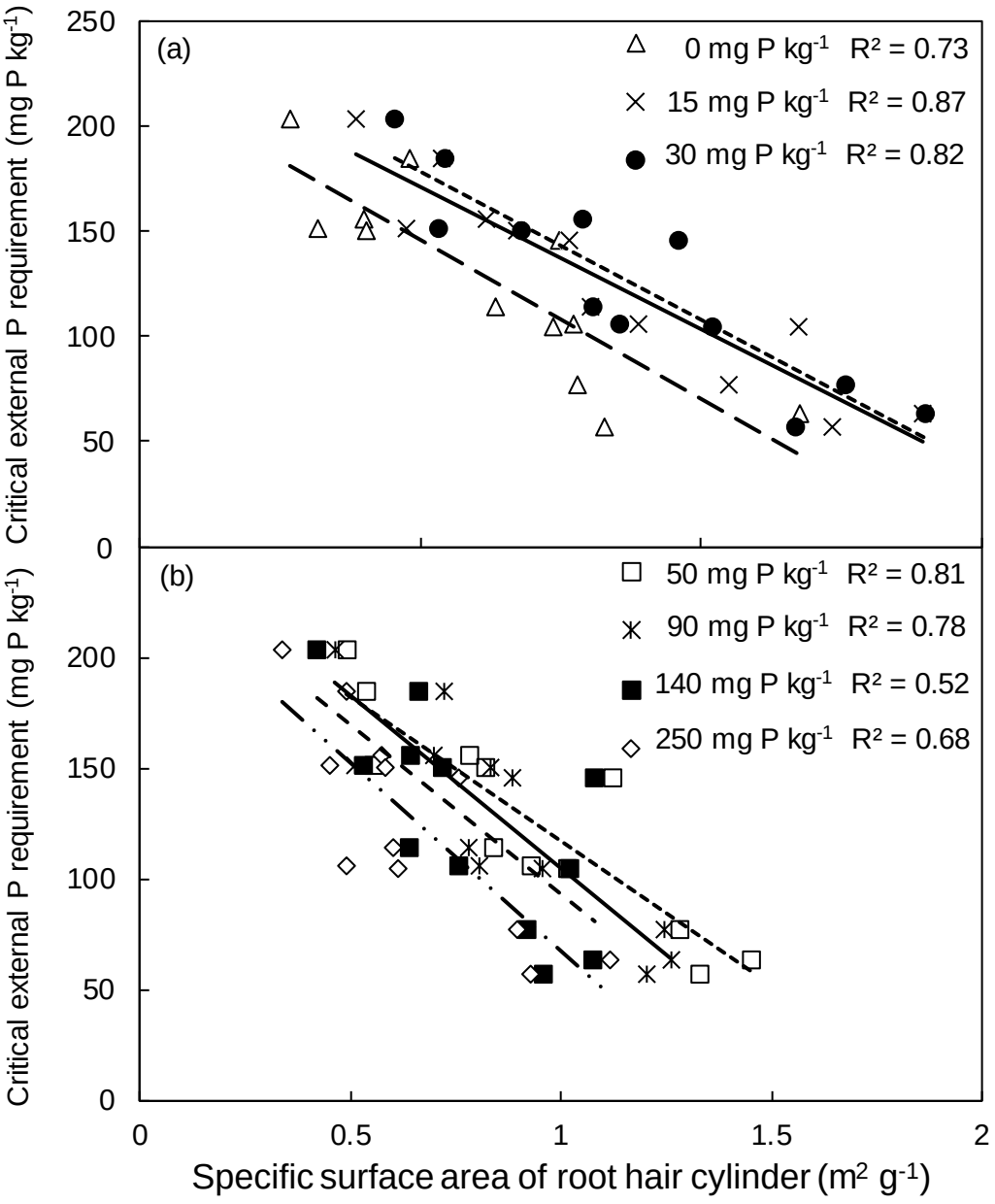


Figure 8.9 Relationship between specific surface area of the root hair cylinder (m^2 g^{-1}) and the critical external phosphorus (P) requirement of 12 legume species grown in soils with P applied to the topsoil layer of the pots at rates of (a) 0, 15, 30 and (b) 50, 90, 140 and 250 mg kg^{-1}. Each of the 12 legumes is represented within each P treatment.

8.4.2 Root phenes associated with a low critical P requirement

The range in critical P requirements of the legumes observed in the present experiment was an asset for determining which root system characteristics were required by pasture legumes to achieve a low critical P requirement. It has been surmised using a subset of the alternative legumes (Chapters 6 and 7), that a large root hair cylinder (as a result of long roots with long root hairs) was characteristic of species that achieved low critical external P requirements. High specific root length was often characteristic of species able to achieve a high root length density (long roots) in the nutrient-rich layer of a soil. However, subterranean clover (which has low specific root length) developed high root length density by allocating more dry mass to root growth. However, long roots without long root hairs was not an efficient way to acquire more P from low P soil. Among the wider range of species in the present experiment, the *Ornithopus* spp. and *B. pelecinus* demonstrated the greatest ability to achieve a large root hair cylinder. *Trifolium subterraneum* was again ranked amongst the poorest species for nutrient foraging potential because of its very short root hairs.

Among the *Trifolium* spp., there was a range in critical external P requirements and nutrient foraging traits. *Trifolium michelanium* had a high nutrient foraging potential relative to most other *Trifolium* spp. as a consequence of its high root length density, high specific root length and, for this genus, relatively long root hairs. Some other *Trifolium* spp. had single root traits that were advantageous for nutrient foraging, they were unable to achieve the combination of high nutrient foraging traits observed for the *Ornithopus* spp. and *B. pelecinus*.

While there was some variation in the critical shoot P concentration among the species, all species appear to be adequate in terms of nutrition for livestock production (Ozanne 1980). Furthermore, the significant linear relationships between specific surface area of the root hair cylinder and critical external P requirement suggests a direct link between the ability of a legume to forage for P and achieving a low critical external requirement for P – rather than being driven by differences in internal efficiency. Interestingly, while the relationship between nutrient foraging and critical external P requirement was strongest at lower concentrations of applied P, relationships were still significant at high concentrations of P. This suggests that a species' intrinsic ability to forage for nutrients may be used as an initial screen for identifying pasture legume species that are likely to yield well in low P soil and to have a low critical external P requirement.

8.4.3 Conclusions

The results support findings from both field (Chapter 4) and controlled environment experiments (Chapters 6 and 7) and indicate that *Ornithopus* spp. could potentially be used to achieve productive, low P input pasture systems. However, further work is required to assess whether the *Ornithopus* spp. can be deployed in a greater range of pasture environments across temperate southern Australia. These species have had consistently low critical P requirements in all experiments. The very low ranking of their critical P requirements relative to *T. subterraneum* suggests that pastures based on the *Ornithopus* spp. may be capable of the level of P-fertiliser savings estimated in Section 1.3.

Biserrula pelecinus has root characteristics suited to P-efficiency but its critical P requirement in field trials has not reflected this. The reason(s) for this should be investigated

further with the objective of capitalising on the anticipated P efficiency of this species. Further examination of germplasm of *B. pelecinus* may be required to achieve this.

Likewise, the P efficiency of some of *Trifolium* spp. with lower critical external P requirements than *T. subterraneum*, could also prove useful in the niche farming environments to which they are suited.

9 Intra-specific variation in the root foraging traits of subterranean clover (*Trifolium subterraneum*).

Zongjian Yang, Richard A. Culvenor, Megan H. Ryan, Daniel R. Kidd,
Adam Stefanski, Rebecca E. Haling, Hans Lambers,
Graeme A. Sandral, Richard J. Simpson

9.1 Introduction

The experiments examining the root morphology of alternative pasture legumes and their acclimation responses to P supply (Chapter 6, 7 and 8) demonstrated that intrinsic and adaptive capacity for exploration of soil by roots was the key to improved growth in low-P soil and a low critical P requirement. For pasture legumes, the key root morphology traits required for P efficiency were long root hairs and ability to achieve a high root length density in nutrient-enriched patches of soil. This in turn was facilitated by having a high specific root length and/or the ability to proliferate roots in the nutrient patch. Subterranean clover was more effective at proliferating root length in a nutrient enriched soil layer than any other legumes species but only achieved this by allocating a large mass of roots to the nutrient foraging activity because it had relatively low specific root length. However, subterranean clover was unable to acquire P from low P soil at the rates achieved by other species with similar high root length density. This was attributed to the fact that subterranean clover had very short root hairs and this prevented the species from capitalising on its extraordinary ability to develop high root lengths in low P soil.

Variation in these key root morphology traits was examined consequently to evaluate whether there were opportunities for improved P foraging within subterranean clover. In addition, variation in lateral root angle and root mass fraction (root mass expressed as a proportion of plant mass) were examined as these traits are also known to assist nutrient foraging in other species (e.g. Lynch 2007).

A collection of heritage and modern subterranean clover cultivars and the 'core' collection of subterranean clover lines that has been developed to represent ~80% of the genetic variation in subterranean clover (Ghamkhar *et al*. 2008; 2015) were used to assess the ranges over which these traits varied in subterranean clover. The standard root trait screening methods were based on work published by Hill *et al*. (2006; 2010). Most root trait screening was conducted using a sandy loam topsoil collected from a site near Canberra. The soil was limed to $pH_{(Ca)}$ 5.3 and steam pasteurised to remove root pathogens without complete removal of arbuscular mycorrhizal fungi (AMF) spores which survive the soil treatment and germinate to colonise plants roots. The soil treatments allow screening of root traits to be conducted in the absence of aluminium toxicity and root disease, and in the presence of some level of AMF root colonisation. Parallel screening of root traits was also conducted in independent experiments at The University of Western Australia (UWA) using unpasteurized, low-P soil selected for low root pathogen symptoms. The objective of measuring traits in diverse soils and under differing plant growth conditions was to test the stability of subterranean root trait phenotypes and the confidence with which the traits might be selected in a plant improvement program.

9.2 Root hair length

9.2.1 Background

Root hairs are an important trait for P acquisition in many plant species. They act to increase the surface area of the root system available for P uptake and reduce the distance over which phosphate must diffuse to the root in the soil solution. Many species are reported to increase root hair length in response to low P supply; this is an effective strategy for increasing P uptake and comes at a low carbon cost to the plant. However, subterranean clover has been found, so far, to have among the shortest root hairs of the pasture legumes (Chapter 5) and did not increase its root hair length in low P soil (Chapters 7 and 8).

9.2.2 Materials and Methods

9.2.2.1 Methods for measuring root hair length

A range of methods for measuring root hair length were investigated initially. Seedlings were grown (approx. 5 days) in an artificially lit cabinet at 22^{o}C in various media to determine the most suitable media for measuring root hair lengths. Media were: petri dish with 1.4 g/L thiram in the dark; sand watered with 20% strength nutrient solution; low-P, acid soil (Colwell-extractable P ~9 mg/kg; pH$CaCl_2$ 3.9, Al ~25% of CEC); low-P, acid soil+1 g lime/kg (pH $CaCl_2$ 5.3); and low-P acid soil+1.5 g lime/kg (pH 6.1).

Plant materials used were subterranean clover cultivars: Leura, Mt Barker, Woogenellup, Riverina and Goulburn. Alternative legumes: biserrula (cv Mauro) and *Lotus corniculatus* (cv Granger) and phalaris (cv Holdfast GT). For convenience, whole seedlings were stored in 50% ethanol prior to measurements.

Root hairs were measured in the zone in which they were fully elongated (i.e. distal from the root tip, Fig. 9.1a) using a compound microscope, according to the method of Haling *et al*. (2010). Root hairs grown in the different growth media were not always uniformly distributed on the roots. For example, root hairs on petri dishes were often missing from sides of the root in contact with the filter paper in the base of the dish (Fig. 9.1b). As this occurred occasionally in all media, the longest (fully expanded) root hairs were always targeted for measurements. The length of ten root hairs judged to be at right angles to the field of vision were measured on each replicate. The co-efficient of variation (COV) for root hair length on an individual root is often quite large and was found to be characteristic of root hairs on any particular root. The COV for root hair lengths on replicate roots were usually similar. It was, consequently, essential to measure root hair lengths on replicate roots. The COV for the mean of the replicates was similar to that expected of other plant traits.

A typical measurement regime adopted for the work was to measure the length of ten randomly-selected root hairs (with orientation at right angles to the field of vision) on at least three roots of each replicate plant.

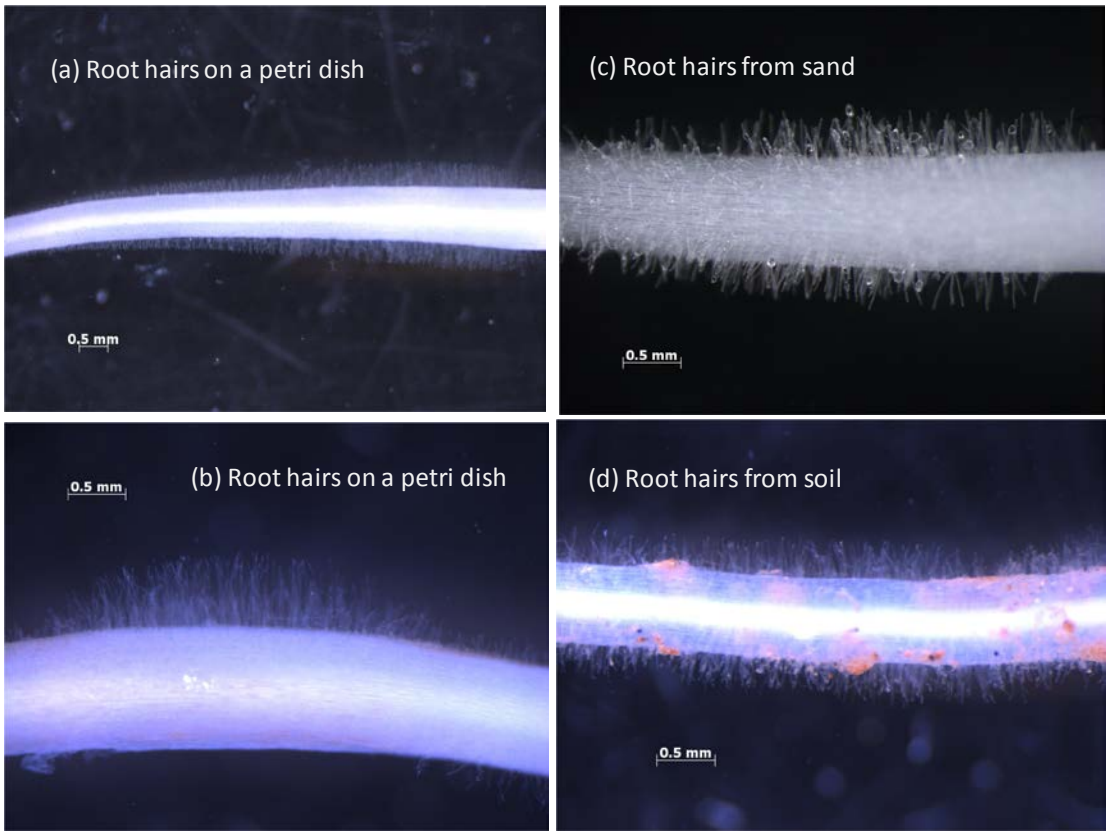


Figure 9.1 Some examples of root hairs observed after growth in different media. Note – root hairs from sand often appeared to have mucilage deposits on them. This had to be removed prior to length measurements by washing with 50% ethanol.

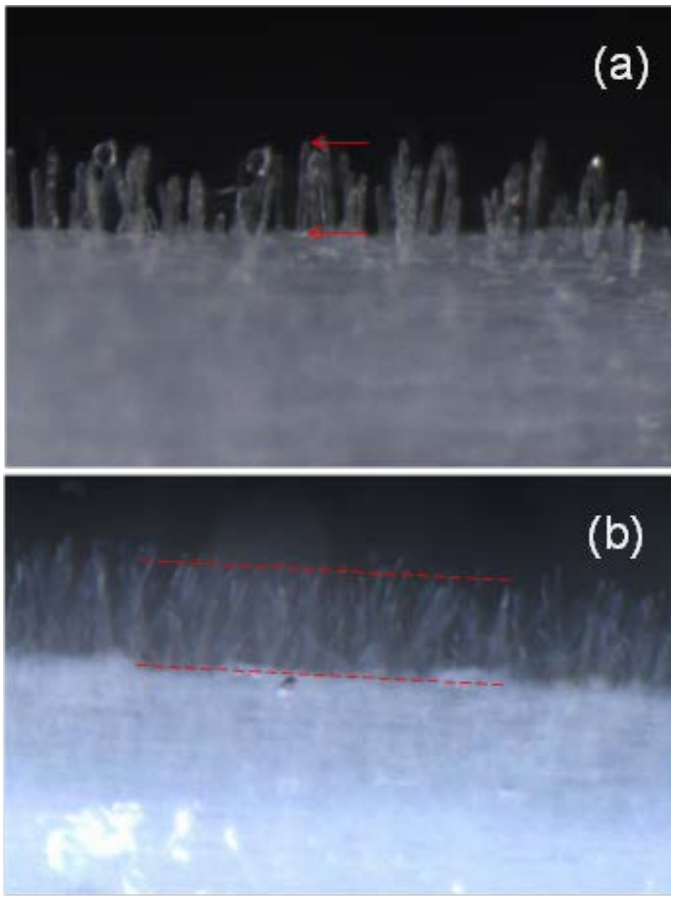


Figure 9.2 Measurement of root hair length as:
(a) individual root hairs, or (b) an estimate derived from the dimensions of the root hair cylinder

9.2.2.2 Root hair length of core and reference collections of subterranean clover

Root hair length of the reference and core collections of subterranean clover were screened on 1-week old seedlings grown in a low-P soil (Colwell-extractable P ~5-9 mg/kg) limed to pH$CaCl_2$ 5.3 (1 g lime kg^{-1} soil). A subset of cultivars in the reference collection (known to differ in root hair length) were also grown for 6 weeks to test whether the seedling-based screening method was a robust indicator of root hair length on older plants.

9.2.3 Results and Discussion

9.2.3.1 Methods for measuring root hair length

Root hair length achieved in soil was effectively a control treatment and it was hoped that there would be an alternative medium that would allow very rapid and easier screening of root hair length on large numbers of plants. Seedling growth on petri dishes in the dark was very easy and quick, provided precautions were taken to minimise microbial growth. Seedling growth in sand was very rapid and uniform.

Root hairs grown on petri dishes were generally longer than those grown in soil (one exception: biserrula) and were generally shorter when grown in the sand (two exceptions: phalaris, lotus) (Fig. 9.3). Among the five subterranean clovers, root hair lengths grown in all

media were correlated. Measurements of root hairs using ten individual root hairs (a long and tedious technique) and an estimate of the dimensions of the root hair cylinder (a relatively rapid and easy approach) were found to be highly correlated (Fig. 9.4). It was concluded that future screening would be best undertaken on petri dishes (using black filter papers + thiram solution), and in soil limed to pH 5.3 – these conditions allowed for the largest differentiation in root hair lengths among cultivars of subterranean cultivars. Root hair length could be reliably estimated in subterranean clover using the dimensions of the root hair cylinder.

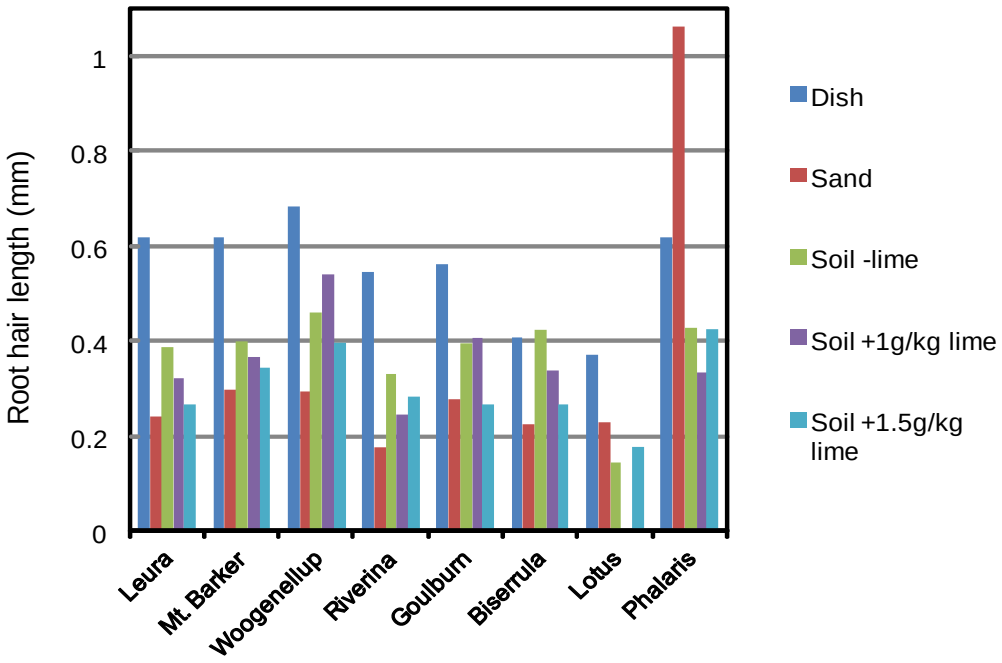


Figure 9.3 Root hair length of five subterranean clover cultivars and three other species grown in five different media.

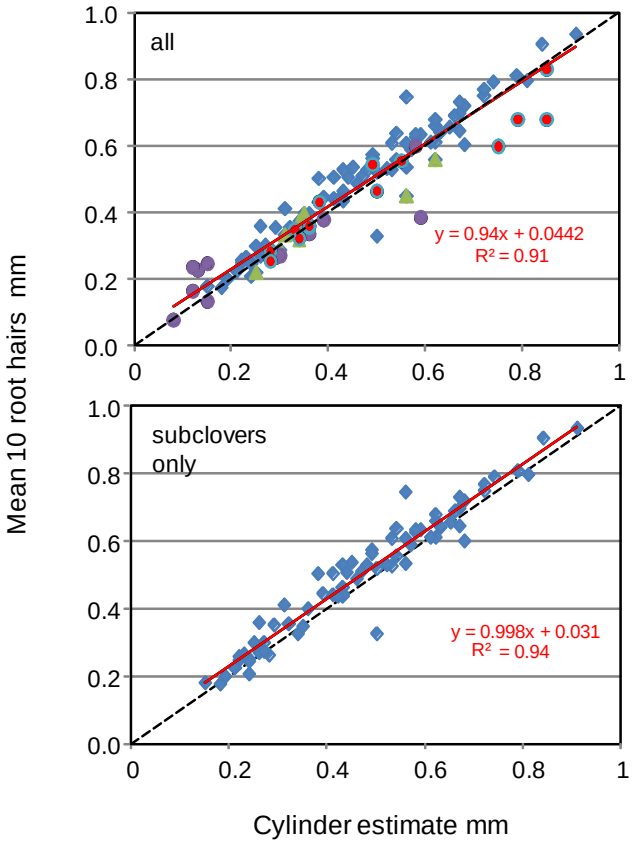


Figure 9.4 Correlation between root hair length measured as an estimate of the dimensions of the root hair cylinder with that as the mean of ten individual root hairs (see Fig. 2): (a) all species and (b) subterranean clover only.

9.2.3.1.1 Root hair length in the 'reference' and 'core' collections of subterranean clover

The results revealed significant intra-specific variation in root hair length in subterranean clover. In the reference collection, root hair length measured on 1-week old seedlings ranged from 0.18 mm to 0.40 mm (Fig. 9.5). The range in the core collection was essentially the same as that observed for the reference collection: 0.21 to 0.43 mm (Fig. 9.6). Root hairs on 1-week old seedlings were longer, but highly correlated to that measured on 6-week old plants (Fig. 9.7). This provides confidence in the value of screening 1-week old plants to provide an assessment of the relative variation in the root hair length of subterranean clover.

9.3 Specific root length and average root diameter

9.3.1 Background

High specific root length (SRL: root length per unit root mass) is an important trait for P acquisition efficiency and is also a trait known in many plant species to respond to P supply. It allows plants to increase the surface area of the root system per unit of dry matter allocation.

9.3.2 Materials and Methods

9.3.2.1 Specific root length in the 'reference' and 'core' collections of subterranean clover

Specific root length and average root diameter of the reference collection was measured in two separate experiments.

In the first experiment (conducted at CSIRO), the core and reference collections were grown in root boxes (Fig. 8) in a controlled-environment cabinet for approximately 3 weeks. Plants were grown in a thin (5 mm) uniform layer of soil (Colwell P = 10 mg/kg) against a Perspex sheet. The boxes were inclined at 30° from the vertical to encourage roots to grow against the Perspex – this was used to allow measurement of lateral root angles (reported later). Both the core and reference collections were grown in a low soil P concentration treatment (Colwell P = 10 mg/kg). The reference collection was also grown in a high soil P concentration (Colwell P = 28 mg/kg) soil. In the second experiment (conducted at UWA), the core and reference collections were grown in pots of a very P-deficient soil in a glasshouse for 6 weeks. Roots were scanned using a flatbed scanner and root length and average root diameter analysed using WinRHIZO. Roots were then dried at 70°C and dry mass determined. Specific root length was calculated as root length divided by root dry mass. For the reference collection grown in the first experiment, roots were washed from the soil and stored at 4°C in 50% ethanol prior to analysis. For the core collection grown in the first experiment, and both collections in the second experiment, roots were washed from soil and immediately analysed and dried.

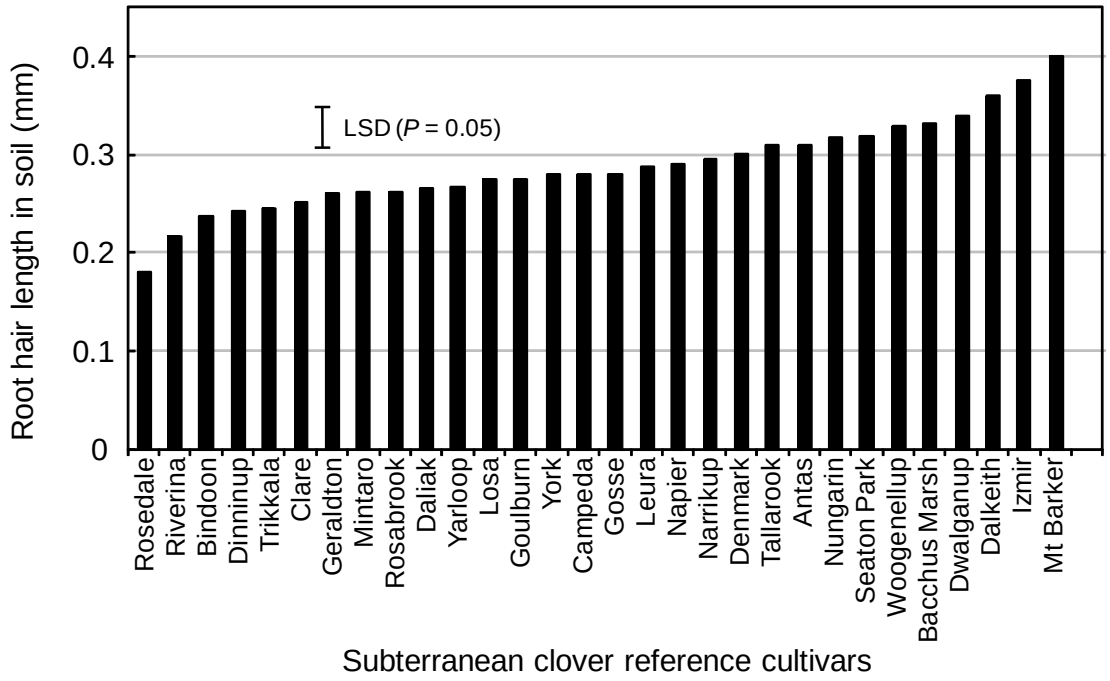


Figure 9.5 Average length of root hairs on the cultivars of subterranean clover in the “reference” collection.

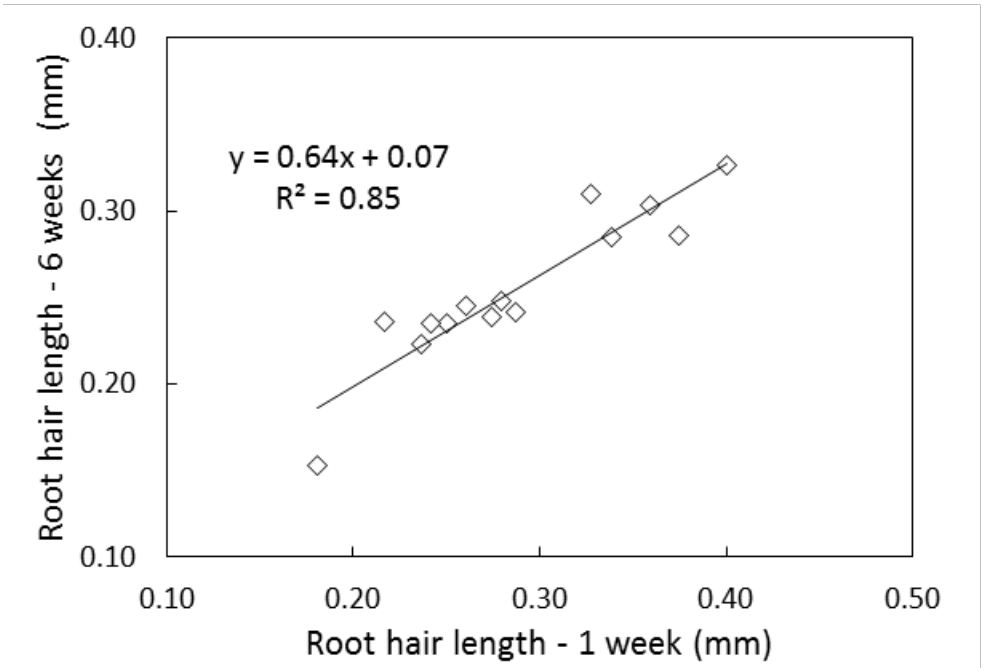


Figure 9.7 Correlation between root hair length measured on 1 week old and 6 week old plants of subterranean clover. Plants are a subset of cultivars from the reference collection.

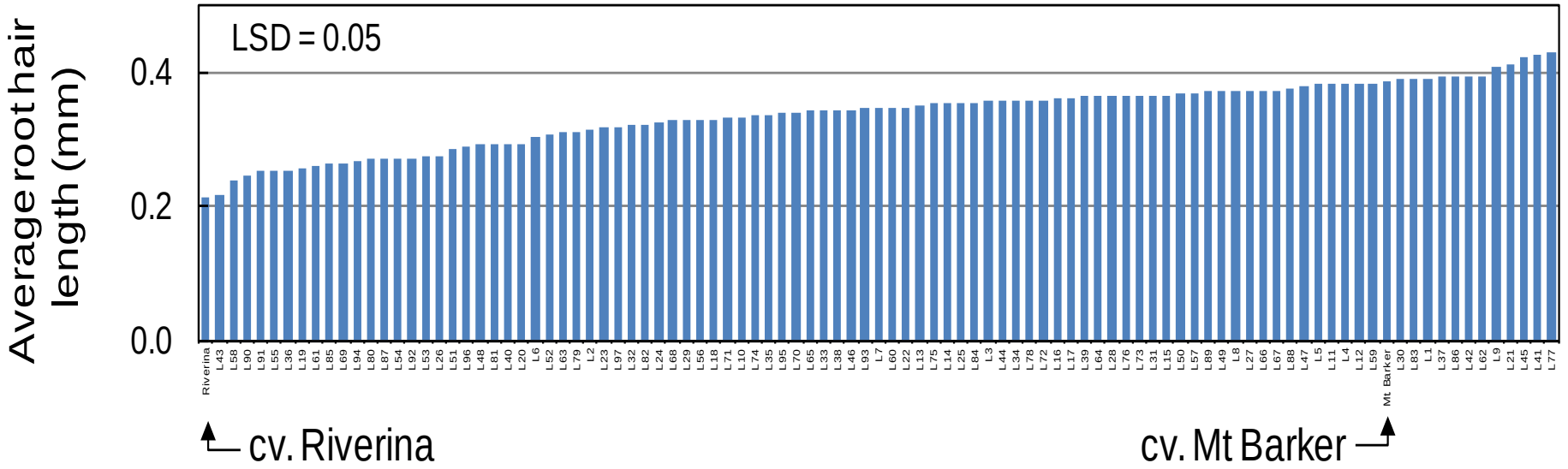


Figure 9.6 Average length of root hairs on the subterranean clovers in the “core” collection which represent 85% of the variation in the subterranean clover genome. Two control cultivars (Riverina and Mt Barker) were included in the assessment so that the data may be related to the previous assessment of root hair length in the “reference” collection of subterranean clover cultivars.

9.3.2.2 Effect of storing roots in ethanol on dry mass

It is often necessary in this work to store roots in 50% ethanol solutions until they can be scanned and analysed for length, diameter and branching using WinRhizo software. However, storage in ethanol results in loss of root dry mass which is most probably due to loss of cell contents (sugars, amino acids, etc.). Eight cultivars of subterranean clover were selected to represent a range of specific root lengths and to represent the three subspecies of *Trifolium subterraneum*. The clovers were grown at three soil P levels (Colwell P = 6, 12 and 31 mg/kg soil) in root boxes in a controlled-environment cabinet. The impact of root storage in an ethanol solution was examined by comparing the dry weight of roots freshly harvested from soil after 3 weeks growth, with the dry weight of similar roots after they had also been stored for 34 days in 50% ethanol at 4^{o}C.

9.3.3 Results and Discussion

Specific root length of the reference collection of subterranean clover ranged from 54-103 m g^{-1} (stored in ethanol; Fig. 9) and 85-175 m g^{-1} (Fig. 9.10), in the respective experiments conducted at CSIRO (root box) and UWA (pot experiment). Differences

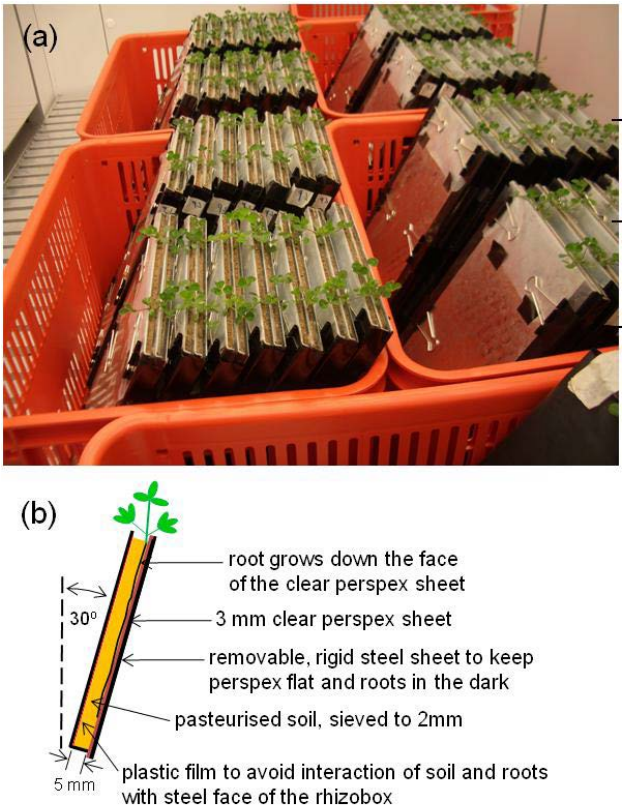


Figure 9.8 (a) Subterranean clover growing in root boxes in a controlled-environment plant growth cabinet; (b) schematic diagram of a root box.

in the absolute measurements of specific root length are likely a consequence of differences in the proportions of tap and lateral roots in root systems of different age/ size. Nevertheless, a significant, two-fold range in specific root length was found within the reference collection in both experiments. In both experiments, specific root length was highly correlated to average root diameter (Figs 9.12 and 9.13). A significant correlation (R^2=0.41) was also found between the measurements of specific root length between the two independent experiments (Fig. 11a). However,

comparison of the measurements of average root diameter in the two independent experiments indicated that it might be a better trait to select if aiming to increase specific root length because the correlation for average root diameter measurements in the two experimental systems was higher (R^2=0.55) (Fig. 9.11b).

Specific root length of the core collection ranged from 36-105 m g^{-1} (Fig. 9.14) and 86-161 m g^{-1} (Fig. 9.15), in the respective experiments conducted at CSIRO (root box) and UWA (pot experiment). For the plants grown in the root box experiment at CSIRO, this 3-fold range was larger than that measured in the same core collection in a pot experiment conducted at UWA, and larger than the range measured in both assessments of the reference collection (see above). However, the larger range is due mainly to three lines with relatively low specific root length (Fig. 9.14). These lines are unlikely to be of benefit for improving P acquisition efficiency of subterranean clover because higher rather than lower specific root length values are required to improve root foraging. In all experiments, specific root length was highly correlated to average root diameter (Figs 9.11, 9.12 and 9.18). Significant correlations were also found for the measurements of specific root length (R^2=0.37) and average root diameter (R^2=0.41) when comparing independent experiments conducted under plant growth dissimilar conditions (Figs 9.13 and 9.19).

As reported above, the variation in specific root length was correlated with differences in average root diameter. In some plant species, specific root length can also vary as a result of variation in root tissue density. Aerenchyma formation (air spaces in the root tissue) in roots reduces tissue density and is reported to be an effective way for plants (e.g. maize; Lynch 2007, Richardson *et al*. 2011) to lower the costs of root proliferation. Only a limited examination of aerenchyma in roots of two cultivars grown in low P soil at UWA has been possible (data not shown). Aerenchyma were only observed occasionally leaving open the question of whether arenchyma are an important variable in specific root length variation in subterranean clover.

The mean multiplier needed to correct root dry mass after storage in 50% ethanol, to that of freshly-harvested roots, was 1.40 ± 0.06 (mean ± se) with only one cultivar differing significantly from this (cv. Riverina CF= 1.26) (Fig. 9.20). There was no evidence that soil P fertility or subterranean clover subspecies had any impact on the correction factor and it was concluded that even for cv. Riverina, the error caused by using the average correction factor would be small. The average correction factor for subterranean clover roots is the same as the correction factor reported for white clover roots stored in a 70% ethanol solution (Crush *et al*. 2010). This correction factor only applies for values of specific root length reported for the reference collection grown in the root box experiment (CSIRO). All other measurements of specific root length were based on root samples that were immediately scanned for analysis of root length and dried.

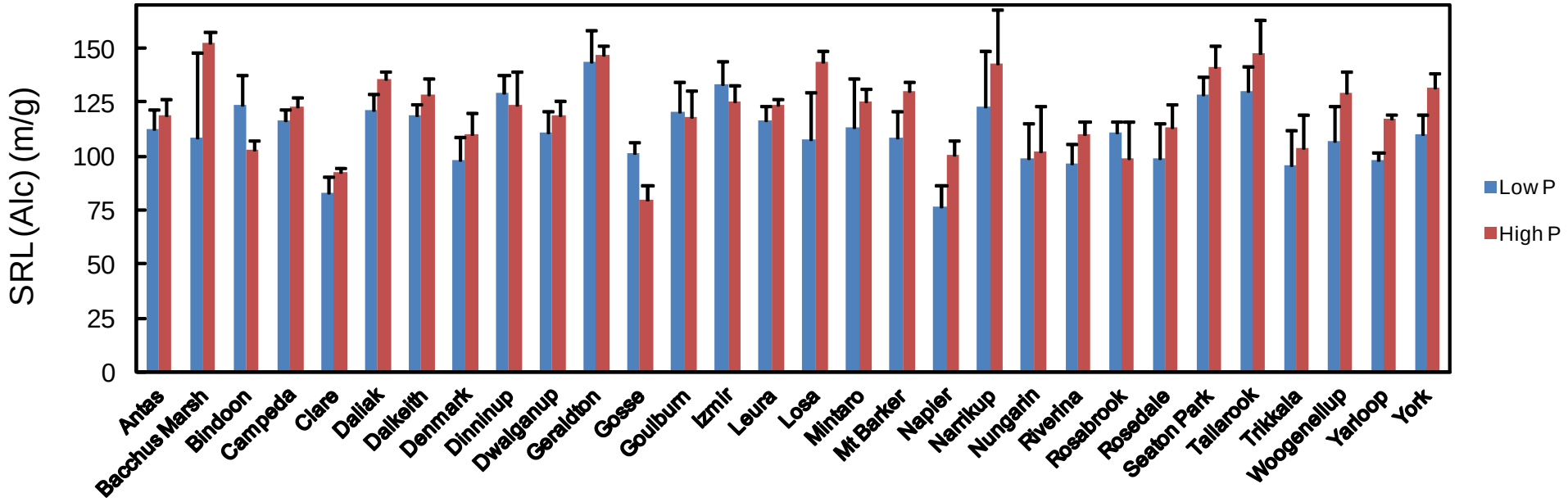


Figure 9.9 Specific root length (root length per unit of structural root mass (ex 50% ethanol solution) for 30 cultivars of subterranean clover grown in root boxes with thin (5 mm) layers of uniform soil at a low soil P concentration (Colwell P = 10 mg/kg) or a high soil P concentration (Colwell P = 28 mg/kg) soil. It has been estimated that values need to be divided by 1.4 to correct for loss of mass associated with storage in ethanol.

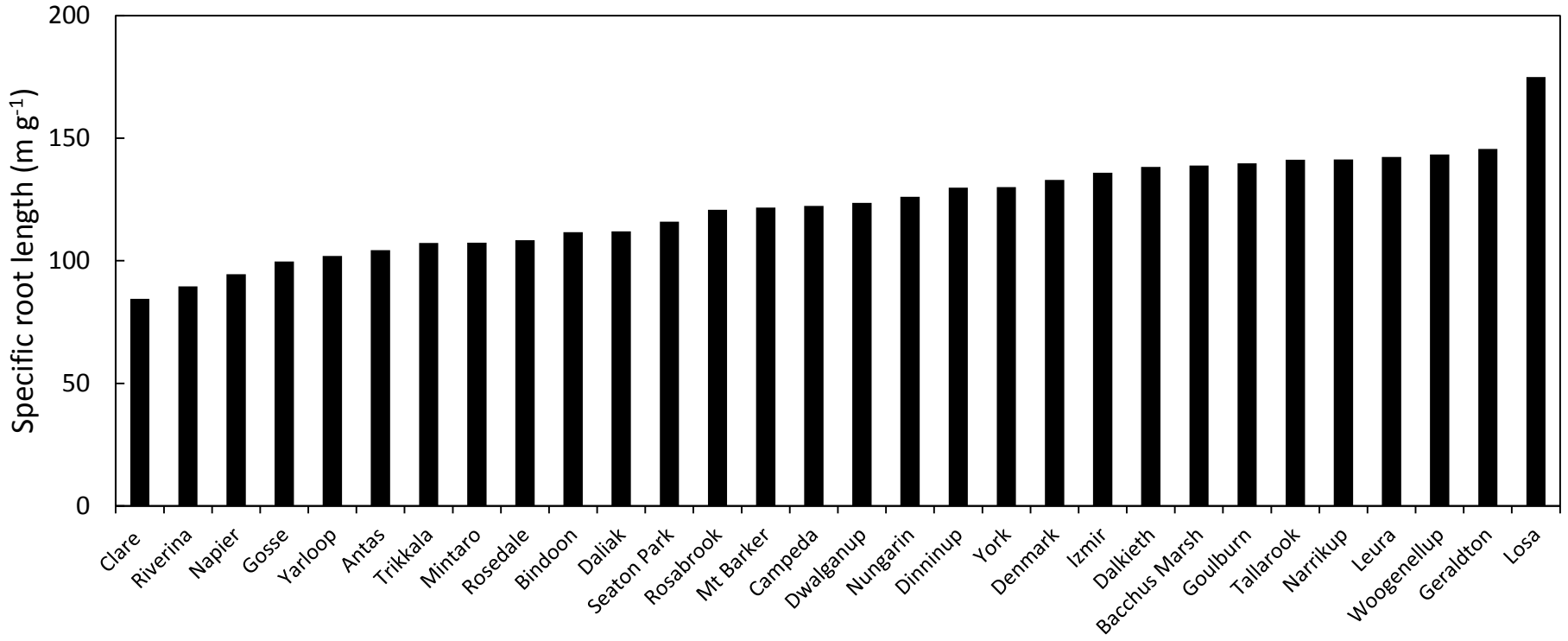


Figure 9.10 Specific root length (root length per unit of structural root mass) for cultivars of subterranean clover grown for 6 weeks in pots of very P-deficient soil in a glasshouse. LSD = 15.4 (P<0.05).

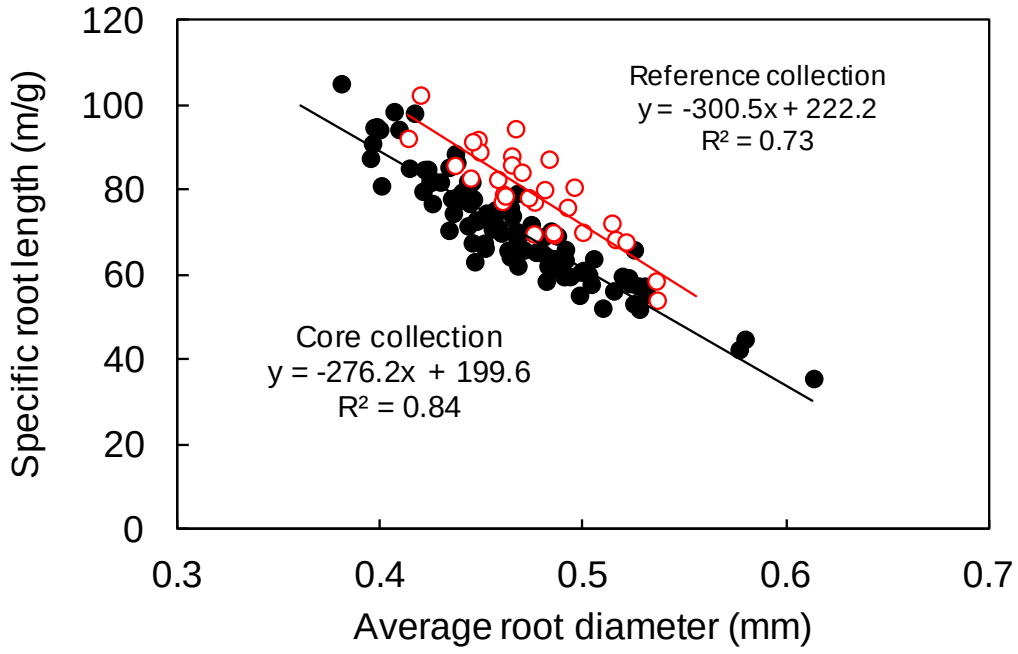


Figure 9.11 Relationship between the average diameter of roots and specific root length of the whole root system for the core collection (closed circles) and, for comparison, the reference collection (open circles). Each collection of subterranean clover lines were grown for 3 weeks in a root box in a controlled-environment cabinet. Each collection was grown in a separate experiment with some difference in growth conditions. The parallel regression relationships recorded for the core and the reference collections are believed to be due to differences in the light conditions of the two experiments and not to be a reflection of genotypic differences. Both experiments were conducted in a controlled environment, plant-growth cabinet under identical conditions except for light levels which were increased from 310-350 (reference collection experiment) to 680-720 µmoles quanta (visible light)/m^2/s (core collection experiment) at plant height.

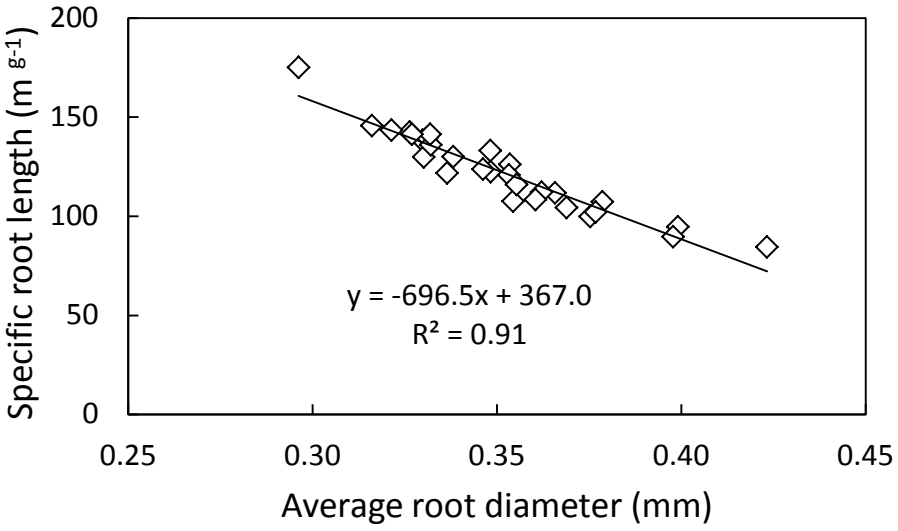


Figure 9.12 Relationship between the average diameter of roots and specific root length of the whole root system for the reference collection as measured on 6 week old plants grown in pots of very P-deficient soil.

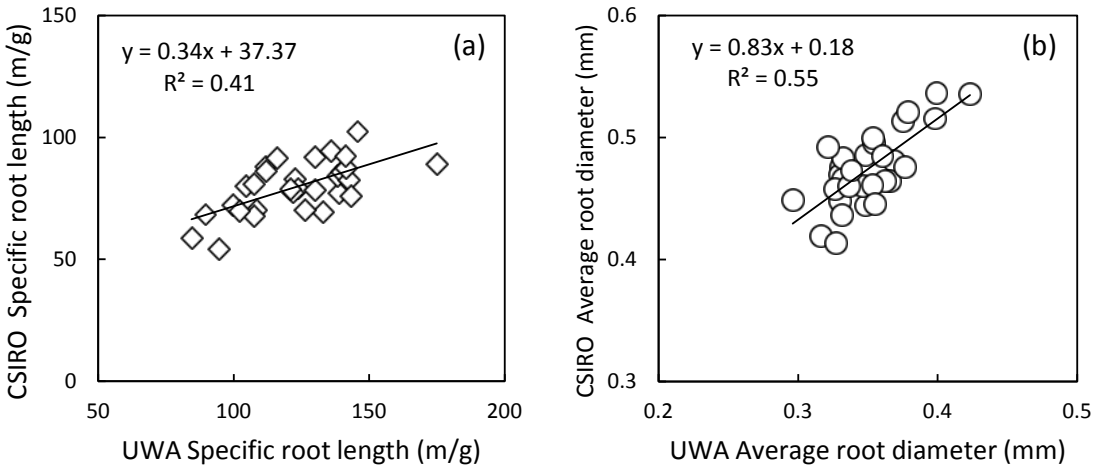


Figure 9.13 Correlations for (a) specific root length and (b) average root diameter measured in independent screens of 30 cultivars of subterranean clover at UWA and CSIRO. Measurements are of plants grown for 6 weeks in pots in a glasshouse (UWA) or 3 weeks in a root box in a controlled-environment, plant-growth cabinet (CSIRO). Variation in absolute measures of specific root length and average root diameter is likely to reflect the effects of different experimental conditions, plant age, etc.

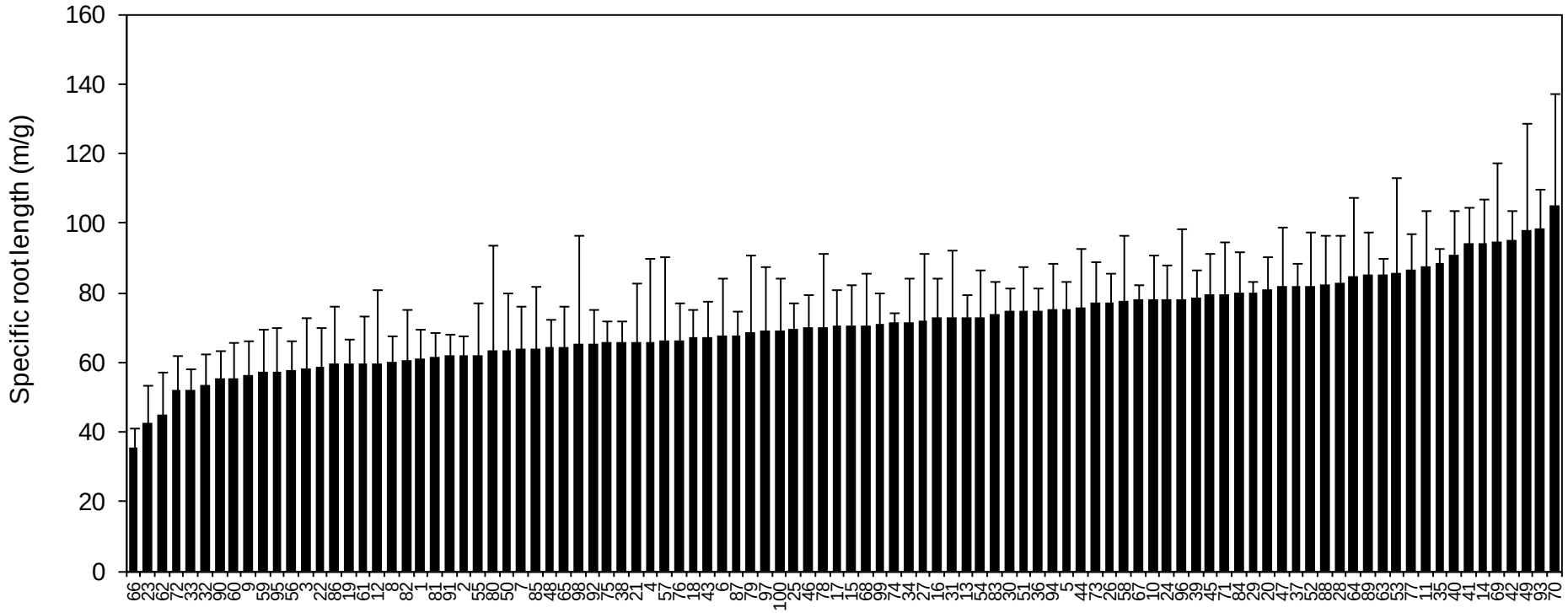


Figure 9.14 Specific root length of 96 lines and 4 cultivars of subterranean clover measured on roots of plants grown in root boxes with thin (5 mm) layers of uniform soil at a low soil P concentration (Colwell P = 10 mg/kg). Presented in order of increasing specific root length. The cultivar controls included in this experiment were: Coolamon (line 96), Urana (line 97), Riverina (line 98), Woogenellup (line 99), and Daliak (line 100). Bars = 1xSD.

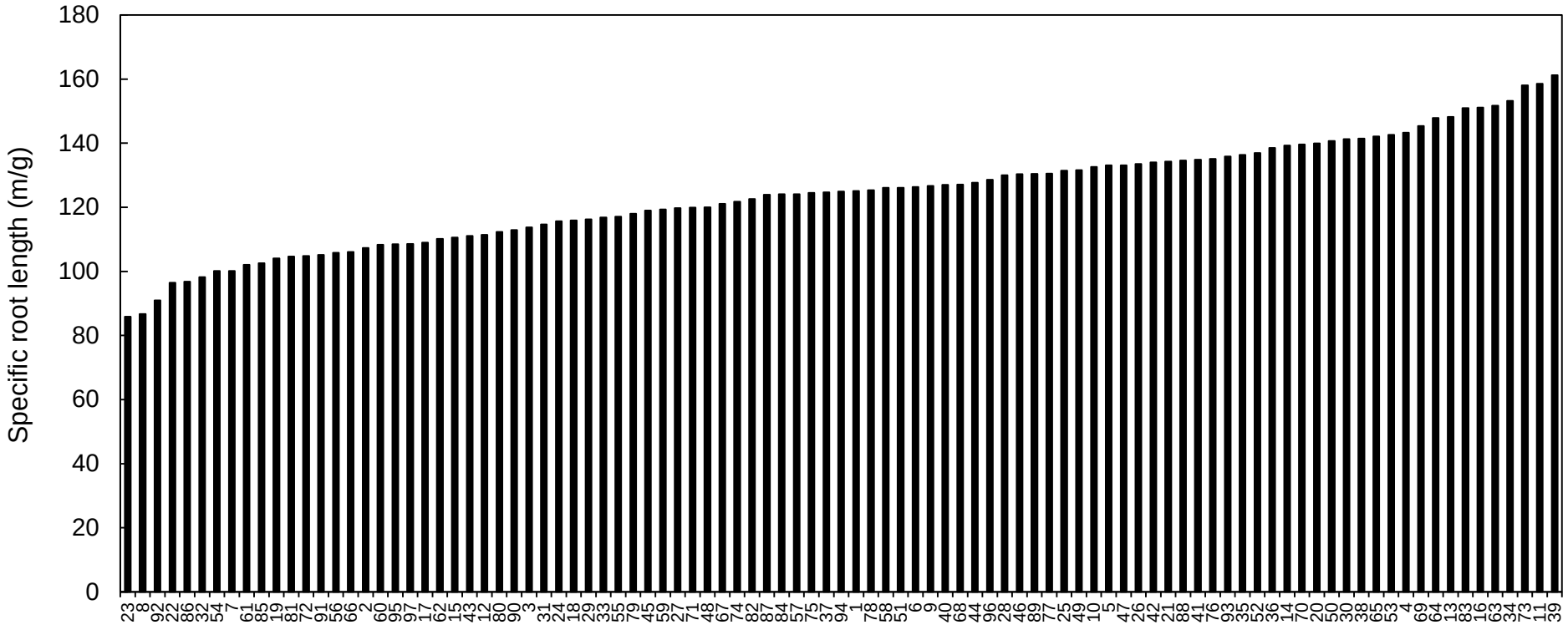


Figure 9.15 Specific root length of 96 lines of subterranean clover measured on roots of plants grown in a glasshouse in pots of P-deficient soil for 6 weeks. Presented in order of increasing specific root length. The cultivar controls included in this experiment were: Coolamon (line 96) and Urana (line 97). LSD is 18.7 (P<0.05).

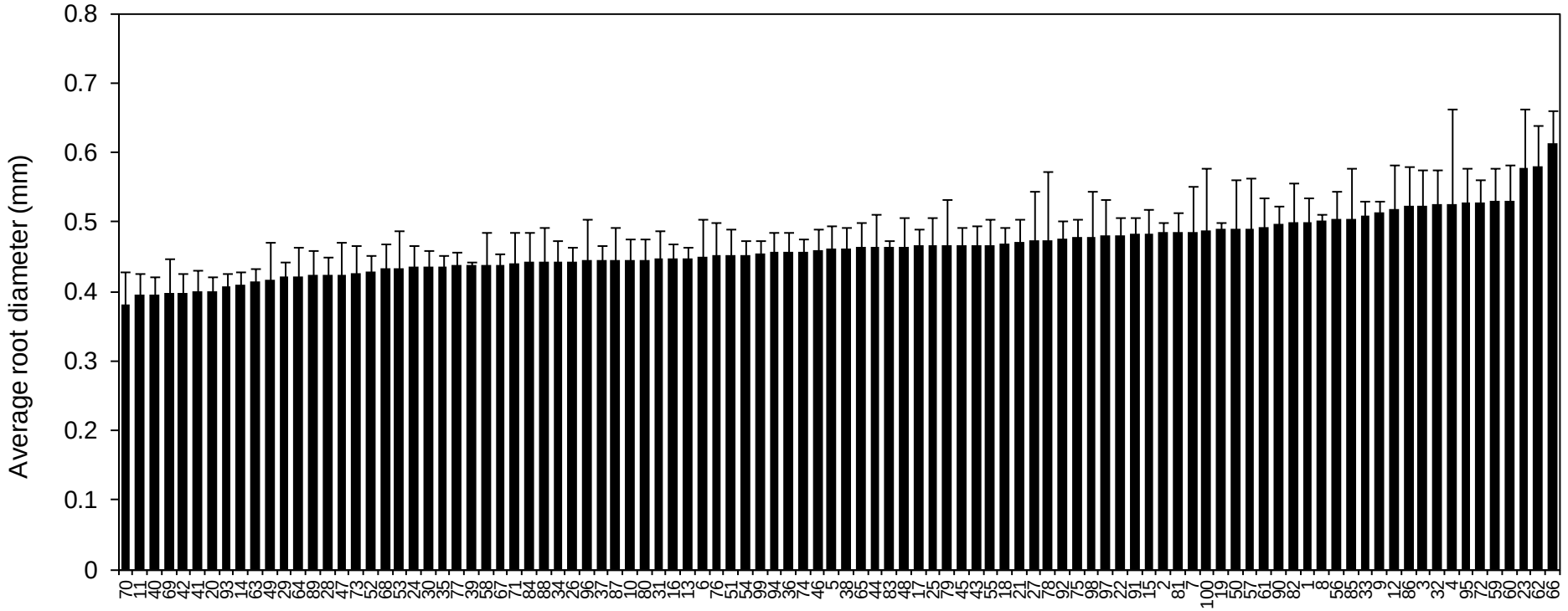


Figure 9.16 Average root diameter of 96 lines and 4 cultivars of subterranean clover measured on roots of plants grown in root boxes with thin (5 mm) layers of uniform soil at a low soil P concentration (Colwell P = 10 mg/kg). Presented in order of increasing average root diameter. The cultivar controls included in this experiment were: Coolamon (line 96), Urana (line 97), Riverina (line 98), Woogenellup (line 99), and Daliak (line 100). Bars = 1xSD.

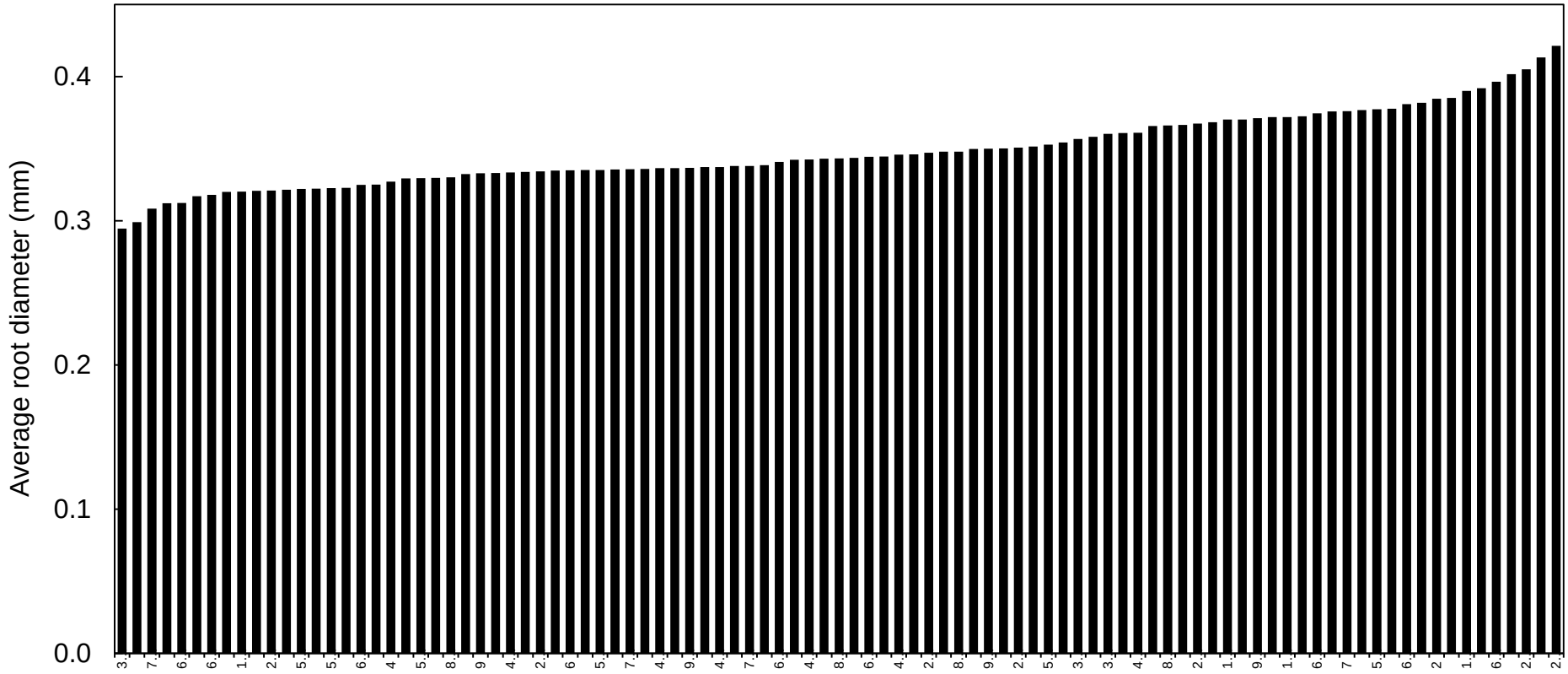


Figure 9.17 Average root diameter of 96 lines of subterranean clover measured on roots of plants grown in a glasshouse in pots of P-deficient soil for 6 weeks. Presented in order of increasing average root diameter. The cultivar controls included in this experiment were: Coolamon (line 96) and Urana (line 97). LSD is 0.025 (*P*<0.05).

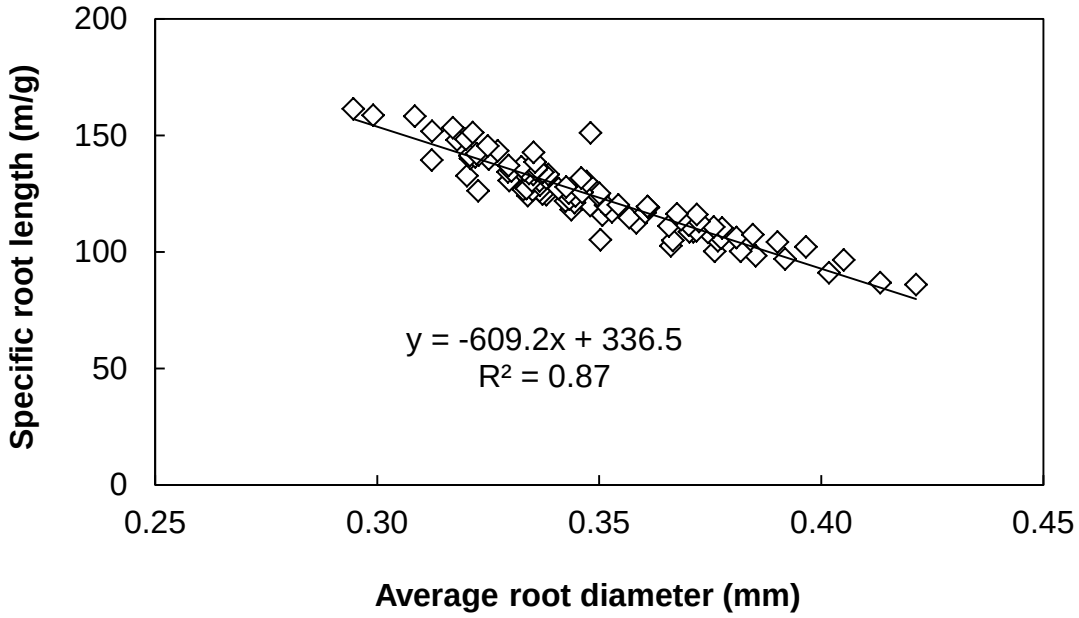


Figure 9.18 Relationship between the average diameter of roots and specific root length of the whole root system for the core collection as measured on 6 week old plants grown in pots of very P-deficient soil.

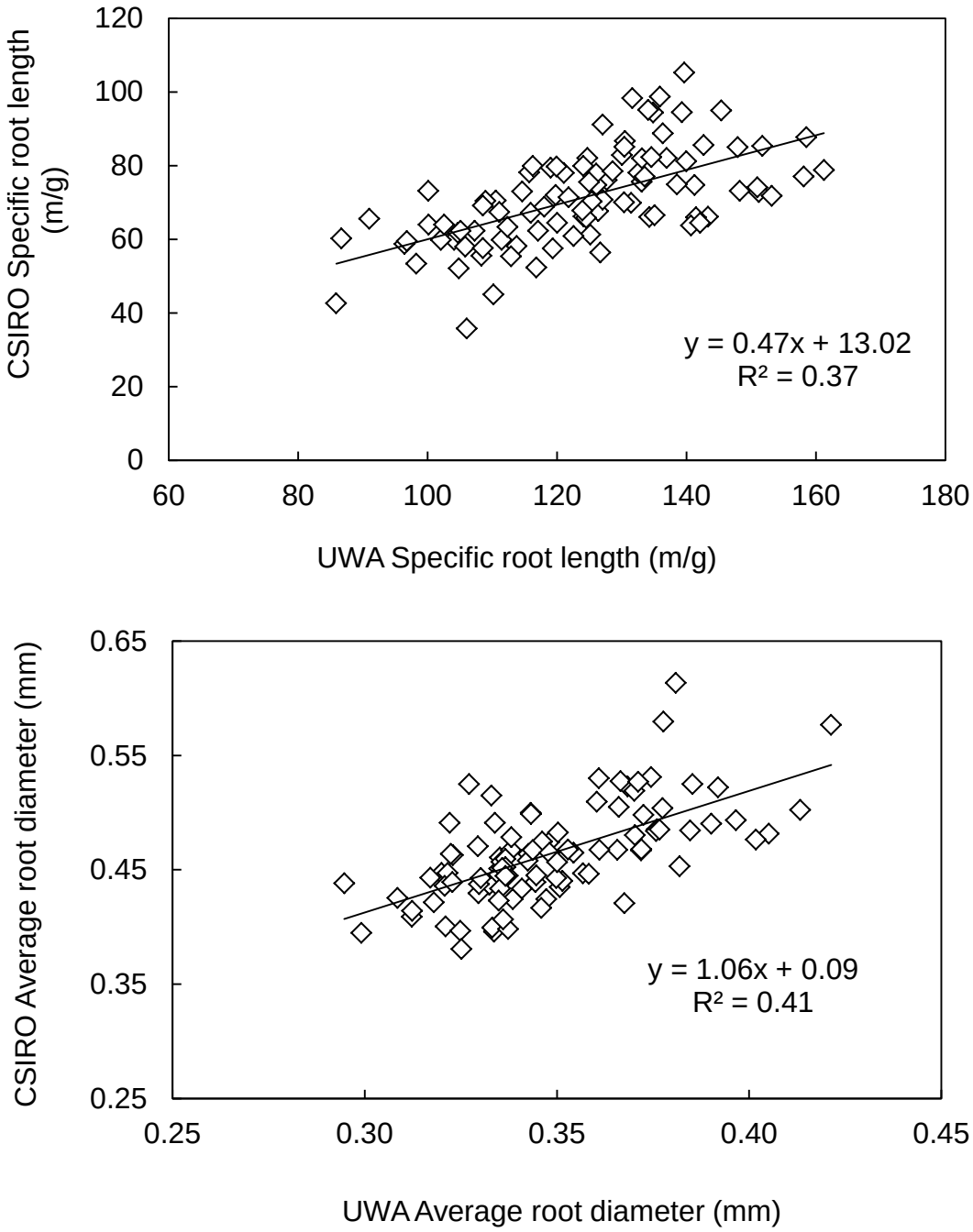


Figure 9.19 Correlations for (a) specific root length and (b) average root diameter measured in independent screens of a core collection of subterranean clover at UWA and CSIRO. Measurements are of plants grown for 6 weeks in pots in a glasshouse (UWA) or 3 weeks in a root box in a controlled-environment, plant-growth cabinet (CSIRO). Variation in absolute measures of specific root length and average root diameter are likely to reflect the effects of different experimental conditions, plant age, etc.

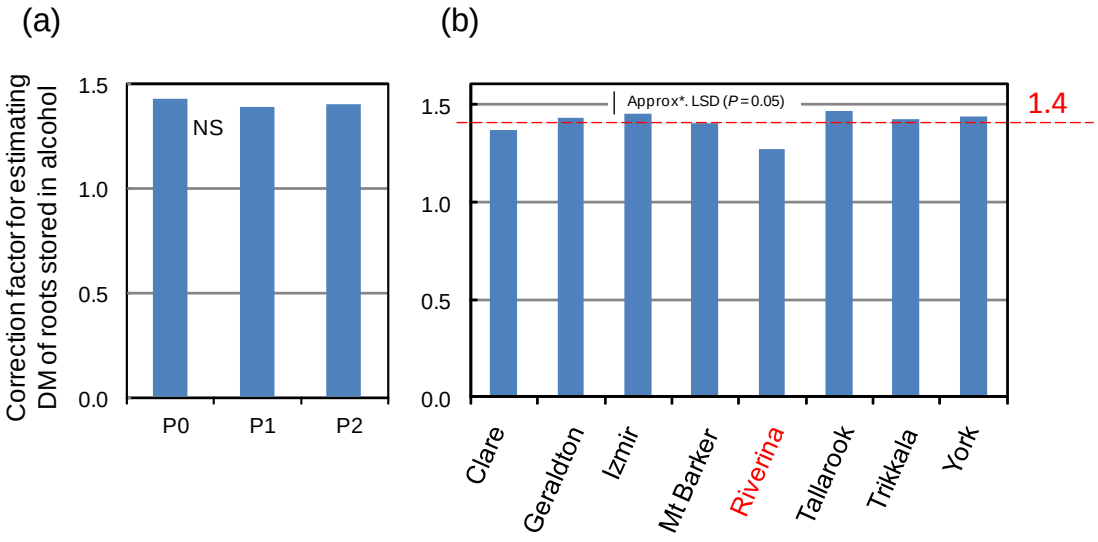


Figure 9.20 Dry mass "correction factors" (CF) calculated for roots after storage in a 50% ethanol solution, where: CF = $DM_{(freshly\ harvested\ roots)}/DM_{(roots\ after\ storage)}$. *Approx. LSD (*P* = 0.05) = 2xSE.

9.4 Lateral root angle

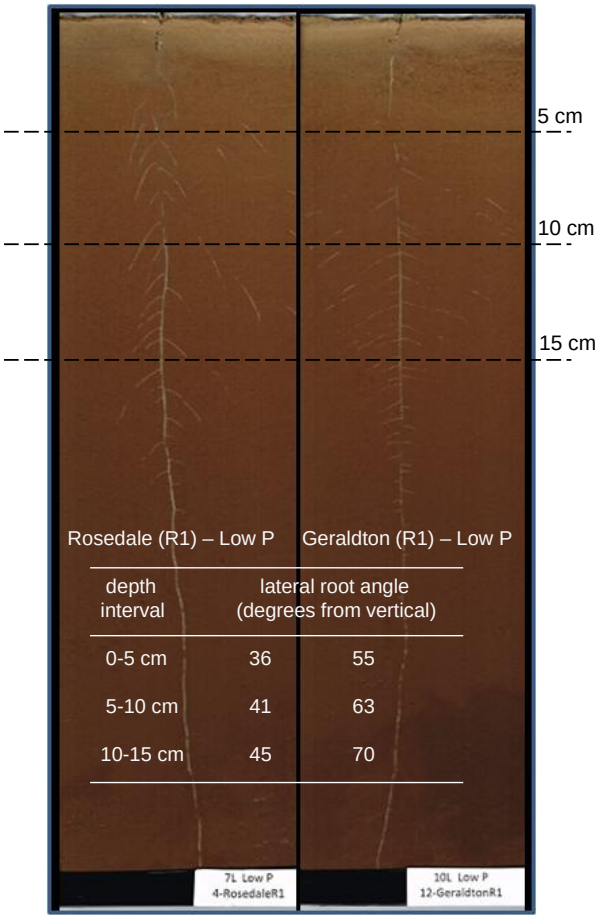
Root angles (i.e. degrees from vertical) that reflect shallow rooting are a potential indicator of the ability of plants to explore nutrient-rich topsoil layers and grow comparatively well in low P soils (Lynch 2007).

9.4.1 Materials and Methods

Root angles of the core and reference collections of subterranean clover were screened in root boxes as reported for specific root length. Briefly, plants were grown in a thin (5 mm) uniform layer of "low P" soil (Colwell P = 10 mg/kg) against a Perspex sheet. The boxes were inclined at 30° from the vertical to encourage roots to grow against the Perspex to allow measurement of lateral root angles. Both the core and reference collections were grown in a low soil P concentration treatment (Colwell P = 10 mg/kg). The reference collection was also grown in a high soil P concentration (Colwell P = 28 mg/kg) soil. Root boxes were scanned to capture images of the roots growing against the Perspex (Fig. 9.21). Lateral root angle was measured as the angle formed by the intersection of the lateral root with a point 1 cm from the tap root. Root angles of genotypes in the core collection were measured at four depth increments from the surface of the root box (0-5; 5-10; 10-15 and 15-20 cm). However, root angles in the top 10 cm of the soil profile are considered most likely to be relevant for P foraging because P is concentrated at the top of the soil profile under Australian pastures.

The core collection was screened in three batches of 120 boxes (which is the maximum capacity of the growth cabinet) to provide an adequate number of replicates. This included extra plantings of some lines when problems with seedling establishment

and growth were experienced. It should be remembered that the core collection is selected to represent the subterranean clover genome (Ghamkhar *et al*. 2008) and is unselected for any agronomic traits. Some lines suffered from poor establishment, poor vigour and/or appeared to be susceptible to disease or growth conditions that do not affect cultivars of subterranean clover. These issues inevitably caused some delays in screening the core collection lines and had the potential to complicate interpretation of the results if the issues were not recognised and repeat analyses performed.



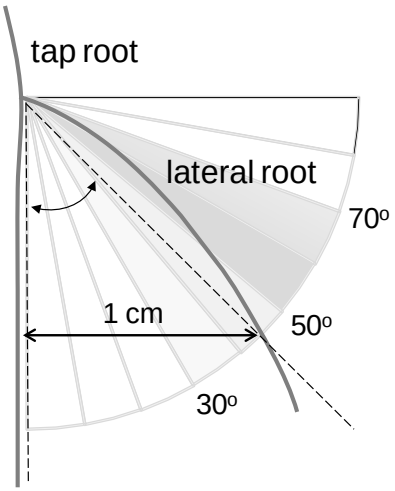


Figure 9.21 Growth of subterranean clover in root boxes to enable the visual assessment of root traits, particularly lateral root angles. Lateral root angles are measured as the angle formed by the intersection of the lateral root with a point 1 cm from the tap root as proposed by Bonser *et al*. (1996).

9.4.2 Results

Lateral root angle of the reference collection ranged from 39 to 66° and 39 to 71° at 0-5 cm, and 48 to 78 and 41 to 73° at 5-10 cm, when plants were grown in low P and high P soil, respectively (Fig 9.22). Lateral root angle of the core collection ranged from 41 to 68° at 0-5 cm and 54 to 68° at 5-10 cm depth, which was consistent with that of the reference collection (Figs 9.23 and 9.24). In both collections, lateral root angle measured at 0-5 cm was highly correlated with that measured at 5-10 cm and root angle was found to increase incrementally with depth (i.e. lateral roots, especially those lines with the lowest angles in the 0-5 cm soil layer, became more horizontal with depth) (Figs 9.24, 9.25 and 9.26). Data collected from the 0-5 and 5-10 cm layers were the strongest and were considered to give the most reliable and useful

assessment of variation in this trait (e.g. Fig. 9.22). This was in part because roots at depth were less visible and hence more difficult to measure. Plants were assessed when the main root axis reached the bottom of the box (~3 weeks growth). At this age, lateral roots deeper in the profile are still developing and there are usually fewer and smaller roots in the deeper soil layers.

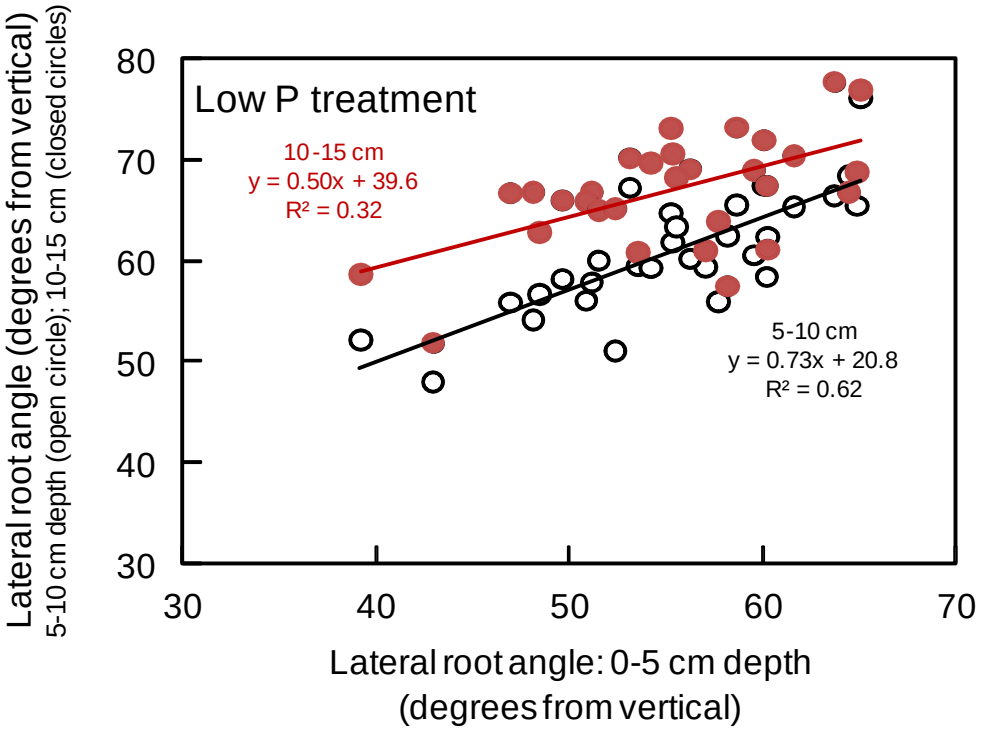


Figure 9.22 Relationships between the orientation of lateral roots in topsoil layer (0-5 cm depth) and that of lateral roots produced deeper on the taproot.(5-10 cm depth - open circles; 10-15 cm depth closed circles).

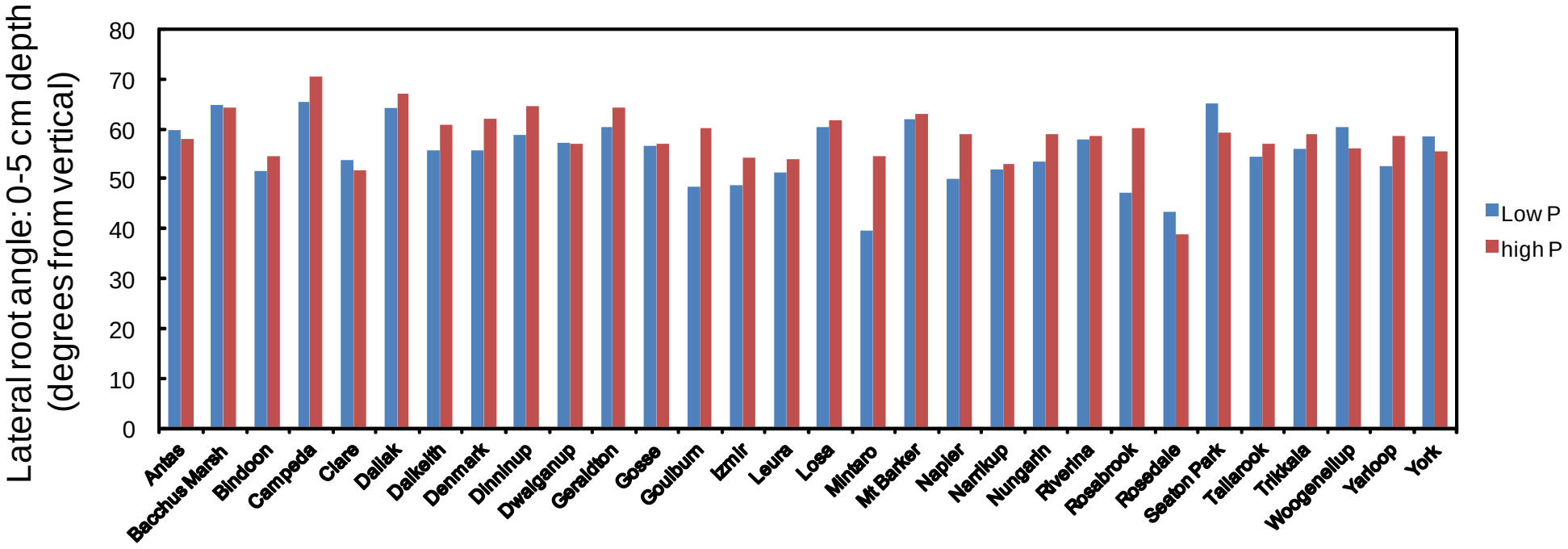


Figure 9.23 Lateral root angles of 30 cultivars of subterranean clover measured on roots (0-5 cm depth) of plants grown in root boxes with thin (5 mm) layers of uniform soil at a low soil P concentration (Colwell P = 10 mg/kg) or a high soil P concentration (Colwell P = 28 mg/kg) soil.

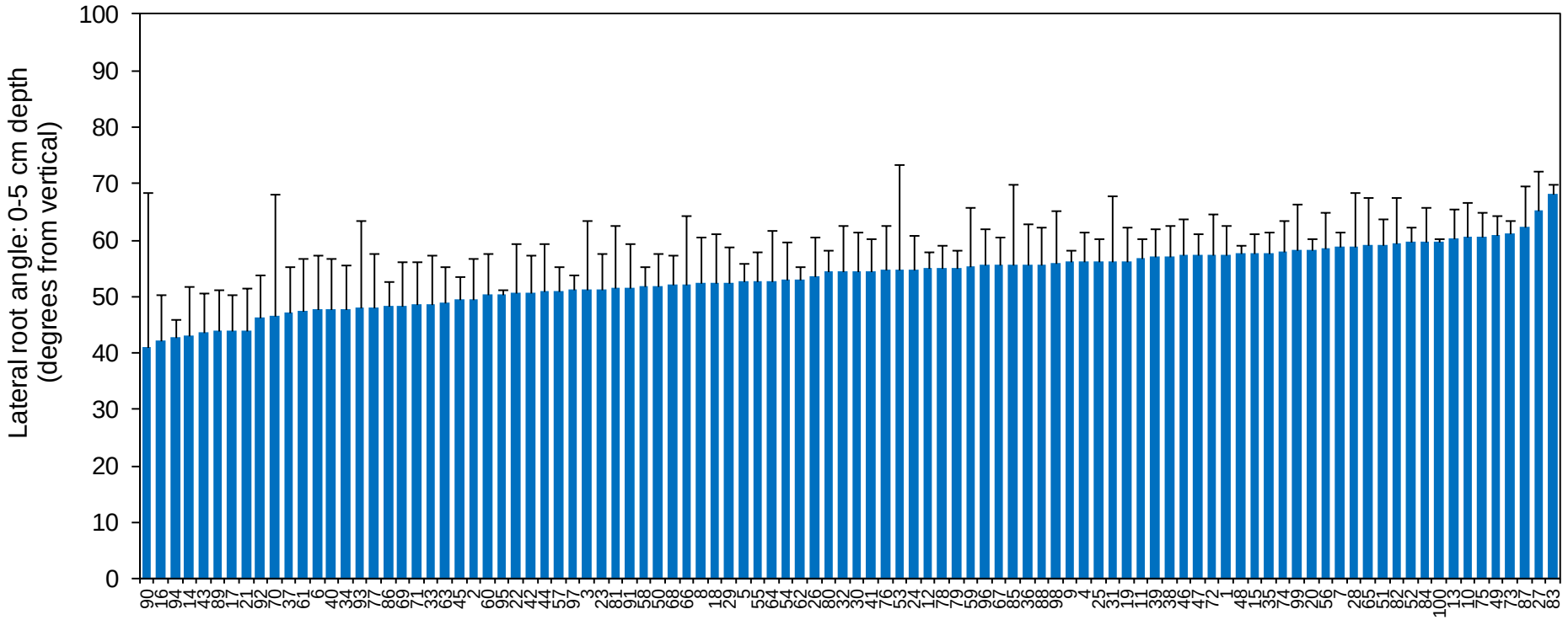


Figure 9.24 Lateral root angles of 96 lines and 4 cultivars of subterranean clover measured on roots (0-5 cm depth) of plants grown in root boxes with thin (5 mm) layers of uniform soil at a low soil P concentration (Colwell P = 10 mg/kg). Presented in order of increasing lateral root angle for 0-5 cm depth. The cultivar controls included in this experiment were: Coolamon (line 96), Urana (line 97), Riverina (line 98), Woogenellup (line 99), and Daliak (line 100). Bars = 1xSD.

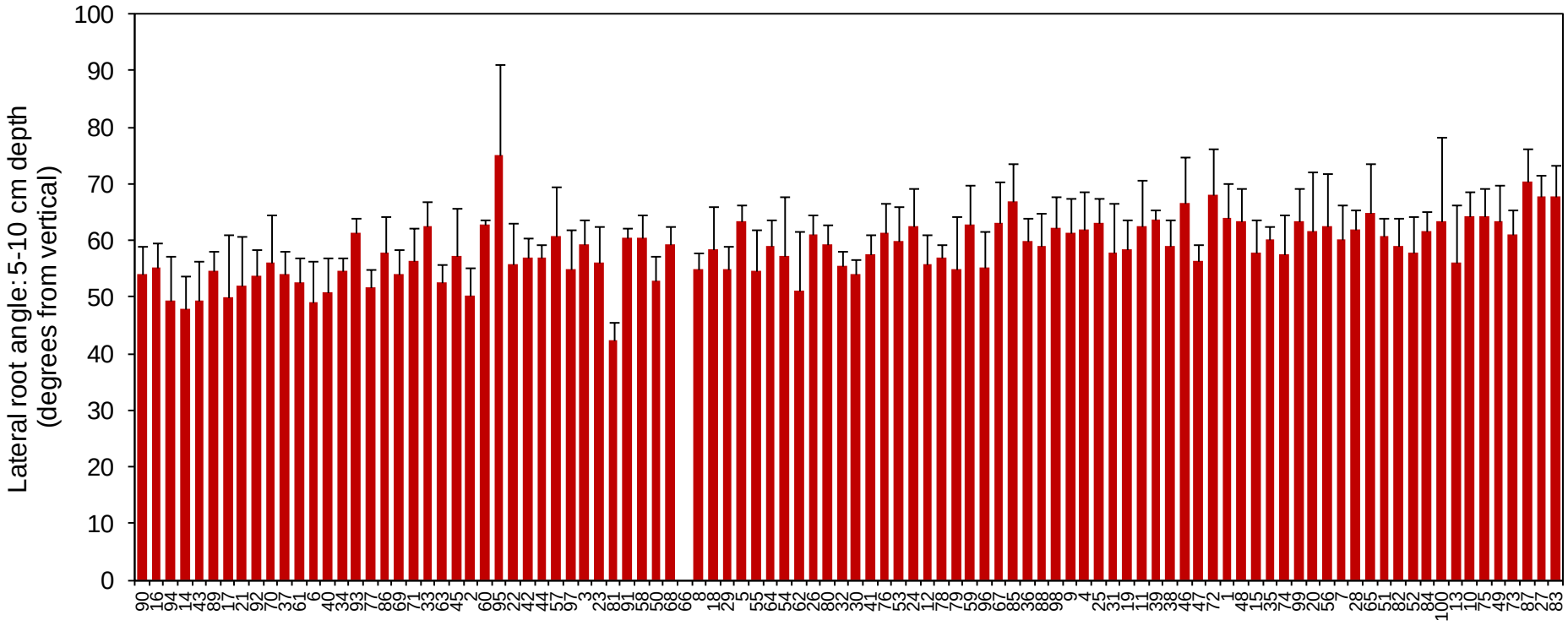


Figure 9.25 Lateral root angles of 96 lines and 4 cultivars of subterranean clover measured on roots (5-10 cm depth) of plants grown in root boxes with thin (5 mm) layers of uniform soil at a low soil P concentration (Colwell P = 10 mg/kg). Presented in order of increasing lateral root angle for 0-5 cm depth. "Missing" data for line 66 reflects very slow root growth by this line and, consequently, inadequate numbers of lateral roots in the 5-10 cm soil layer. The cultivar controls included in this experiment were: Coolamon (line 96), Urana (line 97), Riverina (line 98), Woogenellup (line 99), and Daliak (line 100). Bars = 1xSD.

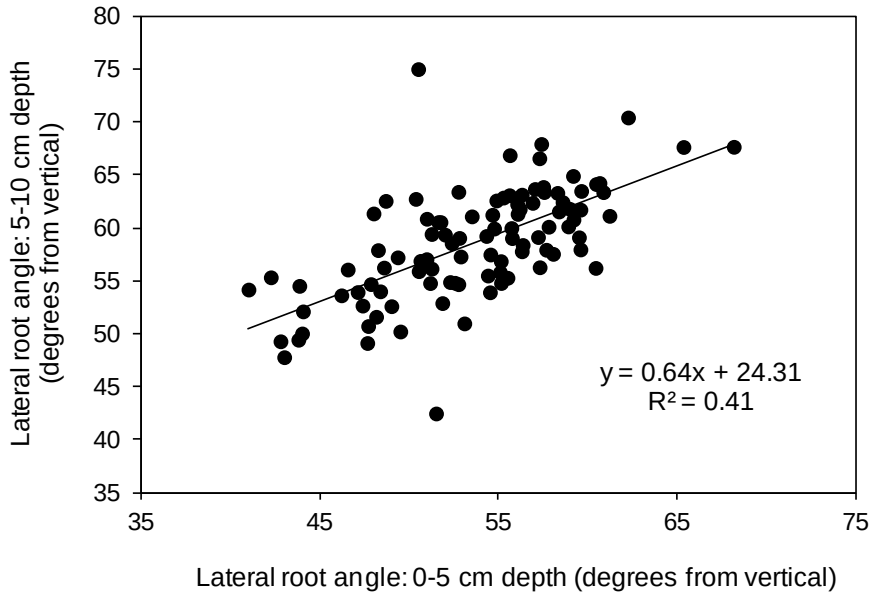


Figure 9.26 Relationship between the orientation of lateral roots in topsoil layer (0-5 cm depth) and that of lateral roots produced deeper on the taproot (5-10 cm depth) for subterranean clover lines from the core collection.

9.5 Root mass fraction

9.5.1 Materials and Methods

Root mass fraction (root mass expressed as a proportion of total plant mass) was assessed on the reference and core collections of subterranean clover in experiments as outlined for specific root length i.e. root boxes (CSIRO) and pot experiments (UWA).

9.5.2 Results

Root mass fraction of the reference collection of subterranean clover cultivars ranged by 1.5-fold from ~0.24-0.40 when measured in root boxes (Fig. 9.27) and ranged 1.7-fold from 0.32 to 0.55 when measured in pots (Fig. 9.28). In the root box experiment, the root mass fraction of some cultivars varied significantly with soil P level. While it is normally expected that root mass fraction will increase when a plant is growing in P-deficient soil, there was no evidence that root mass fraction was consistently changed by the soil P levels used in this experiment.

Root mass fraction of the core collection ranged 1.5-fold from 0.42 to 0.64 when measured in low P soil in root boxes (Fig. 9.29). A similar range was measured when plants were grown in a pot experiment; 0.39 to 0.59 (Fig. 9.30). Measurements of root mass fraction of the core collection were correlated between the two experiments (Fig. 9.31).

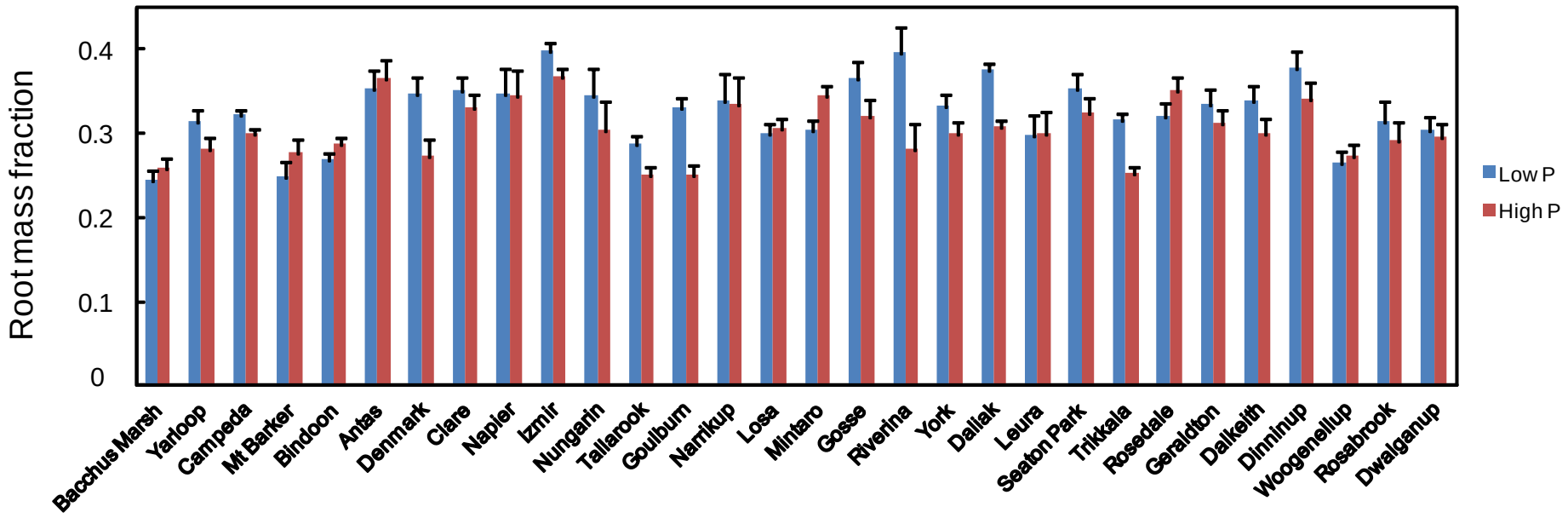


Figure 9.27 Root mass fraction (root dry mass expressed as a proportion of total plant mass) of 30 cultivars of subterranean clover grown in root boxes with thin (5 mm) layers of uniform soil at a low soil P concentration (Colwell P = 10 mg/kg) or a high soil P concentration (Colwell P = 28 mg/kg) soil.

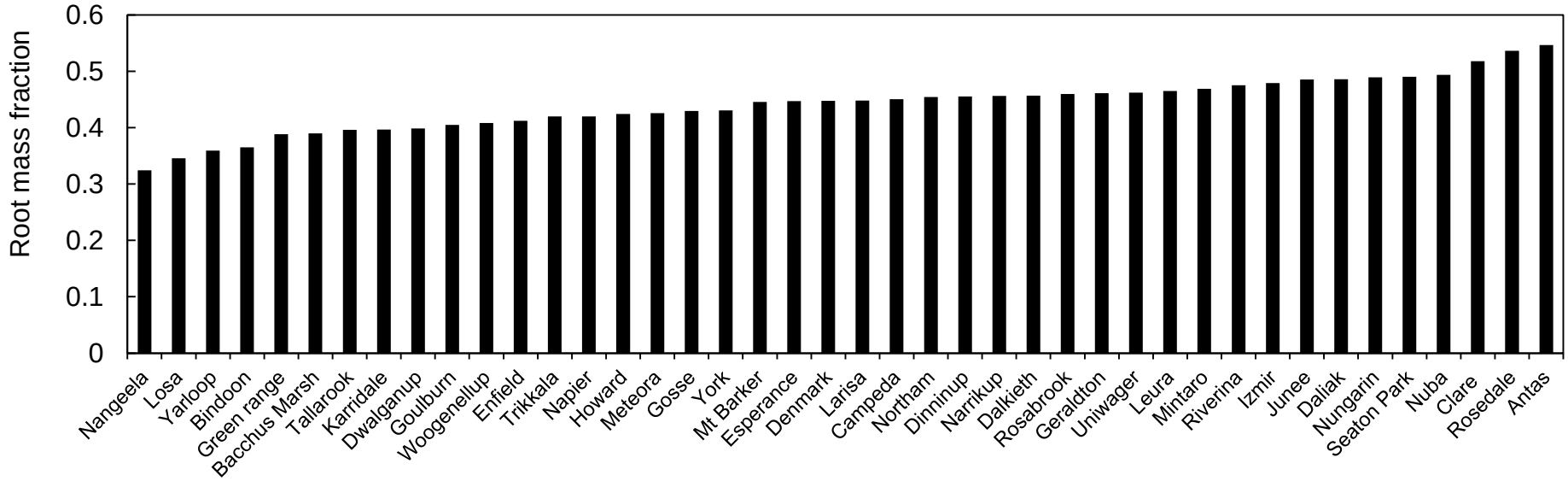


Figure 9.28 Root mass fraction of 42 cultivars of subterranean clover measured on roots of plants grown in a glasshouse in pots of P-deficient soil for 6 weeks. Presented in order of increasing root mass fraction. LSD is 0.057 (*P*<0.05).

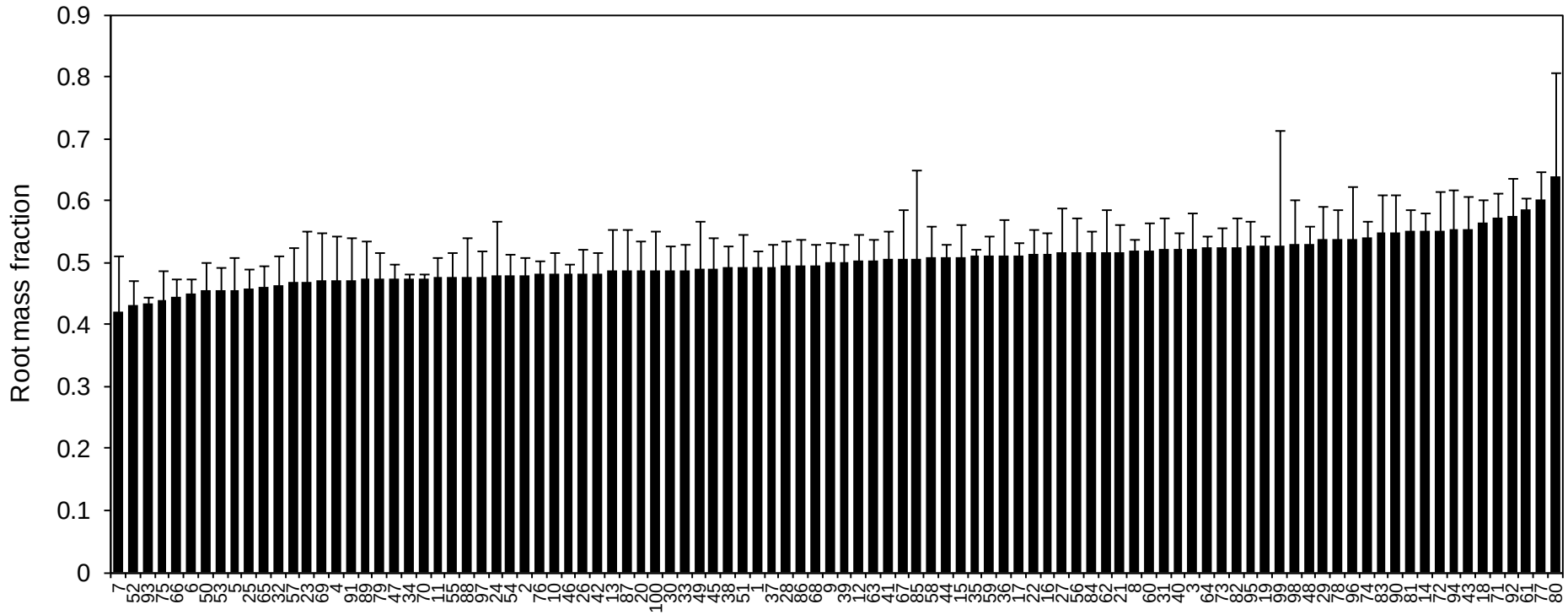


Figure 9.29 Root mass fraction of 96 lines and 4 cultivars of subterranean clover measured on roots of plants grown in root boxes with thin (5 mm) layers of uniform soil at a low soil P concentration (Colwell P = 10 mg/kg). Presented in order of increasing average root diameter. The cultivar controls included in this experiment were: Coolamon (line 96), Urana (line 97), Riverina (line 98), Woogenellup (line 99), and Daliak (line 100). Bars = 1xSD.

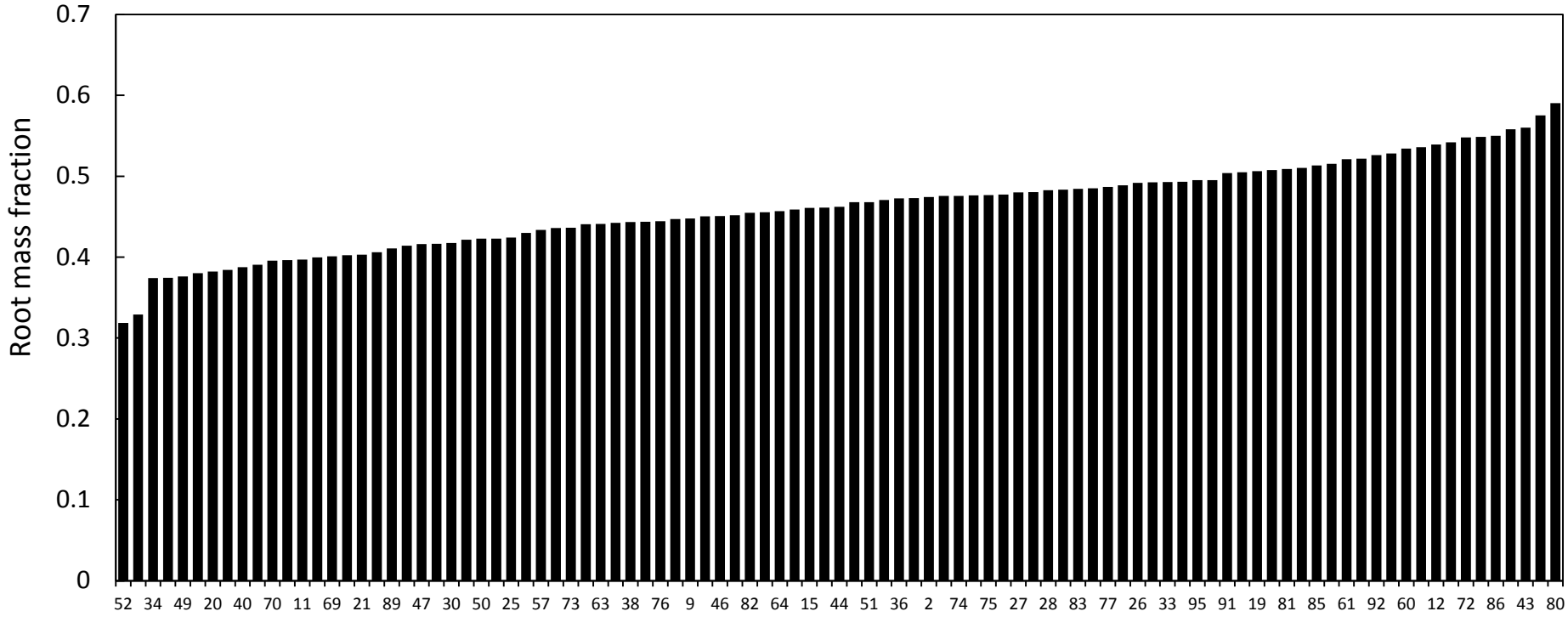


Figure 9.30 Root mass fraction of 96 lines of subterranean clover measured on roots of plants grown in a glasshouse in pots of P-deficient soil for 6 weeks. Presented in order of increasing root mass fraction. The cultivar controls included in this experiment were: Coolamon (line 96) and Urana (line 97). LSD is 0.084 (P<0.05).

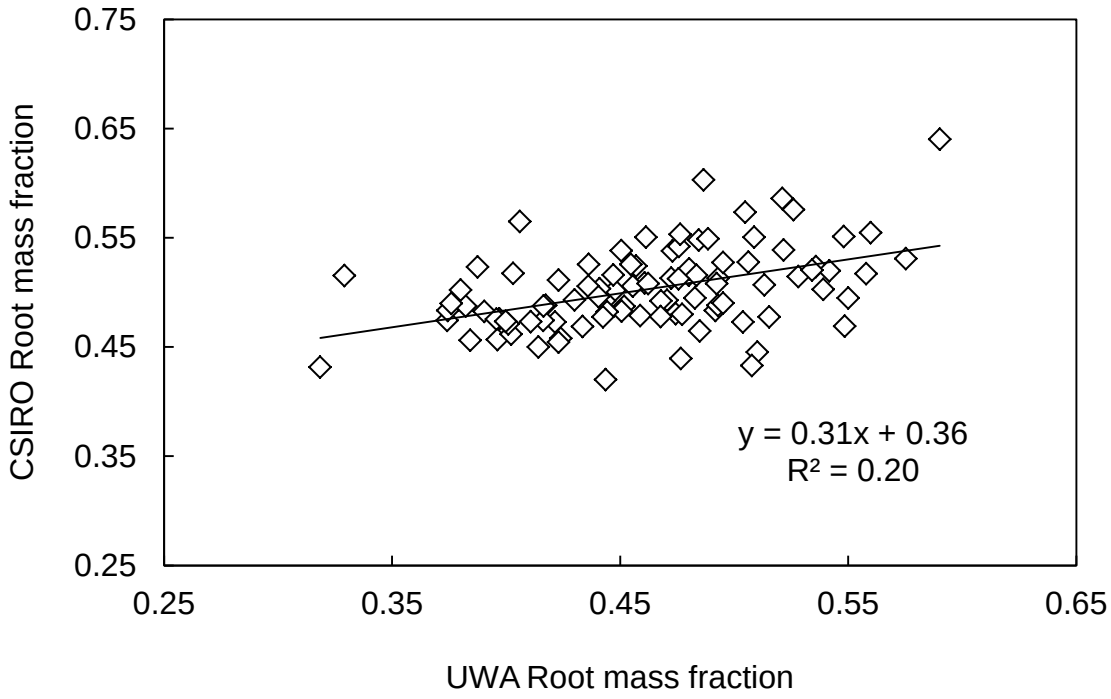


Figure 9.31 Correlations for root mass fraction measured in independent screens of a core collection of subterranean clover at UWA and CSIRO. Measurements are of plants grown for 6 weeks in pots in a glasshouse (UWA) or 3 weeks in a root box in a controlled-environment, plant-growth cabinet (CSIRO).

9.6 Rhizosheath

9.6.1 Background

The rhizosheath is defined as the soil that adheres to the roots when they are gently excavated from soil. The soil is anchored by the root hairs and bound by mucilage from the roots or microorganisms in the rhizosphere. In cereal breeding, the size of the rhizosheath on seedling roots has been used as a surrogate measure of root hair length (RHL) to improve the ease and speed with which screening for this trait can be conducted (Delhaize *et al*. 2012). Screening for RHL is usually done under the microscope and is both tedious and time consuming. The rhizosheath is measured as the weight of root plus adhering soil per unit length of the root, but semi-quantitative, visual assessments can also be made for rapid initial screening. Root mass is often small in comparison with the weight of the rhizosheath soil and errors associated with this screening method are usually not significant. This work investigated whether the size of the rhizosheath could be used as a rapid screen for RHL.

9.6.2 Materials and Methods

Five subterranean clover cultivars known to differ in RHL were grown for 9 days in a Ferrosol pasture soil (pH 6.5) from Robertson, NSW. The soil was self-mulching with a fine aggregate structure that was known to form stable rhizosheaths on wheat roots when maintained with a soil moisture content in the range 70-90% of field capacity (Delhaize *et al*. 2012).

The mass of the rhizosheath plus fresh root, and length of root hairs was measured.

We gratefully acknowledge the assistance of M. Delhaize and T. Rathjen (CSIRO) in this analysis.

9.6.3 Results and Discussion

Measurements of rhizosheath size were very consistent within cultivars and significant differences existed between some cultivars (Figs 9.32 and 9.33). However, in comparison with the rhizosheath size of other plant species, the rhizosheaths of subterranean clover were very small. The correlation measured between rhizosheath size and RHL was relatively poor (Fig. 2b). While the cultivars were able to be correctly ranked for RHL using visual assessment of rhizosheath size (Fig. 3a), the small range in RHL and associated rhizosheath size is likely to limit the use of this as a screening technique for RHL in subterranean clover.

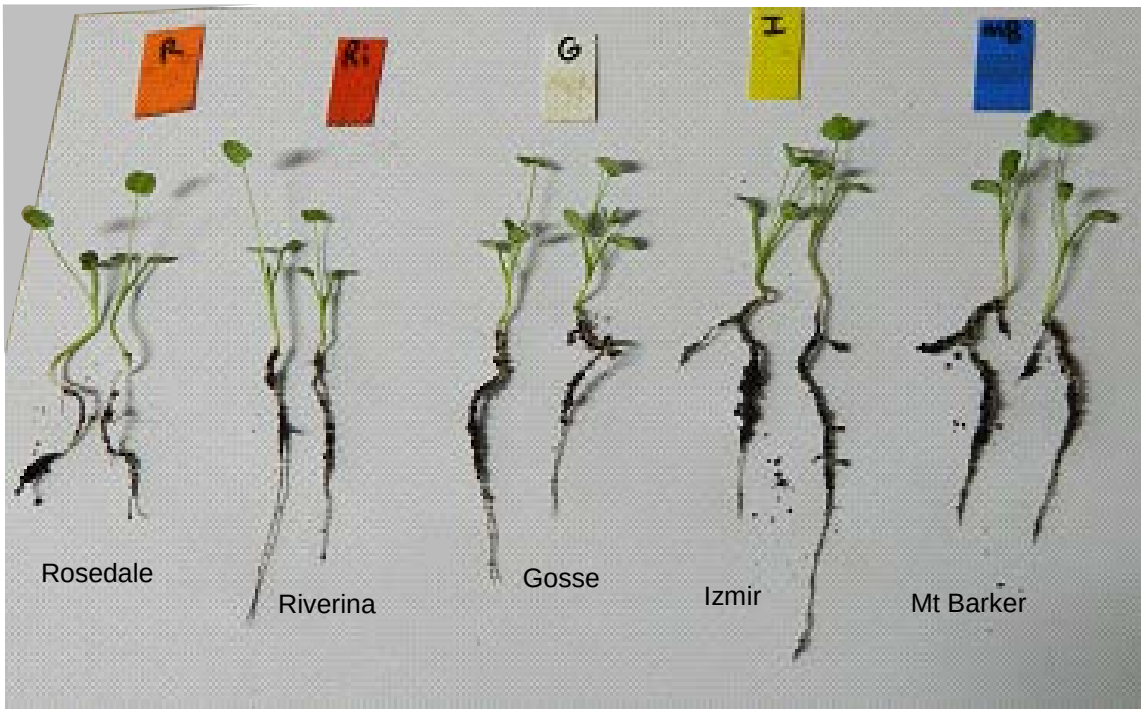


Figure 9.32 Rhizosheaths on roots of subterranean clover after 9 days growth.

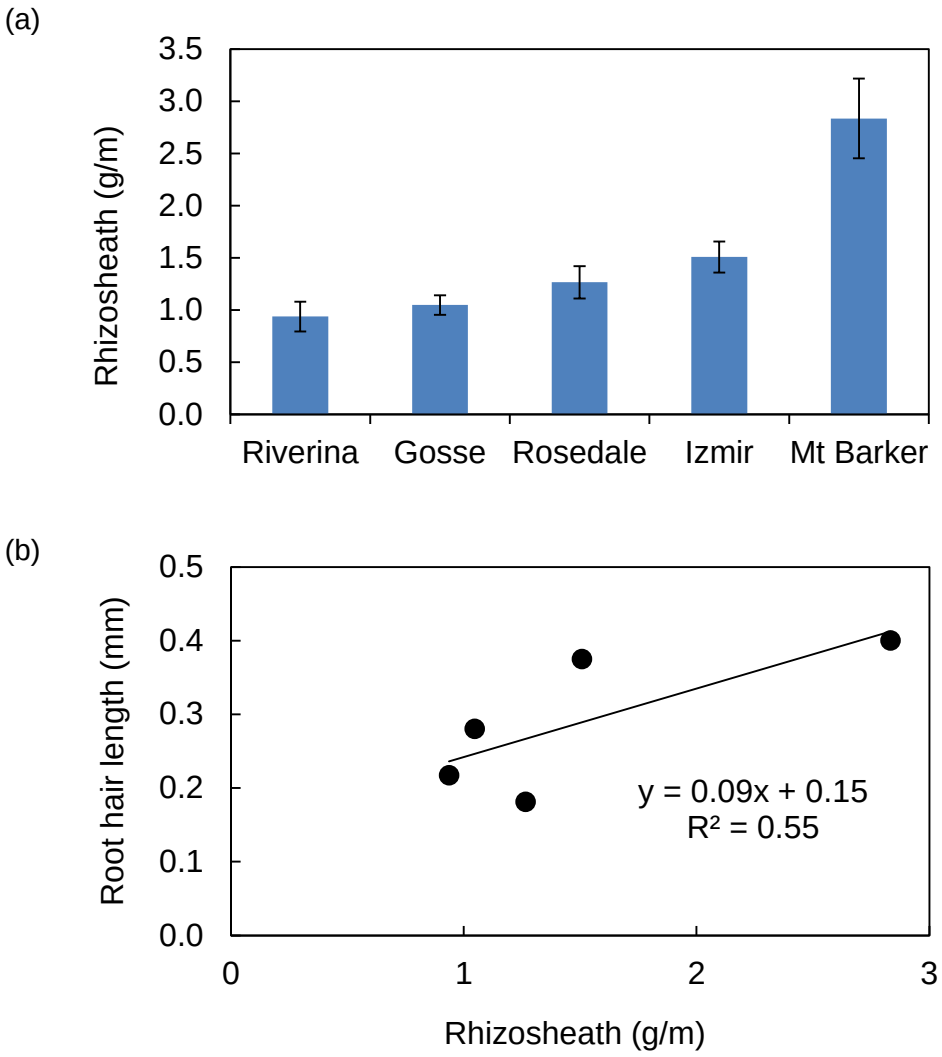


Figure 9.33 (a) Rhizosheath size on each of five cultivars of subterranean clover (Bars= 2xSE), and (b) the relationship between rhizosheath size and root hair length.

9.7 Discussion

There was a large variation among the subterranean clover cultivars and the lines of the core collection for most of the root traits. Generally speaking, the variation in root trait dimensions across the cultivar collection was similar to that observed among the lines of the core collection. This is a particularly helpful observation for a number of reasons. The lines of the core collection represent the potential range in subterranean clover genes and phenotypes but are not selected for agronomic fitness. It was noted in some experiments that some lines failed (e.g. succumbed to root or other diseases) confounding attempts to measure their root traits. In contrast, the cultivars have all been selected for yield, agronomic type and, at least, for some degree of disease resistance. This makes them more robust and better suited for root physiology experiments planned to examine the relative importance of roots traits for P acquisition by subterranean clover. This observation also indicates that selection for improved P efficiency traits can readily build on the existing legacy represented by the modern subterranean clover cultivars.

9.7.1 Root hair length

Short root hairs were clearly identified as a P-efficiency weakness in subterranean clover. However, across the species root hair lengths varied over a 2-fold (range ~0.2 - 0.4 mm). On face value, this suggested a large scope for selecting for subterranean clover genotypes with longer root hairs but even the longest root hairs of subterranean clover fell well short of the lengths observed on serradellas (0.6-0.8 mm) and grasses (0.7-1.1 mm).

It is hypothesized that there is an interaction between the net effectiveness of root hair length for P uptake and the presence of arbuscular mycorrhizal fungi (AMF) (Schweiger *et al*. 1995). AMF usually colonise the roots of subterranean clover and improve P acquisition by the clover in very low P soils. Based on the experiment of Schweiger *et al*., (1995) it remains unclear whether selecting for longer root hairs alone (i.e. lengths up to 0.40 mm) would positively influence P acquisition in subterranean clover when AMF are present. This hypothesis is also backed by divergent selection of white clover for root hair length in which plants with root hairs about 0.3 mm in length were no more effective at acquiring P from low P soil than plants with smaller root hairs (~0.2 mm) unless they were grown in the absence of AMF (Caradus 1981).

There studies indicate that further work is required:
(i) to clarify the benefit or otherwise of breeding for longer root hairs alone unless lengths greater than ~0.5 mm can be achieved on subterranean clovers,
(ii) to assess whether there are other sources of genes for long(er) root hairs that may be utilised in a subterranean clover breeding program, and
(iii) to determine whether “long” root hairs, in combination with other desirable root morphology traits could deliver a additive benefit for P acquisition efficiency in subterranean clover.

The methods by which a plant breeder might screen genotypes were also examined briefly. Root hair length measurements are tedious and time consuming. In wheat breeding, a surrogate measure (rhizosheath size) has been used to screen for longer root hairs. However, the relationship between root hair length and rhizosphere size in subterranean clover was weak and rhizosphere size did not appear a suitable method for screening lines for root hair length in this species. At this stage, visual screening of root hair length under a low-power microscope using the size of the root hair cylinder as a guide to root hair length appeared to be reliable and reasonable rapid. This could be conducted on roots of young seedlings (e.g. 1-week old) washed quickly from the soil medium in which they are grown.

9.7.2 Specific root length

Specific root length (SRL) of subterranean clover is low compared with some other pasture legumes and this means that subterranean clover must commit relatively high dry matter resources to root growth when exploring soil for P. Relative to other species, this means that P acquisition comes at a relatively high carbon cost (i.e. high dry matter allocation to roots; potentially high root maintenance respiration costs). Across the species, there was also a large variation in SRL (~2-fold range). However, again the highest SRL in subterranean clover (~150 m/g) was substantially

lower than that of the serradellas (200-300 m/g). SRL of subterranean clover was highly responsive to plant growth conditions and was adjusted by the clover in response to P supply and sward density (Fig. 7.8b). Under sward conditions, the adjustments in SRL were in the opposite direction to that considered advantageous for low-cost P acquisition: i.e. SRL was lower in low P soil. This was most probably a consequence of a complex interaction between the response of root morphology to P supply and etiolation due to self-shading in pasture swards grown with high P supply (Chapter 8). This interaction may be the reason why SRL differed so much between different experiments. Nevertheless, the SRL of the subterranean clover genotypes were always correlated when grown under very different plant growth and soil conditions (range in R^2: 0.40 - 0.55) indicating that the ranking genotypes was relatively stable.

The variation in SRL in response to plant growth environments was considered to be potentially problematic for root trait screening in a plant improvement program. SRL is also a very time-consuming trait to measure as root length and root mass are required for its measurement. A solution to this problem may be to select lines using root diameter as a surrogate measure for SRL. Average root diameter was high correlated with SRL of the subterranean clover lines (range in R^2: 0.73-0.91). It did not vary when SRL was altered by the interaction of P supply and sward growth conditions provided soil P levels were in the moderate to high P supply range (Fig. 7.8b) suggesting that root diameter would prove to be a more stable trait for screening clover genotypes.

9.7.3 Lateral root angle

Lateral root angle is a proven P acquisition efficiency trait in common bean (*Phaseolus sativus*) *and* soybean (*Glycine m*ax) (Bonser *et al*. 1996; Liao *et al*. 2001). Genotypes that develop shallow roots can be substantially more effective acquiring P from low P soils because P is often concentrated in the topsoil layers as a consequence of leaf litter and excreta deposition and as a consequence of topdressing with fertiliser.

Large variations in lateral root angle (38^o - 68^o from vertical) were observed within the subterranean clover species. For any particular genotype, the smallest lateral root angles were observed in the uppermost soil layers and root angles became larger with root system depth. However, the root angles of the genotypes were correlated between depths indicating that shallow-rooted lines tended to maintain higher root angles at all soil depths than lines with roots that had a deeper rooting orientation.

On face value there appeared to be considerable scope for selecting lines with large differences in lateral rooting angle. However, caution is advised before such a step is undertaken. The experiments reported in Chapters 5 and 8, demonstrate that subterranean clover has an exceptional ability to proliferate roots and to achieve high root length densities in the nutrient-rich layers of a soil profile. This attribute may negate any apparent advantage of roots with a favourable orientation for topsoil rooting. In addition, Ho *et al*. (2005) have reported a significant trade-off between having high P acquisition (shallow roots) and tolerance of drought (deep roots) in beans selected for these contrasting root orientation patterns.

The principle mechanisms used for drought tolerance/survival in subterranean clover are selection of cultivars that fit the available growing season (maturity type selection) and hardseededness (survival of seeds over summer). In this annual clover, trade-offs between deep and shallow roots systems may not be as significant as they will be for a crop where high yield is only achieved if an early terminal drought can be tolerated. Nevertheless, the potential for trade-offs (Richardson *et al*. 2011) also demonstrates that the true benefit of a shallow root system for P acquisition must be established in subterranean clover before attempting to select for the trait in a plant improvement program. Indeed, if the P-acquisition benefit for subterranean clover of selecting roots for high root angles is demonstrated to be marginal because the species has a high capacity to proliferate roots in nutrient patches, it may more generally beneficial (for reasons of seed production and general pasture resilience) to select for deeper roots (i.e. low root angles).

9.7.4 Root mass fraction

Root mass fraction is a proportional measure of a plant's allocation of dry mass to root growth. All plants acclimate to P-deficient soil by increasing root mass fraction. In Chapter 6 it was observed that pasture legume genotypes differed in the intrinsic allocation of mass to roots (Fig. 6.4a). However, the rate at which species with contrasting abilities to acquire P from low P soil adjusted root mass fraction in response to low P soil was surprisingly similar (Fig. 6.4a). The effectiveness of the allocation of root mass for P uptake varied substantially among legume species because of large differences in their specific root length (e.g. Fig. 5.1a), root hair lengths (e.g. Fig. 5.2), and their capacity or propensity to proliferate roots in a soil nutrient patch (Fig. 6.4b).

The objective of the assessment of root mass fraction reported here was to establish whether subterranean clover lines differed in their intrinsic allocation of dry mass to roots. Assessments were made using P-deficient soils because of the known root mass acclimation response of plants to low P, but subsequent experiments demonstrated that the assessment could most likely have been made under high soil P conditions without any impact on the relative rankings of the lines (e.g. Fig. 6.4a). The range in root mass fraction within the subterranean clover species (1.5-fold) was not as large as found for other root traits but was, nonetheless, significant.

The value of the RMF trait for P acquisition is not as easily determined as other root traits. This is because allocation of dry matter towards roots will improve the potential for root foraging but will also compete with shoot yield for carbon as a direct result of dry mass allocation and indirectly as a result of the respiration required for growth and maintenance of additional root mass. Root mass fraction must, therefore, also be assessed in combination with the traits that modify the effectiveness of root mass for P acquisition such as specific root length, root hair length and proportions of root mass allocated specifically to nutrient foraging (root proliferation in nutrient patches). No significant relationship was found between root mass fraction and specific root length. Likewise, there were no significant relationships between specific root length and lateral root angle, or lateral root angle and root mass fraction (data not shown). This suggests that it is likely that the key P-efficiency traits may potentially be modified independently.

9.8 Conclusions

Substantial variation existed within the subterranean clover genome in all of the key P acquisition phenes examined to date indicating potential for selection of more P-efficient root systems. Variation in root proliferation is presently still being investigated.

However, it was also clear that the range in specific root lengths or root hair lengths of the subterranean clover lines does not encompass the fineness of roots and length of root hairs found in the serradella species which set a P-efficient benchmark for pasture legumes.

Based on the range in root phene dimensions measured in the core collection of subterranean clover lines and the equivalent variation measured among the cultivars, it is predicted that significant differences in the P-acquisition efficiency and critical-P requirements of subterranean clover cultivars will exist and may potentially be enhanced by breeding. However, it is likely that a source of genes that can confer higher specific root lengths and substantially longer root hairs than presently found among the subterranean clover lines may be required to achieve the very low critical P requirements and high P-acquisition efficiency of the serradella species.

10 Intra-specific variation in root proliferation and yield in response to low soil P supply by *Trifolium subterraneum*.

Rebecca E. Haling, Adeline Becquer, Adam Stefanski, Daniel R. Kidd, Megan H. Ryan, Graeme A. Sandral, Hans Lambers, Phillip G.H. Nichols, Richard J. Simpson

10.1 Background

In this project, we have identified an extraordinary response of subterranean clover in low-P soil to proliferate root length in zone(s) of the soil where extractable-P concentrations are elevated above the general soil P level (i.e. an increase in length of roots per unit soil volume - also termed increased root length density; Chapter 7). Although all of the pasture legumes reacted to P deficiency by proliferating root length in zones where the soil available P concentration was higher, the response by subterranean clover was very pronounced and exceeded that observed in most of the other pasture legumes (Chapter 8). Root length proliferation assists the clover to increase P acquisition despite generally low soil P availability. Experiments with subterranean clover (cv. Leura) showed that it was able to achieve an even higher maximum root length density in a nutrient “patch” than two very P-efficient, benchmark species of serradella (Chapters 6, 7 and 8).

We surmise that root length proliferation is a particularly important strategy for P acquisition in subterranean clover which does not have long root hairs (Chapter 9); a root morphology trait that is key to the efficient P-acquisition capability of the serradellas. The range in root hair lengths measured among lines that represent ~80% of the subterranean clover genome was limited (0.2-0.4 mm) and root hairs on the cultivars examined so far, do not increase in length in response to low P supply as found in some other species. By contrast, the serradellas have root hairs that are ~0.7 mm long.

Given these observations, it is important to know whether there is significant intra-specific variation in subterranean clover for (i) root proliferation in response to low soil P, (ii) the achievement of high root length density, and (iii) specific root length as these root characteristics are likely to be key traits for improving the P acquisition efficiency of subterranean clover cultivars.

10.2 Materials and Methods

10.2.1 Plant material

The reference collection of 30 subterranean clover cultivars (Chapter 9) and the core collection of 97 lines of subterranean clover (Chapter 9) were assessed in separate experiments for the ability of subterranean clover lines to proliferate roots in response to low soil P supply and for shoot yield under the low and adequate P supply..

Plant growth conditions

It was not feasible, within the resources of the project, to examine the response of this number of lines over the full P response range for subterranean clover. However, in previous experiments (Chapters 6 and 7) it was shown that subterranean clover develops roots preferentially in zone(s) of a soil profile where the concentrations of plant-available P are highest. This proliferation of roots was enhanced when P supply from the soil as a whole was limiting for shoot growth; more root length being grown in the nutrient patch when P supply was more limiting for shoot growth. However, a point was reached at very low P supply where the clover was unable to continue proliferating root length and the acclimation of the plant to low P soil "failed" (Chapters 6 and 7). Consequently, root proliferation and yield were assessed at a P supply rate close to the threshold low P level for maximum root proliferation in low P soil and were compared with root length and shoot yield achieved with non-limiting P supply.

The soil was a sandy loam (Yellow Chromosol; Isbell 1996) with a low concentration of extractable P (8.3 mg kg^{-1} P; Colwell, 1963; method 9b in Rayment and Lyons 2011) and was collected from Ginninderra Experiment Station Canberra, ACT, Australia (35°10'30"S, 149°02'33.4"E). The soil was pasteurised to reduce disease inoculum (65°C for 1 h), sieved to < 5 mm and mixed with lime (1.06 g $CaCO_3$ kg^{-1}) to raise the pH (1:5 w/v, 0.01 M $CaCl_2$) to 5.2. Nutrients were mixed into the soil at rates of 41.1 mg kg^{-1} soil $MgSO_4.7H_2O$, 43.0 mg kg^{-1} $CaSO_4.2H_2O$, 169 mg kg^{-1} KNO_3, 27.5 mg kg^{-1} $(NH_4)_2SO_4$, 16.7 mg kg^{-1} NH_4NO_3, 119 µg kg^{-1} H_3BO_3, 759 µg kg^{-1} $MnCl_2.4H_2O$, 359 µg kg^{-1} $ZnSO_4.7H_2O$, 33.3 µg kg^{-1} $CuSO_4.5H_2O$, 72.1 µg kg^{-1} $(NH_4)_2MoO_4$, 19.8 µg kg^{-1} $CoCl_2 \cdot 6H_2O$ and 1530 µg kg^{-1} Fe-EDTA.

Pots (cylindrical PVC; 87 mm internal diameter) were filled with a bottom layer of the soil (0.9 kg oven dry basis) that was not fertilised with P (the subsoil) and then with a topsoil layer using P-fertilised soil (0.3 kg oven dry basis; 47 mm settled soil height) to achieve two contrasting rates of P supply. The P-fertilised topsoil was generated by mixing KH_2PO_4 with subsamples of the amended low-P soil at rates of 40 mg P kg^{-1} topsoil (to achieve an "intermediate" level of P stress considered to be close to the threshold low P level for maximum root proliferation) and 250 mg P kg^{-1} topsoil (sufficient to achieve maximum plant growth). The stratification of P in the pots was intended to mimic the concentration of P that occurs in topsoil under pastures fertilised by broadcasting P fertiliser onto the soil surface. The boundary of the fertilised topsoil and unfertilised subsoil layers was marked by placing small alkathene beads around the interior edge of the pot.

Micro-swards of each cultivar were established by sowing 50 mg pot^{-1} of viable seed and the plants were inoculated with Group C rhizobium (strain WSM 1325, NewEdge Microbials, Albury, NSW, Australia).

Plants were grown in a controlled-environment growth cabinet [12 h light (620 µmol quanta m^{-2} s^{-1})/ 12 h dark; 25/15°C]. Pots were arranged in a randomised complete block design and rotated within blocks. Reflective sleeves were fitted to the outside of the pots and raised daily to plant height to reproduce the light conditions that occur in the field during peak spring growth. The soil was maintained at 75 to 80% of field capacity by daily watering to a predetermined weight and was watered to reach field capacity once per week. This water regime was designed to avoid the drying of the

subsoil that can occur over the duration of an experiment if pots are only watered from above to maintain 75 to 80% of field capacity.

Three replicates were grown for each combination of P and cultivar. For the reference collection of 30 cultivars, all three replicates were grown and harvested simultaneously. For the core collection, the large number of lines required each replicate to be grown as blocks separated in time. Environmental variation was minimised by conducting the experiment in a large controlled-environment plant growth cabinet. An additional three cultivars (Napier, Leura and Losa) were included in the assessment of the core collection as controls in common with the reference collection.

10.2.2 Plant harvest and measurements

Shoots and roots in the topsoil were harvested after 5 weeks growth from germination. The logistics of these experiments dictated that subsoil roots could not be harvested. However, it had already been shown that the root response of P-deficient subterranean clover was essentially confined to the topsoil layer (Chapter 6). Roots from the topsoil were harvested in 3 sections. A quarter segment of the topsoil layer was washed on 1-2 mm sieves and scanned immediately on a flatbed scanner for determination of root length and average root diameter using WinRHIZO software (Regent Instruments Inc., Quebec, Canada). dried and weighed; a one half segment of the topsoil layer was washed, dried (70^{o}C) and weighed; and the remaining quarter segment was washed and stored at 4°C in 50% ethanol for determination of root hair length and mycorrhizal colonisation of roots. It was only feasible to measure mycorrhizal colonisation of roots for the reference collection due to the large number of samples in the core collection. Specific root length (i.e. length per unit root dry mass) was calculated for the sample that had been washed, scanned and dried. Total length of roots in the topsoil was calculated by multiplying the specific root length of roots from the scanned quarter segment by the estimated total mass of roots in the topsoil ($^{4}/_{3}$ x the combined dry mass of roots from the scanned quarter and the un-scanned half). Root volume and root tissue density were estimated using the estimates of root length, average root diameter and root dry mass.

Root hairs were photographed using a Leica MZFLIII Fluorescence microscope (Leica Microsystems, Sydney, Australia) fitted with a Zeiss AxioCam camera (Zeiss, Sydney, Australia). The length of 15 root hairs per replicate was measured using ImageJ (Rasband 1997–2014).

Mycorrhizal colonisation (40 mg P kg^{-1} treatments) was measured using the grid-line intersect method (Giovannetti and Mosse 1980) after clearing (10% w/v KOH for 2-4 days followed by rinsing in water and 1% v/v HCl) and staining (5% v/v Schaeffer blue ink/ white vinegar solution for 1 h; Vierheilig *et al.* 1998).

All shoots and root subsamples were dried at 70^{o}C and weighed. Shoots and root samples from the reference collection were then milled to a fine powder and 25 to 50 mg samples were ashed in a muffle furnace for 4 hours at 550^{o}C. The ashed material was dissolved in 2M HCl and P concentration determined colorimetrically using malachite green (Irving and McLaughlin 1990).

10.3 Results

Within both the reference collection of 30 cultivars and core collection of 97 lines of subterranean clover there was a two-fold range in shoot yield in the low-P soil (40 mg P kg^{-1}) (Fig. 10.1). This was despite most cultivars or lines having similar maximum yields in the high-P soil (250 mg P kg^{-1}).

Root length density was increased by 1.2- to 2.6-fold among the cultivars, and 1.4- to 3.3-fold among lines of the core collection, in response to low-P supply. The range in root length density among the reference collection and core collection in the low-P soil was large (13-28 cm cm^{-3} and 16-34 cm cm^{-3} for reference and core collections, respectively) (Fig. 10.3). However, most cultivars or lines had similar root length densities in the high-P soil.

There was a 2-fold range in specific root length within both the reference collection and core collection in the low P soil (e.g. 116-253 m g^{-1} and 116-237 m g^{-1} for reference and core collections, respectively) (Fig. 10.4). Specific root length was negatively correlated with average root diameter. However, it was evident that there was significant variation in specific root length within a given root diameter class (Fig. 10.5a,b). Within each root diameter class, there was a negative linear relationship between root tissue density (mass of root per unit root volume) and specific root length (length of root per unit root mass) (Fig. 10.5b). This relationship is expected given that root tissue density and specific root length are related by cross-sectional area and that within a root diameter class the cross-sectional area is effectively constant. In addition, there was a positive linear relationship between “specific root length divided by root tissue density” (i.e. the gradient of the linear relationships presented in Fig. 10.5c,d) and root diameter class (Fig, 10.5e,f), indicating that reduction in root tissue density was more effective for increasing specific root length when average root diameter was smaller.

There was a positive linear relationship between root length density (root proliferation) and shoot dry mass for both the reference collection (R^2=0.37) and core collection (R^2=0.21) (Fig. 10.6a,b). For the reference collection, there was a positive linear relationship between root length density and P uptake into the shoot and topsoil roots (R^2=0.54) (Fig. 10.6c).

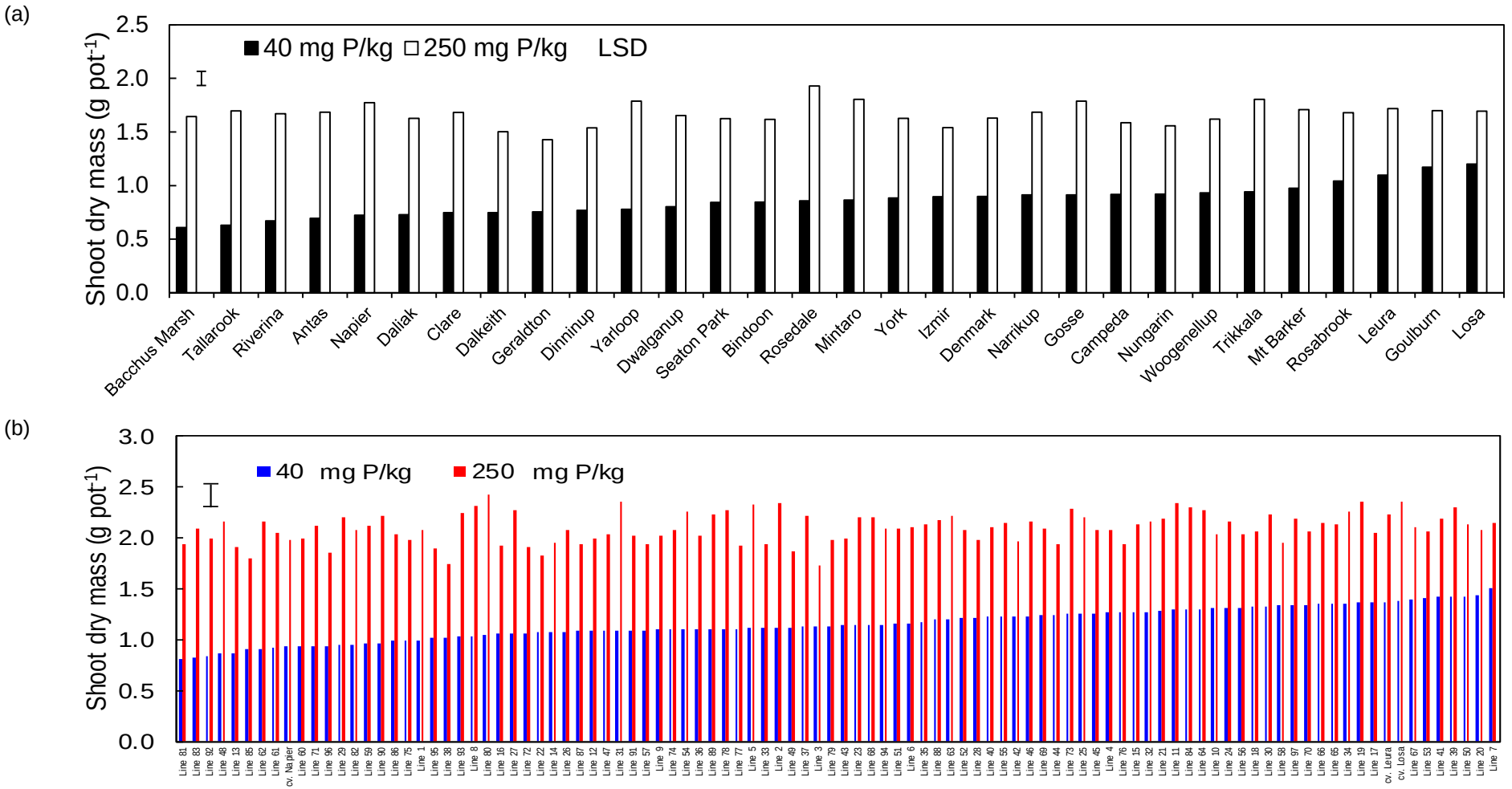


Figure 10.1 Shoot dry mass of (a) the reference collection of cultivars and (b) the core collection of subterranean clover grown with 40 and 250 mg P kg^{-1} applied in the topsoil layer. Bars show LSD (*P*=0.05; n=3) for interaction of genotype x P supply.

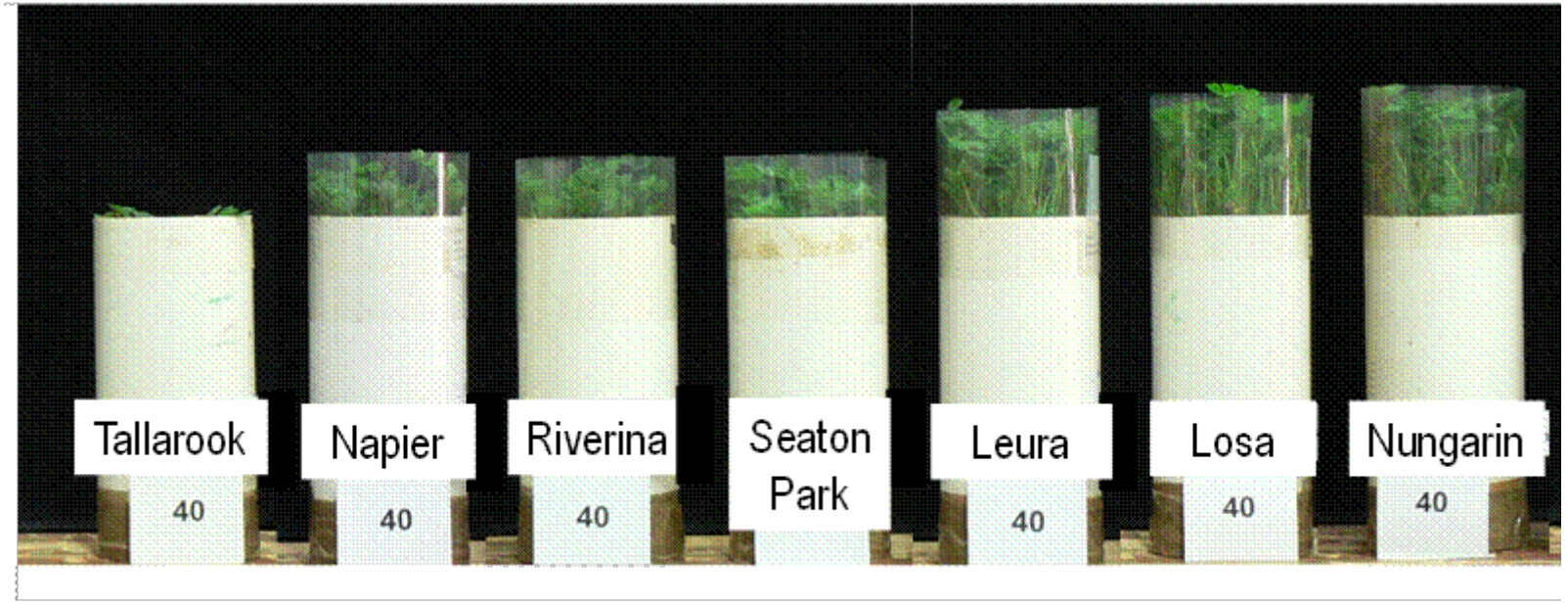


Figure 10.2 A subset of the cultivars of subterranean clover that illustrates the range in shoot yields achieved when the cultivars were grown in soil with the low P supply (40 mg kg^{-1} soil).

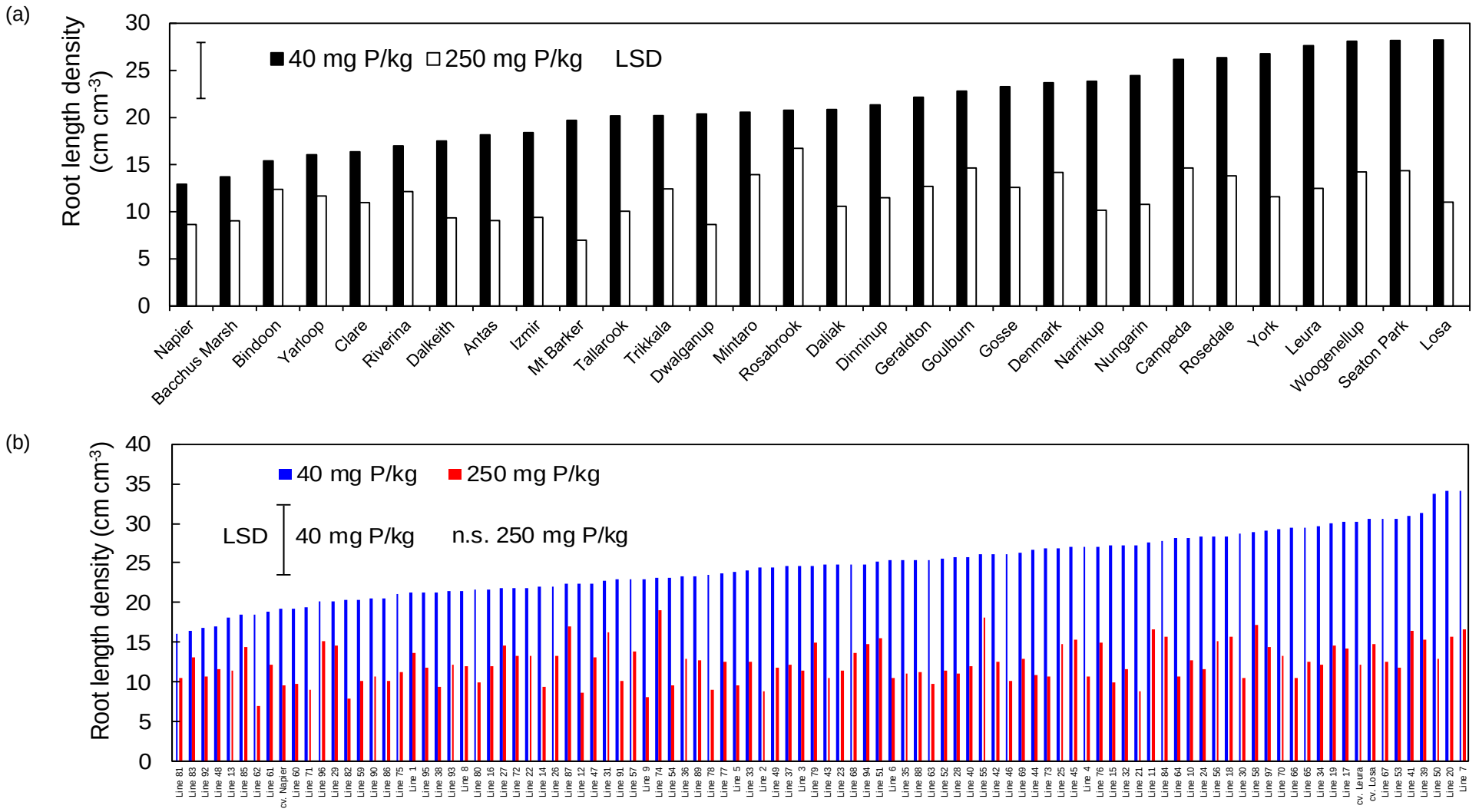


Figure 10.3 Root length density of (a) the reference collection of cultivars and (b) the core collection of subterranean clover grown with 40 and 250 mg P kg^{-1} applied in the topsoil layer. Bars show LSD (*P*=0.05; n=3) for (a) interaction of genotype x P supply and (b) effect of genotype at 40 mg P kg^{-1}; interaction of P supply x genotype, and effect of P supply at 250 mg P kg^{-1} were not significant (n.s.).

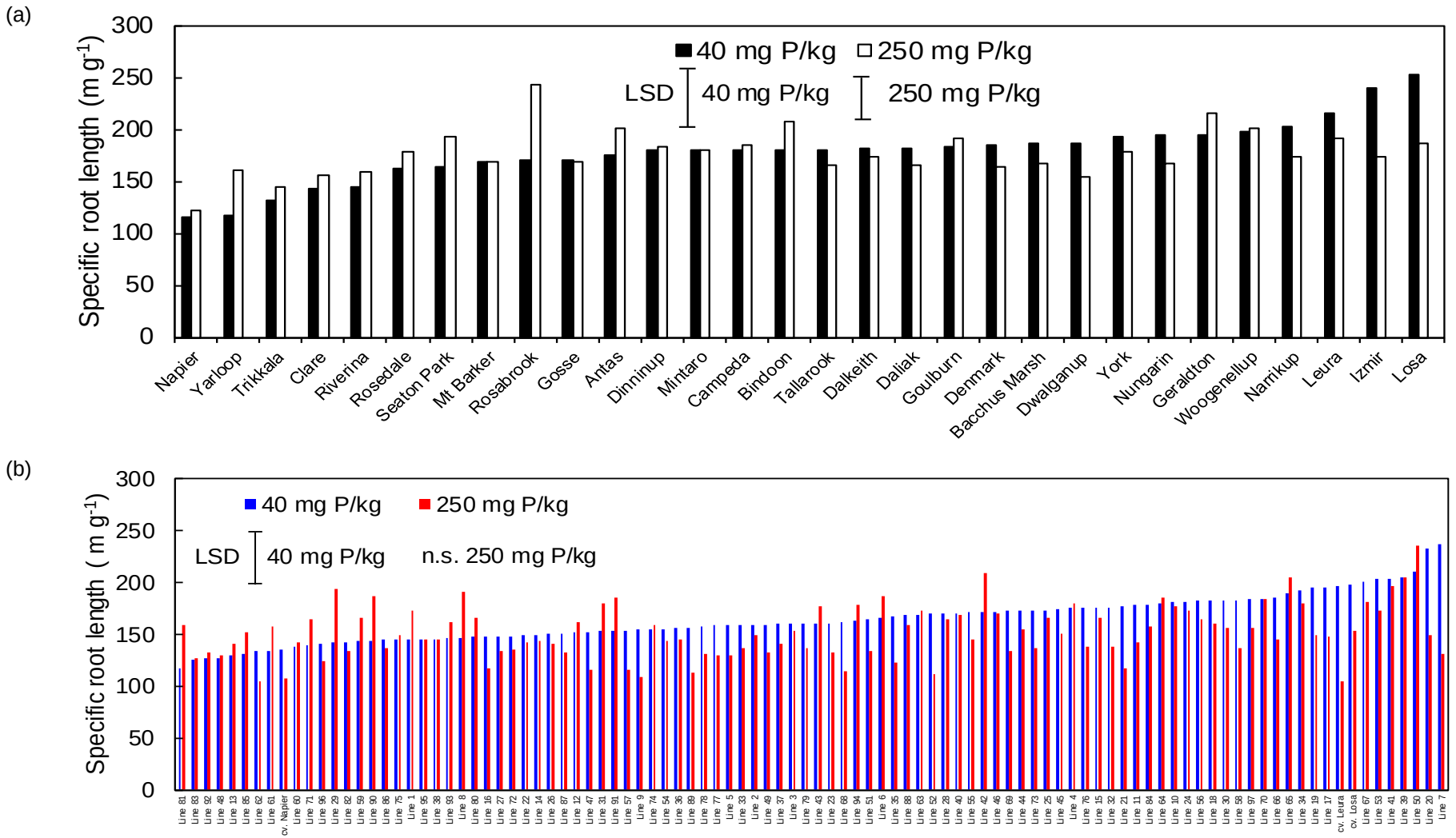


Figure 10.4 Specific root length of (a) the reference collection of cultivars and (b) the core collection of subterranean clover grown with 40 and 250 mg P kg^{-1} applied to the top quarter of pot. Bars show LSD (*P*=0.05; n=3) for effect of genotype at 40 mg P kg^{-1} and 250 mg P kg^{-1}, respectively (n.s. indicates not significant); interaction of P supply x genotype was not significant.

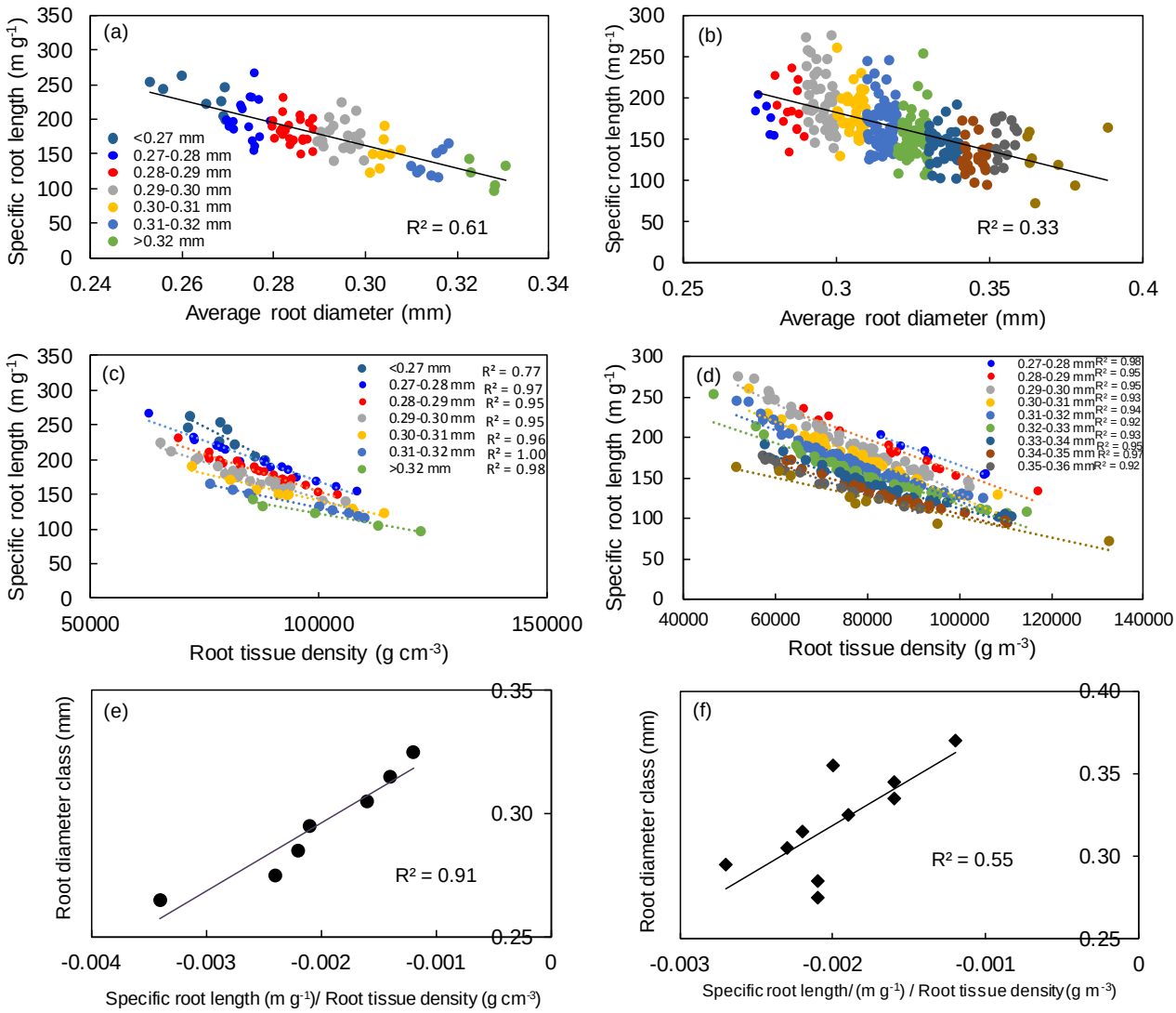


Figure 10.5 Relationship between (a,b) average root diameter and specific root length (c,d) root tissue density and specific root length and (d,e) specific root length/ root tissue density (i.e. gradient of linear relationships in c,d) and root diameter class for (a,c,e) the reference collection of cultivars and (b,d,f) the core collection of subterranean clover grown in pots of soil with the top quarter fertilised with 40 mg P kg^{-1}. In (a,b,c,d), the different symbol colours represent different root diameter classes.

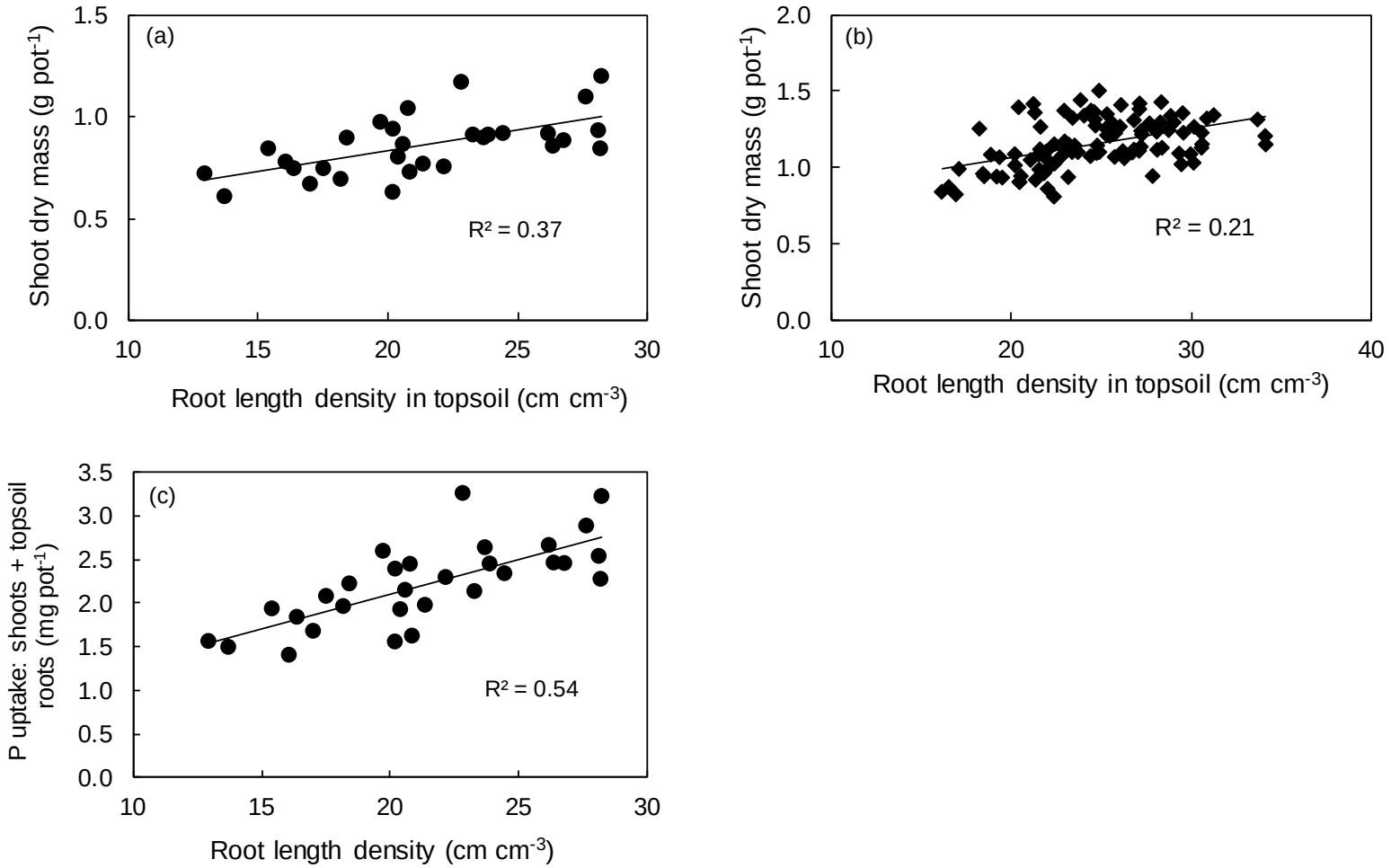


Figure 10.6 Relationship between (a,b) root length density and shoot dry mass and (c) root length density and P uptake (i.e. sum of shoot P and P in roots in P-fertilised topsoil layer) for (a,c) the reference collection of cultivars and (b) the core collection of subterranean clover grown in pots of soil with the top quarter fertilised with 40 mg P kg^{-1}.

10.4 Discussion

There was a wide range in the ability of the subterranean clover cultivars and lines in the core collection to yield in response to low soil P supply and in root proliferation under P deficient conditions. Previously (Chapter 9), the ranges of other root morphology traits (e.g. root length density, specific root length and average root diameter) were found to be similar among the reference collection of cultivars and the core collection of diverse lines. The present experiment has also shown that there is a wide range in the ability of the subterranean clover cultivars and lines in the core collection to proliferate roots in response to low soil extractable-P and that the ranges were similar among the two collections of clover lines.

There was a positive linear relationship between root length density in low P soil and shoot dry matter. However, only ~21 to 37% of the variation in shoot dry mass was explained by variation in root length density for the core and reference collections, respectively. Variation in root length density explained more (~54%) of the variation in plant P uptake into shoots and topsoil roots for the reference collection.

The results indicate that root proliferation is a significant trait for P acquisition efficiency in subterranean clover. However, they also indicate that other factors are also contributing to P uptake and yield. For the reference collection of cultivars, root hairs were measured (data not shown) but did not significantly improve the relationship with P uptake, possibly due to the limited range in root hair length among the cultivars. Variation in factors such as phosphate transporters, release of P-solubilising exudates and possibly interactions with mycorrhiza may contribute to the component of P uptake among the cultivars that was not explained by root length density alone. Variation in internal P-use efficiency and partitioning of biomass to exploring patches of nutrients could explain further variation among the cultivars in their yield in the moderately low-P soil. It is also likely that cultivars do not necessarily express a full suite of desirable root traits and internal efficiencies, and that benefits of one desirable trait could be counteracted by inefficiency in a different trait.

Specific root length was found to vary with both average root diameter (also shown in Chapter 9) and root tissue density. This indicated that selection for low average root diameter alone may not be a reliable surrogate for high specific root length as root tissue density could be high. As mentioned in Chapter 9, a preliminary assessment of the root structure of subterranean clover found few or no aerenchyma. However, further work is warranted to better understand the factors contributing to differences in root tissue density within subterranean clover. In some species (e.g. maize), aerenchyma are an important adaptation to P-deficiency (Fan *et al.*, 2003).

The experiments provide novel insights into the capacity of subterranean clovers to tolerate low P soil conditions. There is only one published experiment that has examined yield responses to P in a range of subterranean clovers (Jones *et al*. 1970). That experiment examined 10 cultivars (9 of these are now out-classed cultivars), but conclusions about P efficiency were confounded by large differences in maximum yield among the lines examined. Nevertheless, some similarities can be reported. For example, cv. Tallarook was identified as very inefficient with respect to P acquisition and this observation has been confirmed.

10.5 Conclusions

Overall, the results of this Chapter have provided:

(i) an initial assessment of the potential variation that exists in P-efficiency among cultivars of subterranean clover when grown in soil with a moderately low soil extractable-P concentration. Indeed, there appeared to be sufficient variation in response to moderate levels of P supply among the subset of cultivars that are in current commercial use, that it is possible that farmers may be able to ensure relatively high production in moderately-fertilised fields by simply selecting the right cultivar of subterranean clover (e.g. compare the 2-fold better yields of Izmir or Losa with those of Napier or Riverina). However, choice of P-efficient cultivars within particular clover sub-species and maturity groups is limited, and further work is essential to verify whether these yield differences are achievable under field growth conditions.

(ii) evidence of significant variation in the root length proliferation response of subterranean clover, and that this is linked with P acquisition and shoot yield. It is initially surprising to find that variation in root proliferation appears to have explained only about half of the variation in P acquisition among the cultivars. This does indicate that root proliferation is a major P-efficiency trait for subterranean clover and suggests that cultivars selected on this basis will have improved ability to acquire P in moderately fertile soils. However, it also indicates that other factors (not yet identified) are important for P acquisition among the cultivars of subterranean clover.

Acknowledgements

We acknowledge Branka Culvenor for technical assistance. AB was funded by an INRA (Montpellier, France) post-doctoral fellowship.

11 Nutrient foraging by *Trifolium subterraneum* roots delivers improved P acquisition efficiency

Rebecca E. Haling, Lawrie K. Brown, Adam Stefanski, Daniel R. Kidd, Megan H. Ryan, Graeme A. Sandral, Timothy S. George, Hans Lambers, Richard J. Simpson

11.1 Background

Trifolium subterraneum (subterranean clover) is the most widely grown annual legume species in mixed temperate pastures in Australia. In these systems, the legume provides a cost-effective source of N for pasture production through biological N_2-fixation. However, relative to the grasses with which it is grown, *T. subterraneum* requires soil with a relatively high concentration of plant-available phosphorus (P) to achieve maximum yield (i.e. a high critical external requirement for P) (Chapter 6, Hill *et al*. 2006; Ozanne *et al*. 1969, 1976). The P-balance efficiency of temperate pastures in Australia is low. For example, median P-fertiliser inputs to sheep-beef pastures exceed removals of P in animal products by 5 to 9-fold (Weaver and Wong 2011). This is associated, at least in part, with the high P requirement of subterranean clover because higher rates of soil P sorption (Barrow 1983) and P accumulation in field soil (Simpson *et al*. 2015; Simpson *et al*. 2014) are associated with soil when it is managed with high concentrations of plant-available P.

We have demonstrated the importance among pasture legume species of root morphology for P acquisition in low-P soil and achievement of a low critical external P requirement (Chapters 5, 6 and 7). *Ornithopus* spp. (serradellas), in particular, have a combination of relatively long root hairs, high specific root length (i.e. root length per unit dry mass) and relatively high root length density (root length per unit soil volume) (Chapters 5, 6 and 13), which allow them to maximise soil exploration and uptake of P in low-P soil (Chapter 6). The critical external requirement for P-fertiliser (i.e. the concentration of applied P required to achieve near-maximum yield) of *Ornithopus* spp. has been found to be less than half that of *T. subterraneum* (cv. Leura). *Trifolium subterraneum* develops a higher root length density, but fails to maximise soil exploration, because it has short root hairs (Chapters 5, 6 and 13). The relatively low specific root length of *T. subterraneum* roots also means that nutrient foraging incurs a relatively large carbon cost (Chapters 5, 6 and 13).

The root morphology of *Ornithopus* spp. represents a potential ideotype for P-efficient annual pasture legumes and indicates that selecting *T. subterraneum* for longer root hairs and/or higher specific root length could improve the potential for P acquisition. However, *T. subterraneum* had an outstanding ability to proliferate roots in in a nutrient-enriched zones of a soil in response to P stress (Chapters 6 and 8) and this may also be an important trait for P-acquisition efficiency in *T. subterraneum*.

Here we describe an experiment that quantified the shoot growth and root morphology acclimation of five cultivars of *T. subterraneum* in response to growth-limiting P supply and investigates the relative importance of root proliferation and the root morphology traits that contributed to their ability to forage for P in a low-P soil profile.

11.2 Materials and Methods

11.2.1 Plant material

The shoot growth and root morphology of five cultivars of *Trifolium subterraneum* L. grown in response to P supply was investigated. The cultivars were Leura (ssp. *subterraneum*), Losa (ssp. *subterraneum*), Napier (ssp. *yanninicum*), Riverina (ssp. *yanninicum*) and Seaton Park (ssp. *subterraneum*).

The experiment was conducted using a similar protocol to that described in Chapters 6 and 7). The soil was a sandy loam (Yellow Chromosol; Isbell 1996) with a low concentration of extractable P (8.3 mg kg^{-1} P; Colwell, 1963; method 9b in Rayment and Lyons 2011) and was collected from Ginninderra Experiment Station Canberra, ACT, Australia (35°10'30"S, 149°02'33.4"E). The soil was pasteurised to reduce disease inoculum (65°C for 1 h), sieved to < 5 mm and mixed with lime (1.06 g $CaCO_3$ kg^{-1}) to raise the pH (1:5 w/v, 0.01 M $CaCl_2$) to 5.2. Nutrients were mixed into the soil at rates of 41.1 mg kg^{-1} soil $MgSO_4.7H_2O$, 43.0 mg kg^{-1} $CaSO_4.2H_2O$, 169 mg kg^{-1} KNO_3, 27.5 mg kg^{-1} $(NH_4)_2SO_4$, 16.7 mg kg^{-1} NH_4NO_3, 119 µg kg^{-1} H_3BO_3, 759 µg kg^{-1} $MnCl_2.4H_2O$, 359 µg kg^{-1} $ZnSO_4.7H_2O$, 33.3 µg kg^{-1} $CuSO_4.5H_2O$, 72.1 µg kg^{-1} $(NH_4)_2MoO_4$, 19.8 µg kg^{-1} $CoCl_2 \cdot 6H_2O$ and 1530 µg kg^{-1} Fe-EDTA. Seven P-fertilised soil treatments were established by mixing KH_2PO_4 with subsamples of the amended soil at rates of 0, 10, 20, 40, 60, 80 and 250 mg P kg^{-1} soil. P application rates from 0 to 80 mg P kg^{-1} were selected to span deficient to moderately P deficient conditions for clover growth, whilst 250 mg P kg^{-1} was known to support the maximum clover growth rate (Haling *et al*. 2016). The $CaCl_2$-extractable P concentrations of the soil after all soil amendment treatments had been applied were: 76, 99, 117, 272, 420, 585 and 5150 µg kg^{-1} (method 9F2 in Rayment and Lyons 2011). Pots (cylindrical PVC; 87 mm internal diameter) were filled with a bottom layer of the soil (0.9 kg oven dry basis) that was not fertilised with P (the subsoil) and then with a topsoil layer using the P-fertilised soils (0.3 kg oven dry basis; 47 mm settled soil height). This stratification of P was intended to mimic the concentration of P that occurs in topsoil under pastures with the surface application of P fertiliser. The boundary of the fertilised topsoil and unfertilised subsoil layers was marked by placing small alkathene beads around the interior edge of the pot. The plants were inoculated with Group C rhizobium (strain WSM 1325, NewEdge Microbials, Albury, NSW, Australia).

Micro-swards of each cultivar were established by sowing 50 mg pot^{-1} of viable seed. Five replicates of each species by P treatment were established. Plants were grown in a controlled-environment growth cabinet [12 h light (620 µmol quanta m^{-2} s^{-1})/ 12 h dark; 25/15°C]. Pots were arranged in a randomised complete block design and rotated within blocks. Reflective sleeves were fitted to the outside of the pots and raised with plant height to reproduce the light conditions in a pasture sward. The soil was maintained at 75 to 80% of field capacity by daily watering to a predetermined weight and was watered to reach field capacity once per week. This water regime was designed to avoid the drying of the subsoil that can occur over the duration of an experiment if pots are only watered from above to maintain 75 to 80% of field capacity.

11.2.2 Harvest and measurements

Plants were harvested five weeks after sowing. Shoots were cut at the soil surface, dried at 70°C and weighed. Soil was removed as an intact core and cut at the interface of the

fertilised topsoil (47 mm height) and the subsoil. The topsoil was accurately cut vertically into two quarter segments and one half segment. Roots from each segment and from the subsoil were washed free of soil on sieves. The roots from one quarter segment of the topsoil were stored in 50% (v/v) ethanol at 4°C for measurement of root hair length and mycorrhizal colonisation. Roots from the second quarter segment were scanned immediately using a flatbed scanner (600 dpi) and root length and diameter were determined by image analysis using WinRHIZO software (Regent Instruments Inc., Quebec, Canada). The scanned root sample, the roots from the topsoil half segment, and the roots washed from the subsoil were dried at 70°C and weighed. Total length of roots in the topsoil was calculated by multiplying the specific root length (i.e. length per unit dry mass) of roots from the scanned quarter segment by the estimated total mass of roots in the topsoil ($^4/_3$ * the combined dry mass of roots from the scanned quarter and the un-scanned half segments).

Root hairs on roots from the fertilised topsoil (0, 10, 20, 60 and 250 mg P kg^{-1} treatments) were photographed using a Leica MZFLIII Fluorescence microscope (Leica Microsystems, Sydney, Australia) fitted with a Zeiss AxioCam camera (Zeiss, Sydney, Australia). The length of 15 root hairs per replicate was measured using ImageJ (Rasband 1997–2014).

Mycorrhizal colonisation (20 and 40 mg P kg^{-1} treatments) was measured using the grid-line intersect method (Giovannetti and Mosse 1980) after clearing (10% w/v KOH for 2-4 days followed by rinsing in water and 1% v/v HCl) and staining (5% v/v Schaeffer blue ink/ white vinegar solution for 1 h; Vierheilig *et al.* 1998).

Shoot and root samples were milled and a 25 to 50 mg sample was ashed at 550°C, dissolved in 2 M HCl and the P concentration was determined colourimetrically using malachite green (Irving and McLaughlin 1990).

The root hair cylinder volume (RHCV) of the roots in the topsoil was calculated as the volume of the cylinder enclosing the root and root hair zone.

$$RHCV = \pi * ([ARD/2] + RHL)^2 * RL \qquad \text{Equation [1]}$$

where: ARD = average root diameter, RHL = average root hair length measured in the topsoil and RL = total root length in the soil layer. RHCVs at 40 and 80 mg P kg^{-1} were determined using root hair lengths averaged across the 20 and 60, and 60 and 250 mg P kg^{-1} treatments, respectively.

Phosphorus content of the shoots, roots in the topsoil and roots in the subsoil were summed to calculate total plant P uptake per pot. Total plant P uptake was divided by the total surface area of the root hair cylinder in the topsoil to determine P uptake per unit surface area of the root hair cylinder.

Linear responses were fitted to the shoot dry matter of each *T. subterraneum* cultivar when grown with P-application rates between 10 and 80 mg P kg^{-1}. Shoot dry matter at 250 mg P kg^{-1} was assumed to represent the maximum growth of the cultivars based on previous experience growing *T. subterraneum* in this soil (Chapter 6). The intersection of maximum shoot yield and the linear response to P-application rates between 10 and 80 mg P kg^{-1} soil was considered to represent the critical external requirement for P.

Critical internal P concentration was determined as the herbage P concentration corresponding to the critical external P-application rate based on curvilinear responses between shoot P concentration and rate of P addition between 10 and 250 mg P kg^{-1}. Internal or physiological P-use efficiency (PUE; g shoot dry matter/ mg shoot P) was determined as the gradient of the linear relationship between shoot dry matter and shoot P concentration for P rates between 10 and 80 mg P kg^{-1}.

Root mass fractions were calculated separately as the mass of roots in the topsoil, the subsoil or the total root system divided by total plant mass.

The effect of P addition and cultivars on shoot dry matter, root dry matter, root mass fraction, specific root length, root hair length, root length density (root length per volume of soil), root hair cylinder volume and P uptake per unit surface area of root hair cylinder was analysed using general analysis of variance in GenStat 16th Edition (VSN International, UK). Data for P uptake per unit surface area of root hair cylinder were log transformed.

11.2.3 Effect of pot size on root proliferation in response to P stress

A second experiment was used to investigate the effect of pot size on shoot and root growth, and relative proliferation response of two cultivars with contrasting proliferation responses. Losa and Napier were selected based on a high and low proliferation response, respectively, as measured in the main experiment. Both cultivars were grown at 40 and 250 mg P kg^{-1} applied to the soil layer representing the top quarter of the mass of soil in pots that differed in their internal diameters (51, 63, 87 and 134 mm). Seed was sown at rates of 17, 26, 50 or 120 mg of viable seed per pot depending on pot diameter, to establish microswards with equivalent density of plants. All other soil preparations and plant growth conditions were as per the main experiment.

Plants were harvested five weeks after sowing. Shoots were harvested and dried as per the main experiment. For the 51 mm (40 and 250 mg P kg^{-1}) and 63 mm (250 mg P kg^{-1}) diameter treatments, roots were washed from the entire topsoil section, immediately scanned and root length and root dry mass determined as outlined above. For the 63 mm (40 mg P kg^{-1}) diameter treatments, the topsoil was divided into half segments; roots washed from one half were analysed for root length and dry mass, and those from the remaining half were used for dry mass determination alone. For the 87 mm diameter pots, the topsoil cores were sectioned into two quarter segments and one half segment. Roots from one quarter segment were analysed for length and dry mass, roots from the other quarter were stored in 50% (v/v) ethanol at 4°C in case analyses of root hair length or mycorrhizal colonisation were required, and roots from the remaining half segment were used for dry mass determination alone. For the 134 mm diameter pots, the topsoil cores were sectioned into two, one-eighth segments and a three quarter segment. Roots from a one-eighth segment were analysed for length and dry mass; roots from the other one-eighth segment were stored in 50% (v/v) ethanol at 4°C, and the remaining three-quarter segment was used for dry mass determination alone. As per the main experiment, the specific root length of the scanned samples was used to estimate the total root length in each sample based on the estimated total root dry matter in the topsoil.

The effect of pot size and P addition on shoot dry matter, shoot dry matter per unit pot area, root dry matter, root length density, root mass fractions and specific root length of the two

cultivars was analysed using general analysis of variance in GenStat 16th Edition (VSN International, UK).

11.3 Results

11.3.1 Shoot dry matter, critical internal P concentration and P-use efficiency

Shoot growth of four of the five cultivars of *Trifolium subterraneum* (Leura, Napier, Riverina and Seaton Park) was slow when no P was applied, but growth then increased linearly for all cultivars in response to P-application rates increasing from 10 to 80 mg P kg^{-1} (Fig. 11.1). Maximum shoot yields (i.e. shoot dry mass at 250 mg P kg^{-1}) among the five cultivars were generally similar; however, cultivars differed in their critical external requirement for P (i.e. the P application rate required for maximum yield) (Table 11.1). This reflected the range (up to a 1.5-fold) in the ability of the cultivars to yield at intermediate levels of P supply.

Among the cultivars, the critical internal P concentration (i.e. shoot P concentration corresponding to the critical external P requirement) was similar (0.34 for Leura to 0.4 for Napier) (Table 11.1). PUE (g shoot dry matter per mg of shoot P) ranged 1.3-fold among the five cultivars (Table 11.1).

11.3.2 Root dry matter and root mass fraction

All cultivars showed greater root dry mass in the topsoil (a 1.4- to 2.2-fold increase) in response to decreased supply of P in the topsoil layer (Fig. 11.2). However, the cultivars differed in the maximum root dry mass achieved in this layer, the level of P supply at which maximum root dry matter was attained, and their ability to maintain topsoil root dry matter at growth-limiting levels of P supply. For example, Leura and Riverina had the greatest root dry matter among the five cultivars (0.49-0.50 g) and this peak was achieved between 40 and 60 mg P kg^{-1}. Losa, Seaton Park and Napier achieved similar maximum root dry matter to each other (0.42-0.45 g), but Losa achieved this between 20 and 40 mg P kg^{-1}, compared with 40 to 80 mg P kg^{-1} for Seaton Park and Napier.

Table 11.1 Critical external requirement for phosphorus (P), critical internal requirement for P and physiological P-use efficiency (PUE; shoot dry matter per unit shoot P) for five cultivars of *Trifolium subterraneum*.

Cultivar	Critical external P requirement (mg P kg^{-1})	Critical internal P requirement (%)	Physiological PUE (g mg^{-1})
cv. Leura	120.4	0.34	255
cv. Losa	102.6	0.37	197
cv. Napier	168.2	0.40	231
cv. Riverina	136.9	0.38	228
cv. Seaton Park	114.5	0.37	238

The cultivars also differed in how their root dry matter in the subsoil responded to P supply in the topsoil. Napier and Riverina demonstrated a gradual increase in dry matter when P supply in the topsoil was lower, and consequently had more root dry matter in the subsoil at low levels of P supply than did other cultivars. In contrast, root dry matter of Leura was less when P supply was lower, while that of Losa showed no significant change. Seaton Park also showed no significant change in root dry matter in response to P supply, with the exception of increased dry matter in the unamended treatment.

The total root mass fraction (proportion of plant dry weight in roots) of all cultivars was increased when P supply was reduced in the topsoil layer (Fig. 11.3a). Maximum total root mass fractions of Losa and Napier was marginally lower (0.46-0.47%) than that of Leura, Riverina and Seaton Park (0.50-0.53%). Maximum total root mass fraction was achieved at rates of P supply equal to or less than 40 mg P kg^{-1}. On average, Losa had the lowest total root mass fraction, which was most evident at rates of P supply between 40 and 80 mg P kg^{-1}. When total root mass fraction was examined in relation to the relative shoot yield of the cultivars (a surrogate measure of P stress) (Fig. 11.3b), it was clear that all cultivars preferentially allocated similar proportions of dry matter to roots and that the lower peak total root mass fraction achieved by Losa and Napier was associated with a change in the preferential allocation of dry matter to roots at a relative shoot yield of approximately 0.45. The other cultivars continued preferential allocation of dry matter to roots until a relative shoot yield of approximately 0.3

Topsoil root mass fraction of all cultivars was increased in response to limited P supply in the topsoil layer. However, this response was not maintained at very low levels of P supply (Fig. 11.3c). At their maximum topsoil root mass fractions, all cultivars allocated approximately a third of their total biomass to roots in the topsoil layer. Seaton Park, Leura and Losa achieved this at lower rates of P supply (10-20 mg P kg^{-1}) than did Riverina and Napier (40 mg P kg^{-1}). This was also evident in the examination of topsoil root mass fraction in relation to relative yield (Fig. 11.3d).

All cultivars increased their allocation of biomass to roots in the subsoil layer (i.e. subsoil root mass fraction) in low-P soil (Fig. 3e). Subsoil root mass fraction increased from approximately 10% of total plant biomass at 250 mg P kg^{-1} to 20 to 30% in the un-amended soil treatment. Riverina and Napier allocated more biomass to their roots in the subsoil layer between 0 and 20 mg P kg^{-1} than the other cultivars. Losa had the lowest or amongst the lowest subsoil root mass fraction. This was also reflected in the examination of subsoil root mass fraction in relation to relative yield (Fig. 11.3f).

11.3.3 Root length density, specific root length and root hair length in the topsoil

The root length density (root length per unit soil volume) of the cultivars increased between 1.4- and 2.1-fold in response to decreased P supply in the topsoil (Fig. 11.4a). Seaton Park, Leura and Losa achieved similar maximum root length densities (25-27 cm cm^{-3}), but the cultivars differed in their ability to maintain high root length density at low P supply. Losa was able to maintain high root length density between 20 and 60 mg P kg^{-1}, while Leura

maintained high root length density between 40 and 60 mg P kg^{-1}. The root length density of Seaton Park equalled that of Leura and Losa at 80 mg P kg^{-1}, but was significantly lower than that of Leura at 40 and 60 mg P kg^{-1}, and significantly lower than that of Losa between 10 and 60 mg P kg^{-1}. Riverina and Napier had significantly lower maximum root length

Table 11.2 Average root diameter in topsoil for five cultivars of *Trifolium subterraneum* grown at seven rates of phosphorus (P) applied to the topsoil of a pot. Interaction of P applied x cultivar was not significant for $P<0.05$ (n=5).

	Average root diameter (mm)				
P applied	cv. Leura	cv. Losa	cv. Napier	cv. Riverina	cv. Seaton Park
0 mg kg^{-1}	0.328 ± 0.002	0.302 ± 0.003	0.360 ± 0.010	0.368 ±0.010	0.327 ± 0.004
10 mg kg^{-1}	0.315 ± 0.003	0.298 ± 0.003	0.337 ± 0.009	0.357±0.006	0.315 ± 0.003
20 mg kg^{-1}	0.318 ± 0.002	0.305 ± 0.003	0.335 ± 0.003	0.365±0.004	0.321 ±0.002
40 mg kg^{-1}	0.325 ± 0.003	0.303 ± 0.002	0.350 ± 0.003	0.353±0.004	0.325 ± 0.002
60 mg kg^{-1}	0.324 ± 0.004	0.299 ± 0.003	0.348 ± 0.008	0.356±0.005	0.333 ±0.004
80 mg kg^{-1}	0.329 ± 0.002	0.299 ± 0.003	0.344 ± 0.002	0.356 ±0.006	0.338 ±0.004
250 mg kg^{-1}	0.303 ± 0.002	0.273 ± 0.003	0.325 ± 0.005	0.338 ± 0.008	0.314 ± 0.004
LSD for P applied ($P<0.05$)	0.006				
LSD for cultivar ($P<0.05$)	0.005				

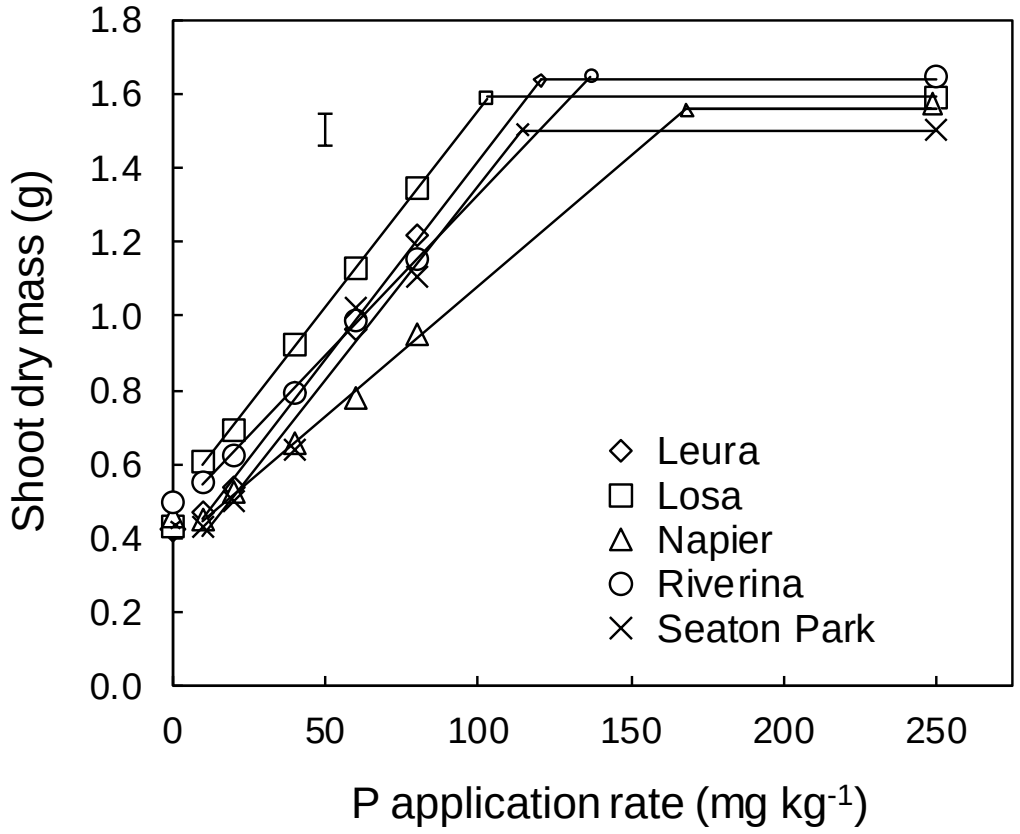


Figure 11.1 Shoot dry mass of five cultivars of *Trifolium subterraneum* grown in response to seven rates of phosphorus (P) applied to the topsoil of a pot (n=5). Bar shows LSD (*P*=0.05) for the Cultivar x P applied interaction. The critical external P requirement of each cultivar (i.e. P required to achieve maximum yield) is shown by the symbol of reduced size and was determined from the intersection of maximum shoot yield (i.e. shoot dry mass at 250 mg P kg^{-1}) and the linear yield response between 10 and 80 mg P kg^{-1}.

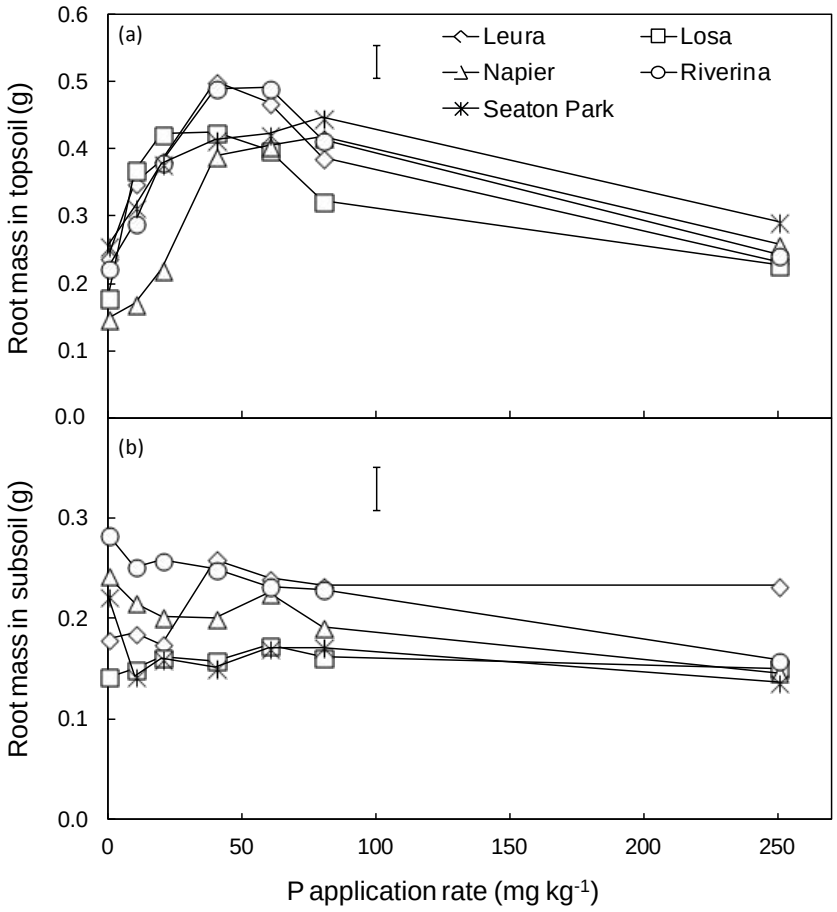


Figure 11.2 Root dry mass in (a) topsoil and (b) subsoil for five cultivars of *Trifolium subterraneum* grown with seven rates of phosphorus (P) applied to the topsoil (n=5). Bar shows LSD (*P*=0.05) for Cultivar x P applied interaction.

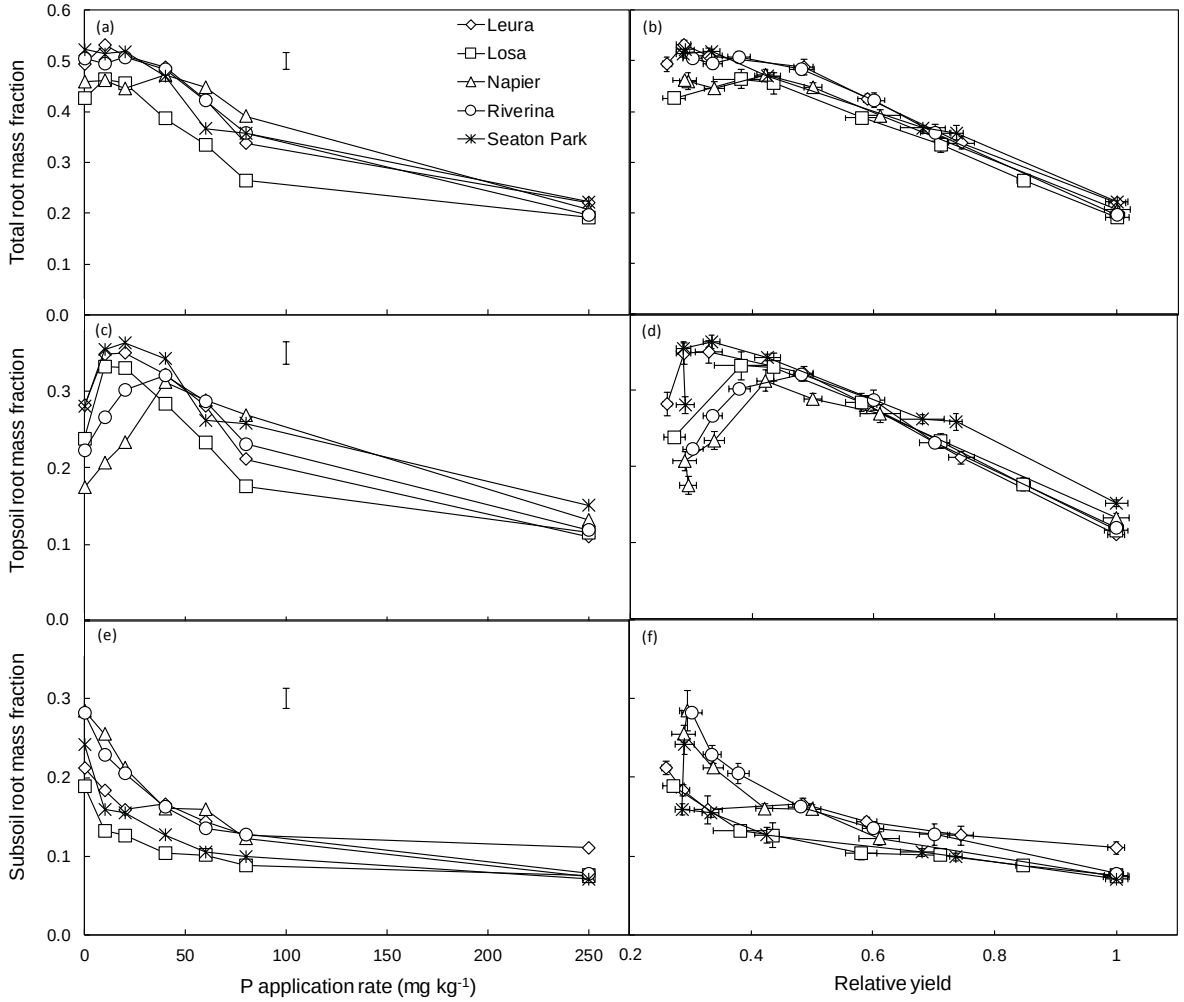


Figure 11.3 The response of the root mass fraction for (a) the total root system (c) the topsoil roots and (e) the subsoil roots for five cultivars of *Trifolium subterraneum* grown with seven rates of phosphorus (P) applied to the topsoil of a pot. The root mass fraction of the (b) whole root system (d) the topsoil roots and (f) the subsoil roots is also graphed in relation to the relative shoot yield achieved at each P supply rate (n=5). Bars in (a,c,e) show LSD (*P*=0.05) for Cultivar x P applied interaction. Error bars in (b,d,f) show ± standard error.

densities (21-22 cm cm^{-3}) than those of Leura and Losa, and root length density of Riverina and Napier was significantly lower than that of Leura and Losa at rates of P supply between 10 and 60 mg P kg^{-1}. Of the two, Riverina maintained a moderately high root length density when P supply was ≥40 mg P kg^{-1}, but displayed a marked decline in its ability to maintain root length density at lower P supply. Napier was unable to maintain moderately high root length density at rates of P supply <80 mg P kg^{-1}. The cultivars showed a similar pattern of change in root length density when the data were graphed against relative shoot yield, that is an initial increase followed by a decrease (Fig. 11.4b). However, the cultivars fell into two categories, with Losa, Leura and Seaton Park able to develop higher root length density than Napier or Riverina for a given level of P stress (i.e. relative shoot yield). High root length density was not maintained when relative yield fell below 0.4-0.5 for all cultivars except Napier which did not maintain high root length density when relative yield fell below 0.6.

Napier and Riverina had the lowest average specific root lengths (112 and 114 m g^{-1}, respectively) while Losa had the highest average specific root length (182 m g^{-1}) (Fig. 11.5a). Average specific root lengths of Leura and Seaton Park were between those of the other cultivars (144 and 145 m g^{-1}, respectively). Specific root length was decreased by 30% at the lower rates of P supply. There was no significant interaction (P>0.05) between cultivar and P supply on specific root length. At each rate of P supply, there was a strong negative linear correlation (R^2 0.75 to 0.98) between specific root length and average root diameter (Table 11.2) among the five cultivars (relationships not shown) indicating high specific root length was associated with smaller average root diameter.

Among the cultivars, average root hair length ranged from 0.36 mm (Leura) to 0.40 mm (Losa) (Fig. 5b). Root hairs were marginally shorter in the unamended soil compared with their lengths in soil to which P had been applied (average across cultivars of 0.36 cf. 0.38-0.39 mm). There was no significant interaction (P>0.05) between cultivar and P supply on root hair length. Mycorrhizal colonisation was measured on roots of plants grown at 20 and 40 mg P kg^{-1} and was very low (<1% of root length colonised; data not shown) and did not differ significantly among the cultivars.

11.3.4 Root hair cylinder volume and plant P uptake

The root hair cylinder volumes (a function of root diameter, root hair length and total root length) of the cultivars (Fig. 11.4c) followed similar trends to that of root length density (Fig. 11.4a) with similar thresholds in P supply rates and relative shoot yields below which the cultivars were not able to maintain a large root hair cylinder volume (Fig. 11.4d). Losa achieved the largest maximum root hair cylinder volume (80 cm^3 i.e. 29% of the volume of the topsoil) and maintained larger root hair cylinder volumes at lower rates of P supply (10-60 mg P kg^{-1}) than did all other cultivars. Napier had the smallest maximum root hair cylinder volume (55 cm^3; 20% of the volume of the topsoil) and had significantly smaller root hair cylinder volumes than that of all other cultivars at P rates between 10 and 60 mg P kg^{-1}. Leura, Seaton Park and Riverina demonstrated responses between those of Losa and Napier. Rating cultivars on the basis of the root hair cylinder developed and maintained in soil with low P supply (< 80 mg P kg^{-1}) was indicative of their potential yield in moderately P-deficient soil (Fig. 11.1) and their critical external requirement for P (Table 11.1).

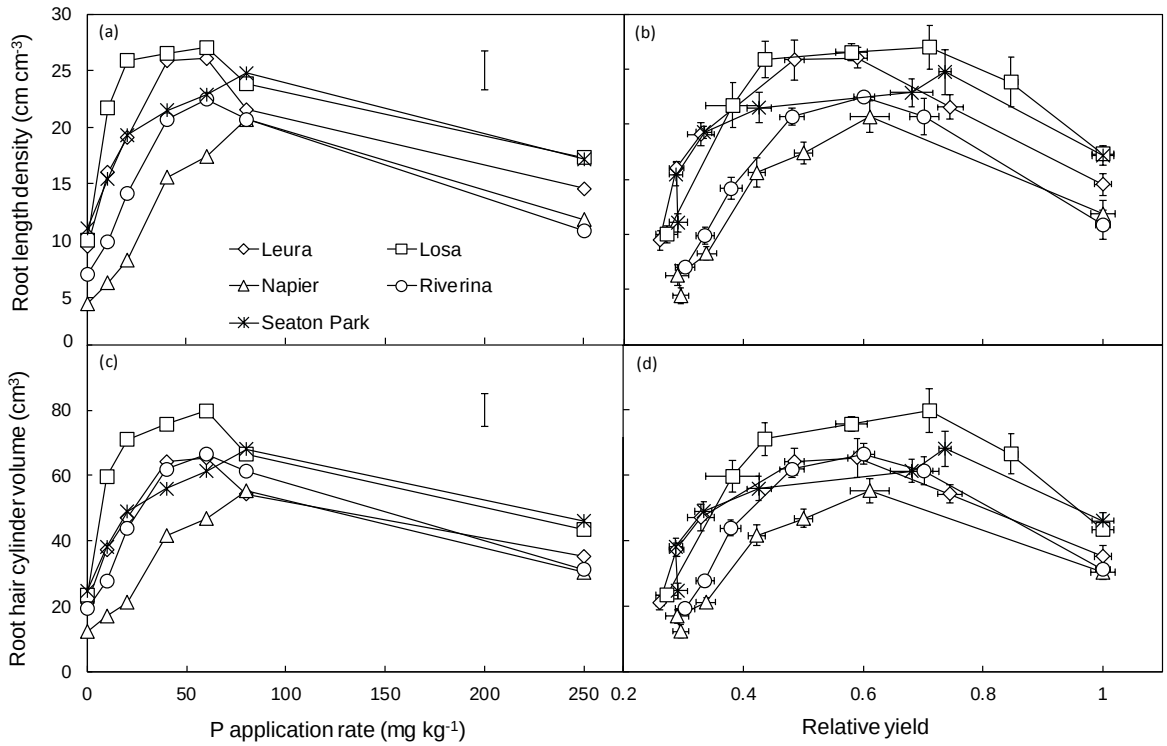


Figure 11.4 The response of (a) root length density in topsoil and (c) root hair cylinder volume in topsoil for five cultivars of *Trifolium subterraneum* grown with seven rates of phosphorus (P) applied to the topsoil of a pot. Topsoil (b) root length density and (d) root hair cylinder volume are also graphed in relation to the relative shoot yield achieved at each P supply rate (n=5). Bar shows LSD (P=0.05) for Cultivar x P applied interaction. Error bars in (b,d) show ± standard error.

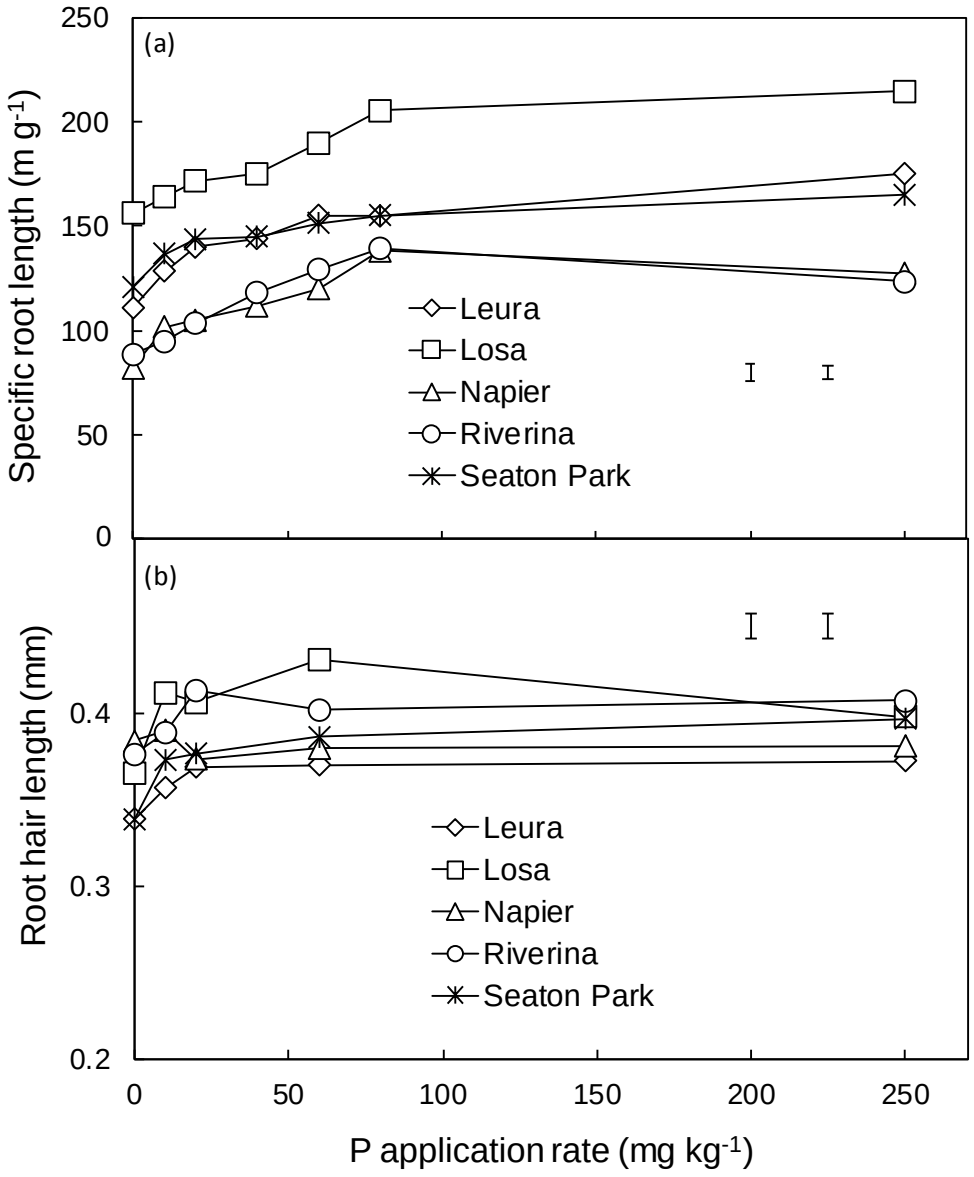


Figure 11.5 (a) Specific root length in topsoil and (b) root hair length in topsoil for five cultivars of *Trifolium subterraneum* grown with seven rates of phosphorus (P) applied to the topsoil of a pot (n=5). Bar shows LSD (*P*=0.05) for P applied and Cultivar, respectively. The interaction of P applied x Cultivar was not significant (*P*>0.05). Root hair length was not determined at 40 and 80 mg P kg^{-1}.

P uptake per unit surface area of the root hair cylinder of the topsoil roots was estimated by assuming that all P in the plants had been acquired from the topsoil. This parameter was generally greater when the plants were grown with P-addition rates above 20 mg P kg^{-1} (Fig. 11.6a). Between 40 and 80 mg P kg^{-1}, P uptake per unit surface area of the root hair cylinder was similar among most of the cultivars at a given P-addition rate. However, at rates of P addition less than 40 mg P kg^{-1} and greater than 80 mg kg^{-1}, Napier and Riverina showed significantly higher P uptake per unit surface area of their root hair cylinders than did the other cultivars. The relatively high rates of P uptake in very low P soil by Napier and Riverina were explored further by dividing P uptake per unit surface area of the root hair cylinder by the initial $CaCl_2$-extractable P concentration of the soil in which the plant was grown (i.e. an estimate of P concentration in soil solution) (Fig. 11.6b). It has been argued previously that the rate of P uptake under the cultural conditions used in this experiment is determined by the rate of P diffusion to the surface area of the root hair cylinder (Chapter 7). Consequently, Fick's Law indicates that P uptake per unit surface area of the root hair cylinder should be directly proportional to the P concentration gradient between the bulk soil and the surface area of the root hair cylinder (Barber 1984). Thus, the quotient of P uptake per unit surface area of the root hair cylinder divided by the $CaCl_2$-extractable P concentration of the soil should be approximately constant. This analysis indicated there was few if any differences in P uptake among the cultivars. The quotient was similar and relatively constant for all five cultivars when they were achieving relative yields <1.0 and >0.4. However, a P-stress threshold was reached at relative yields of about 0.3 - 0.4; below this the rate of P uptake by all cultivars (not just Napier and Riverina), whilst very low, was greater than expected given the concentration of $CaCl_2$-extractable P (Fig. 11.6b).

11.3.5 Effect of pot size on shoot and root response of Napier and Losa

In most instances, the shoot dry mass per unit area of the pot did not differ for a given cultivar grown at a given rate of applied P for pots with internal diameters between 63 and 134 mm (Fig. 11.6a). However, shoot dry mass per unit area was commonly lower when the plants were grown in pots with an internal diameter of 51 mm.

For root length density, there were significant two-way interactions for P applied x Pot size and P applied x Cultivar, but there was no significant three-way interaction or interaction between Pot size and Cultivar (Fig. 11.6b). On average, root length density was higher in the 40 mg P kg^{-1} soil than in the 250 mg P kg^{-1} soil. Root length density in the 250 mg P kg^{-1} soil did not differ across pot sizes, but root length density in the 40 mg P kg^{-1} soil was higher in pots with internal diameters of 63 to 134 mm relative to the 51 mm pot. Root length density of Losa was significantly greater than that of Napier when grown in soil with 40 mg P kg^{-1}, but not at 250 mg P kg^{-1}.

Specific root length increased with pot size, but mostly did not differ significantly between the two P treatments (Table 3 3). Losa had significantly higher specific root lengths than did Napier (Table 3.3).

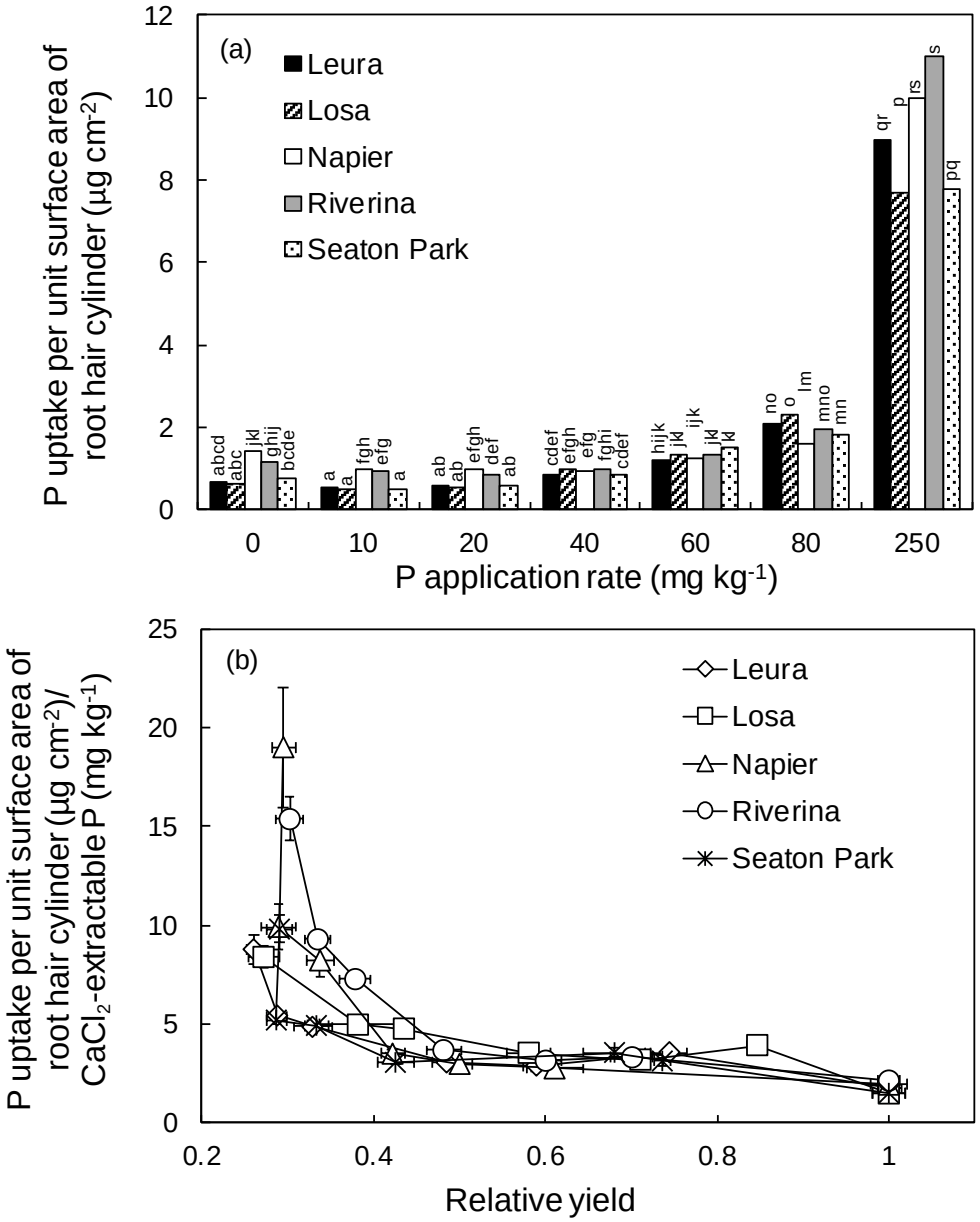


Figure 11.6 (a) Total plant phosphorus (P) uptake per unit surface area of root hair cylinder of topsoil roots for five cultivars of *Trifolium subterraneum* in response to seven rates of phosphorus (P) applied to the topsoil of a pot (n=5). Different letters denote significant differences among treatments (*P*=0.05). (b) P uptake per unit surface area of the root hair cylinder of topsoil roots divided by the initial $CaCl_2$-extractable soil P concentration of the topsoil layer graphed in relation to the relative shoot yield of the five cultivars of *T. subterraneum*; bars show ± standard error.

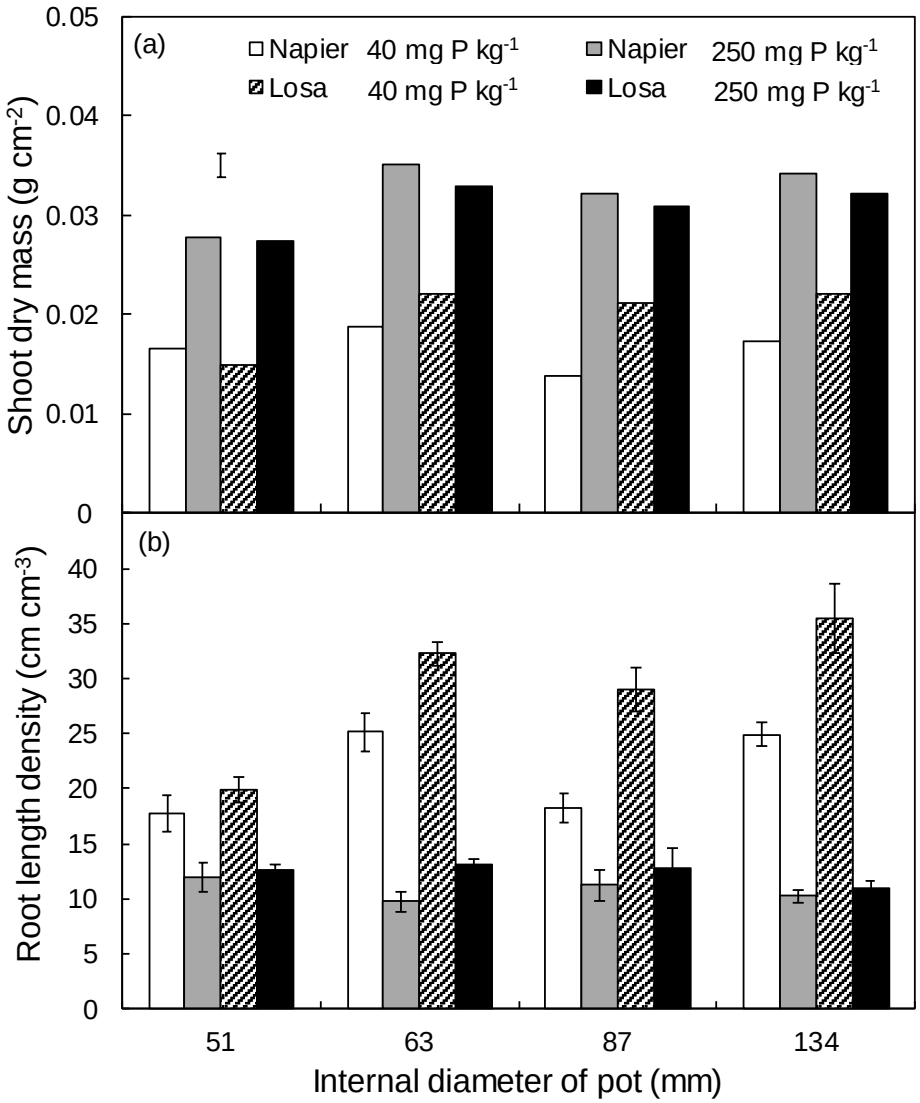


Figure 11.7 (a) Shoot dry mass per unit area of pot and (b) root length per unit volume of topsoil for each of two cultivars of *Trifolium subterraneum* grown at two rates of phosphorus (P) applied to the topsoil, in pots that differed in their internal diameter and, consequently, soil volume (n=5). Bars in (a) show LSD (*P*=0.05) for 3-way interaction for Cultivar x P applied x Pot size. Error bars in (b) show standard error; 3-way interaction was not significant (*P*>0.05).

Table 11.3 Specific root length in topsoil, total root mass fraction, topsoil root mass fraction and subsoil root mass fraction for two cultivars of *Trifolium subterraneum* grown in pots of four internal diameters at two rates of phosphorus (P) applied to the topsoil (n=5). n.s. indicates not significant.

	Specific root length (mg g^{-1})		Total root mass fraction (g g^{-1})		Topsoil root mass fraction (g g^{-1})		Subsoil root mass fraction (g g^{-1})	
	cv. Napier	cv. Losa	cv. Napier	cv. Losa	cv. Napier	cv. Losa	cv. Napier	cv. Losa
P applied 40 mg P kg^{-1}								
Internal diameter of pot (mm)								
51	108	161	0.417	0.410	0.273	0.232	0.144	0.178
63	128	238	0.413	0.307	0.291	0.200	0.122	0.107
87	130	247	0.427	0.282	0.276	0.187	0.152	0.094
134	146	244	0.430	0.322	0.270	0.212	0.160	0.110
P applied 250 mg P kg^{-1}								
Internal diameter of pot (mm)								
51	105	157	0.255	0.244	0.142	0.105	0.113	0.139
63	106	193	0.179	0.164	0.100	0.081	0.079	0.082
87	144	247	0.169	0.145	0.094	0.066	0.075	0.079
134	150	211	0.153	0.156	0.081	0.065	0.072	0.091
LSD for P applied x Pot size	18							
LSD for P applied x Cultivar	13							
LSD Pot size x cultivar	18							
LSD 3-way interaction (*P*=0.05)	n.s.		0.027		0.019		0.023	

Total, topsoil and subsoil root mass fractions were mostly similar for a given cultivar grown at a given rate of P for pot sizes between 63 and 134 mm. However, in pots with an internal diameter of 51 mm, Losa had significantly higher total root mass fractions than at other pot sizes at both rates of applied P, and Napier had significantly higher total root mass fraction than at other pot sizes at 250 mg P kg^{-1}. This was driven by increases in root mass fractions in both the topsoil and subsoil.

11.4 Discussion

11.4.1 Pot size and root proliferation

Inappropriate choice of pot size in plant growth experiments can potentially restrict roots, limit shoot growth and lead to erroneous conclusions about plant growth responses (Poorter *et al*. 2012). Given the focus of the present experiments on root proliferation in response to soil P supply, an auxiliary experiment using the cultivars Napier (which had a relatively low root proliferation response) and Losa (high root proliferation response) was conducted using pots of contrasting internal diameter (51-135 mm internal diameter) to assess whether the pot size used in the main experiment (87 mm) would influence shoot growth or root length proliferation responses. Similar yields and responses in root length density in low P soil were observed for all pot sizes except when the clover swards were grown in the smallest diameter pots (51 mm internal diameter). The very small pots constrained plant growth substantially and the difference between the cultivars in root proliferation was eliminated. This confirmed the need for caution when using very small soil volumes for assessing root responses to soil nutrients (Poorter *et al*. 2012). However, the experiment also indicated that shoot yield and root growth were not influenced by the size of pot in which the main experiment was grown.

11.4.2 Dry matter allocation to nutrient foraging roots

All of the *T. subterraneum* cultivars achieved similar maximum shoot yields in the high-P soil (250 mg P kg^{-1}), but the largest yields in low-P soil were associated with development of high root length density and a large root hair cylinder volume in the P-amended topsoil. In part, this was because dry matter was partitioned preferentially to the growth of nutrient-foraging roots and in the most P-efficient cultivars to their ability to maintain dry matter allocation to the P-enriched topsoil layer when P supply was insufficient for maximum clover growth.

11.4.3 Specific root length and root hair length modify the effectiveness of dry matter allocation to nutrient foraging

The relative effectiveness of dry matter partitioning to nutrient foraging was determined by differences among the *T. subterraneum* cultivars in their specific root length and root hair length. Many plant species respond to low soil P by increasing specific root length and root hair length (Chapters 5 and 7; Datta *et al*. 2015; Bates and Lynch 1996). However, the *T. subterraneum* cultivars did not make favourable adjustments in these root traits (i.e. root hair length was not increased significantly in response to low-P soil, and specific root length was reduced in low-P soil). The effectiveness of dry matter allocation to roots for nutrient foraging was, instead, dependent on the intrinsic specific root length and root hair length characteristics of each cultivar.

This is illustrated particularly clearly by examining the contrasts in development of root length and root hair cylinder volume by Losa and Napier. Losa was notable for its relatively high shoot yield at intermediate levels of P supply. This was associated with its ability to develop and maintain the highest root length density and root hair cylinder volume in the topsoil layer at low rates of P application. However, Losa did not allocate the largest mass of roots to the topsoil. High potential for soil exploration was a consequence of allocating root mass to nutrient foraging combined with its relatively high specific root length. In contrast, the poorest performing cultivar (Napier) had a much lower maximum root length density and root hair cylinder volume and did not maintain its root length density or root hair cylinder volume even in moderately P-deficient soil. This was associated with low specific root length and an apparent failure to continue to allocate plant biomass to the fertilised topsoil layer at rates of P application <60 mg P kg^{-1} soil. Instead, Napier allocated root mass to the subsoil, even when P supply was greater in the topsoil. This phenomenon was also observed for the cv. Riverina. It is not known whether this was a cause or effect of the inability to acquire sufficient P for plant growth. However, it warrants further investigation, as it may reflect differences in the sensing of marginal (but still potentially beneficial) concentrations of P in the soil.

The root hair lengths of the five *T. subterraneum* cultivars were short (<0.45 mm) and differences in root hair length among the cultivars were relatively small. For example, the longest root hair lengths (Losa and Riverina) were only ~10% longer than those of Leura (shortest). Nevertheless, the impact on the development of root hair cylinder volume was significant, as shown by the differences in maximum root hair cylinder volume achieved by Losa and Leura, which developed similar maximum root length densities in the topsoil. Comparative studies of the root morphology and P-acquisition attributes of *Ornithopus* spp. (which have relatively long root hairs and are able to achieve equivalent maximum shoot yields to *T. subterraneum,* but with substantially lower rates of applied P), indicate that the short root hairs of *T. subterraneum* limit the P acquisition capacity of the clover (Chapters 5, 7 and 13). However, presently root hair lengths have only been reported for a limited range of clover lines and a wider survey to define the extent of intra-specific variation in root hair lengths among *T. subterraneum* genotypes is warranted.

11.4.4 Acclimation of roots to low P supply

The soil P thresholds for root mass (and consequently root length) allocation to nutrient foraging differed substantially among the cultivars. We assessed whether the degree of P stress being experienced by each cultivar was a trigger for these adjustments in root mass and length by graphing root mass fraction, root length density and root hair cylinder volume against relative shoot yield. We assumed relative shoot yield was a reasonable surrogate measure of the nutrient stress being experienced by each cultivar. Initially, all cultivars deployed equivalent root mass to nutrient foraging as P stress impacted their growth (Fig. 11.3d). However, the cultivars with the poorest ability to acquire P in low P soil (i.e. Napier, Riverina) stopped allocating mass to foraging roots when less P-stressed (i.e. at a relative yield of ~0.4) than the cultivars with the best P-acquisition abilities (Fig. 11.3d). The superior cultivars (e.g. Losa, Leura) benefitted from having: (i) higher specific root length (i.e. more root length and capacity for P uptake for every unit root mass deployed to foraging) which delayed their experience of P stress; and (ii) the ability to continue allocating mass to foraging roots even when highly P-stressed (relative yields >0.3).

Interestingly, for Napier and Riverina, P uptake per unit surface area of root hair cylinder was greater than that of the other three cultivars at the three lowest rates of P application (Fig. 11.6a). This may potentially indicate additional low-P acclimation responses by these genotypes (e.g. release of root exudates that facilitate elevated P availability). The quotient of P uptake per unit surface area of the root hair cylinder divided by the $CaCl_2$-extractable P concentration was graphed to examine whether these relatively high rates of P uptake were greater than might be expected by P diffusion in the soil solution alone (Fig. 11.6b). Indeed, this was confirmed, but it was also evident that P uptake rates per unit root hair cylinder surface area were elevated for all of the cultivars when they were extremely P-stressed (i.e. relative yields 0.3-0.4; Fig. 11.6b). We concluded that a marginal enhancement in P uptake rate under these circumstances was of limited value for the P-stressed plants and was probably a consequence of extreme P distress, rather than a useful acclimation trait. For this reason, it was also more obvious in cultivars with the poorest P-acquisition capabilities which experienced greater P stress in moderately P-deficient soil.

11.5 Conclusions

This experiment demonstrated that dry matter partitioning to nutrient foraging roots and the specific root length of these roots were the major factors determining the differential ability of five cultivars of *T. subterraneum* to acquire P and yield well in soil with low concentrations of plant-available P. The primary result of these root system attributes was development of a high root length density in the P-enriched zone of the soil profile. The cultivars that were most effective at acquiring P also exhibited a superior ability to maintain high root length density when soil P fertility levels were very low. The results from this study and Chapters 9 and 10 demonstrate that there is potentially useful variation within *T. subterraneum* for root proliferation and specific root length that could be used to breed cultivars with improved P-acquisition efficiency. However, short root hairs on *T. subterraneum* remain as a significant constraint to more effective nutrient foraging and useful variation in root hair length within the species is also needed if levels of P efficiency equivalent to that of the *Ornithopus* spp. is to be achieved.

Acknowledgements

The authors thank Branka Culvenor for technical assistance. The experiments were completed within the current project with data analysis continuing under “RnD4P-15-02-016 Phosphorus Efficient Pastures” a project supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme and from MLA, Dairy Australia, and AWI. Megan Ryan was also funded by an ARC Future Fellowship (FT140100103). Participation in the experiment by Laurie Brown and Tim George (The James Hutton Institute, Dundee, UK) was funded by an International Society of Root Research travel grant and supported by the Scottish Government through RESAS workpackage 3.3. The collaboration was possible as a result of a visit to CSIRO coinciding with the 9th Symposium of the International Society for Root Research (Canberra 2015).

12 High variation in the percentage of root length colonised by arbuscular mycorrhizal fungi among 139 lines representing the species subterranean clover (*Trifolium subterraneum*).

Megan H. Ryan, Daniel R. Kidd, Graeme A. Sandral, Zongjian Yang, Hans Lambers, Richard A. Culvenor, Adam Stefanski, Phillip G.H. Nichols, Rebecca E. Haling, Richard J. Simpson

Published 2016, Applied Soil Ecology **98**, 221-232.

12.1 Introduction

Most pastures used for sheep and beef production in southern Australia are based on annual pasture legumes, the most widespread being subterranean clover (*Trifolium subterraneum* L.) (Nichols *et al*. 2012). Colonisation of subterranean clover roots by arbuscular mycorrhizal fungi (AMF) is ubiquitous and a diverse population of these fungi is likely to be present (Abbott and Robson 1982; Ryan and Kirkegaard 2012; Simpson *et al*. 2011a; Tibbett *et al*. 2008). Phosphorus (P) fertiliser is routinely applied to these pastures (McLaughlin *et al*. 2011) as soils often contain insufficient plant-available P for rapid growth of annual pasture legumes and/or accumulate P in forms unavailable to plants when fertilised. The P-balance efficiency (i.e. the percentage of P inputs harvested as outputs) of pasture systems is low, and this is a concern given the non-renewable nature of rock phosphate reserves (Cordell *et al*. 2009; Scholz *et al*. 2013). Indeed, across southern Australia, Weaver and Wong (2011) found the median P-balance efficiency to be as low as 11% for sheep, 19% for beef and 29% for dairy enterprises. The major source of P inefficiency in these pasture systems is the loss of fertiliser P into soil pools not accessible by plants, although loss of P by erosion, run-off and leaching may also occur (McLaughlin *et al*. 2011; Simpson *et al*. 2014). Substantial savings in P-fertiliser use, and improvements in fertiliser P-use efficiency, may be possible if target levels of plant-available P in these pasture soils could be reduced, while maintaining yields (Simpson *et al*. 2014). A key strategy to achieve this would be to develop pasture cultivars with lower P requirements than present cultivars (Simpson *et al*. 2014). Selection of cultivars better able to forage P from soil is a key way forward (Lynch 2011; Richardson *et al*. 2011).

Subterranean clover may have a higher P requirement than some other annual pasture legumes. For instance, in a series of field trials in Western Australia with a large range of P-addition levels, subterranean clover had a critical P requirement (the level of plant-available soil P required for 90% of maximum yield) greater than that of serradella (*Ornithopus* spp.) (Bolland and Paynter 1992). Thus, one strategy to produce more P-efficient pastures may be to aim for widespread adoption of alternative annual pasture legumes with a lower critical soil P requirement than subterranean clover. A number of alternative legume species are increasingly being adopted in southern Australia, but little is known about their P requirements (Loi *et al*. 2005; Nichols *et al*. 2012). However, the broad adaptation of subterranean clover to the soils and climatic conditions across southern Australia, and its suitability to common management practices such as continuous grazing and ley farming

(where pastures naturally regenerate following a cropping phase) (Nichols *et al*. 2012), make reduction of its critical soil P requirements also a priority.

An important initial objective, therefore, is to identify subterranean clover germplasm with morphological or physiological root traits suggestive of a superior ability to forage for P in soil. Morphological traits include increased average root hair length, greater specific root length (which could be achieved through a lower average root diameter) and increased root distribution in topsoil, where soil P tends to be concentrated in pastures (Crush *et al*. 2008; Hill *et al*. 2006; Ho *et al*. 2005; McLaughlin *et al*. 2011). Physiological traits, such as exudation of organic anions and phosphatases into the rhizosphere, could enhance plant P uptake from sparingly-available soil P (Richardson *et al*. 2011). However, AMF must also be considered. In glasshouse experiments, inoculation of AMF into pasteurised soil reduces the critical soil P requirement of subterranean clover due to an increase in plant P uptake and growth when soil P availability is low (Abbott and Robson 1977; Schweiger *et al*. 1995). Moreover, AMF can change host plant morphological and physiological root traits that affect plant P uptake. For instance, inoculation with AMF may result in a lower root–shoot ratio (Abbott and Robson 1978) or reduced amounts of rhizosphere organic anions (Nazeri *et al*. 2014). Thus, AMF may need to be present when screening subterranean clover for variation in root traits associated with P uptake if the ranking of lines is to be relevant to field conditions.

There are around 10,000 lines of subterranean clover available for screening for root traits associated with P uptake; these are stored in Genetic Resource Centres around the world (Nichols *et al*. 2013). Most of these lines originate from southern Europe, the Middle East and northern Africa (Ghamkhar *et al*. 2015). In Australia, subterranean clover was introduced in the 19th century, and commercialised in the early 20th century (Nichols *et al*. 2012); 45 cultivars have been registered. An active breeding programme continues to release cultivars with updated characters that in recent years have included resistance to insect pests and root pathogens (Nichols *et al*. 1996; 2013). To aid this breeding programme, the 10 000 available lines were used to develop a “core collection” of 97 lines (Nichols *et al*. 2013). These 97 “core lines” are estimated to represent almost 80% of the total diversity of the species (Nichols *et al*. 2013).

Core collections are a useful tool for breeders because they allow the variation in all collections to be represented in a small sample size, making screening for traits of interest practically and economically feasible. Core collections have been developed for other crop and pasture species including soybean (*Glycine max* L.) (Qiu *et al*. 2011), chickpea (*Cicer arietinum* L.) (Upadhyaya and Ortiz 2001), bladder clover (*Trifolium spumosum* L.) (Ghamkhar *et al*. 2008) and biserrula (*Biserrula pelecinus* L.) (Ghamkhar *et al*. 2012). Variation in root traits associated with P-uptake efficiency has been found within the core collection of *Cucumis melo* L. (Fita *et al*. 2011). No core collection has been screened for colonisation level by AMF and genotypic variation in colonisation is poorly understood. For instance, colonisation in 27 cultivars of Welsh onion (*Allium fistulosum*) ranged from 48–81% of root length (Tawaraya *et al*. 2001) and when An *et al*. (2010) assessed colonisation in over 200 lines of maize (*Zea mays* L.) sown in each of two consecutive years in the field, variation in colonisation level was again large (~10–80% of root length). In contrast, colonisation level varied only from 70–75% of root length among 23 wild emmer (*Triticum turgidum* subsp. *dicoccoides*) accessions (Yucel *et al*. 2009), from 10–18% of root length

among 10 cultivars of Canadian hard red spring wheat (*Triticum aestivum* L.) (Kirk *et al*. 2011), and did not differ among 21 newly-bred wheat cultivars in China (Mao *et al*. 2014).

This paper reports three glasshouse experiments. 1) Screening of the 97 lines in the core collection and 42 cultivar lines of subterranean clover in a low-P field soil with an indigenous population of AMF for the percentage of root length colonised by AMF to determine the degree of variation in colonisation and whether it is greater for the core lines than the cultivar lines. 2) Screening of two cultivars for the percentage of root length colonised by AMF in 11 field soils to assess the robustness of rankings in Experiment 1. 3) Inoculation of AMF into pasteurised and unpasteurised field soil to assess whether root traits associated with P uptake of two cultivars changed in the presence of AMF. The overall objectives were to determine whether there is potential to select for subterranean clover with higher or lower colonisation by AMF and to determine whether AMF should routinely be included in screens of subterranean clover germplasm for root traits related to P uptake.

12.2 Materials and methods

12.2.1 Experiment 1. Colonisation by AMF of core lines and cultivar lines of subterranean clover heading

12.2.1.1 Soil

A sandy-loam soil (8% clay, 82% sand, 10% silt) (soil "FH" in Table 12.1) was collected at 0–40 cm depth from remnant bush at The University of Western Australia (UWA) Future Farm located at Pingelly Western Australia (32° 30' S, 116° 59' E) on 25 March 2013.

A subsample of soil was sent to CSBP laboratories (Bibra Lake Western Australia) for analysis. Unless otherwise specified, soil analysis methods followed those of Rayment and Lyons (2011) and codes from this reference are supplied: bicarbonate-extractable P (9B) and potassium (18A1; Colwell (1965)), P-buffering index (912C; Allen and Jeffrey (1990)), mineral nitrogen (N) (ammonium-N plus nitrate-N) (7C2b; Searle (1984)), extractable sulfur (10D1; Blair *et al*. (1991)), pH and conductivity in a soil:solution ratio of 1:5 (4A1, 4B3, 3A1), organic carbon (6A1; Walkley and Black (1934)) and particle size (Indorante *et al*. 1990).

The soil had low extractable P (5.8 mg kg^{-1}), a high P-buffering index (591) and was quite acidic (pH_{CaCl2} 4.7). The soil was chosen because preliminary experiments showed roots of plants grown in it became highly colonised by AMF, but did not show symptoms of the root pathogens that commonly affect pastures of subterranean clover in Australia (O'Rourke *et al*. 2009). The soil was dried at 40ºC for one week and thoroughly mixed; it was not sieved, but large rocks and debris were removed by hand.

12.2.1.2 Experimental design and plant growth

The experiment examined the 97 lines of the subterranean clover core collection (core lines) (Nichols *et al*. 2013), which consisted of 95 lines designated L01-L95 and two cultivars, Urana (L96) and Coolamon (L97), and an additional 42 cultivar lines of subterranean clover, some of which are no longer commercially available (Nichols *et al*. 1996, 2013). Overall, there were 35 lines of ssp. *brachycalycinum*, 97 lines of ssp. *subterraneum* and seven lines of ssp. *yanninicum*. For a description of the three subspecies, see Nichols *et al*. (2013). All seed were supplied by the Department of Agriculture and Food Western Australia (DAFWA)

and originated from a single seed-bulking trial conducted in 2011 at the UWA Shenton Park Field Station (31°57'S, 115°50'E).

The experiment was located in a naturally-lit glasshouse at UWA (31° 98' S, 115° 81' E) which reached an average daily maximum light intensity of 1686 µmol m^2 s^{-1} and had an average diurnal temperature range of 13–24ºC. Sterilised, free-draining, pots (90 × 90 × 180 mm) had a Whatman No. 5 filter paper placed in the bottom before filling with 1 kg of soil. Pots were watered to 100% field capacity two days prior to sowing. Seeds were surface sterilised, scarified and germinated in deionised water in a Petri dish on Whatman No. 5 filter paper at room temperature. Once a radicle had emerged, three germinated seeds were transferred to each pot. There were four replicate pots of each line. One replicate was sown every week for four weeks, commencing on 15 April, 2013 (mid-spring). Pots from each replicate were randomly arranged in a block and re-randomised weekly. Harvest was staggered by replicate to ensure all plants were harvested after six weeks of growth.

After sowing, a shallow layer of alkathene beads was applied to each pot to reduce evaporation. Group C rhizobium peat mix, containing strain WSM1325, (Becker Underwood, Somersby, New South Wales) was added. Plants were thinned to one per pot after one week. To ensure P was the only nutrient limiting plant growth, 1 mL of the following solution was added to each pot after two weeks of growth; 156 K_2SO_4, 151 $MgSO_4$, 1.3 $ZnSO_4$, 0.216 Na_2MoO_4 and 80 NH_4NO_3 (g L^{-1}). To ensure that the pH did not become more acidic by these additions, 0.5 g $CaCO_3$ was applied to each pot. Pots were maintained at field capacity by watering to weight every 2-3 days; watering did not result in loss of water through pot drainage holes.

12.2.1.3 Harvest

At harvest, shoots were removed, washed in deionised water, dried at 60ºC for three days, weighed and finely ground. A 0.1 g representative subsample was then digested in a 3:1 HNO_3:$HClO_4$ solution and total P was measured using a UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan) by the yellow vanadomolybdate method (Kitson and Melon, 1944). Roots were washed in deionised water and then root length (for calculation of specific root length) and average root diameter were measured after scanning fresh roots floating in water using the WinRHIZO 4.1 software package (Regent Instruments Inc., Quebec, Canada, 2000). Roots were then dabbed dry on a paper towel, fresh weight recorded, and a representative subsample taken, weighed and stored in 50% (v/v) ethanol for later analysis of colonisation by AMF. The remaining roots were dried at 60ºC for three days, weighed and total root dry mass calculated. Root mass ratio (g g^{-1}) was calculated as: (total root DM)/(total root DM + shoot DM).

Root subsamples were rinsed in deionised water, placed in 10% (w/v) KOH for ~4 days at room temperature, rinsed again with deionised water and then with a 1% (v/v) HCl solution, before staining in a 5% (v/v) Schaeffer blue ink/vinegar solution for 1 hour and storage in lactoglycerol (Vierheilig *et al*. 1998). The gridline intersect method was used to determine the percentage of root length colonised by AMF (Giovannetti and Mosse, 1980).

Table 12.1 *Characteristics of the 11 soils used in the three experiments*

Soil	Experiment	Location	Paddock use	Extractable phosphorus (Colwell) (mg kg^{-1})	Organic carbon (%)	PBI†	Mineral nitrogen (mg kg^{-1})	Extractable potassium (Colwell) (mg kg^{-1})	Sulfur (mg kg^{-1})	Conductivity (dS m^{-1})	pH ($CaCl_2$)	Clay (%)	Sand (%)	Silt (%)	SARDI qPCR assays for AMF*	Groups of AMF present (out of 6)
FH	1, 2	Pingelly	Bush	5.8	2.5	591	3	79	19.4	0.149	4.7	8	82	10	8 172	2
M	2, 3	Mukinbudin	Pasture	6.3	0.81	45	9	257	3.6	0.024	4.7	11	82	7	2 471	4
P	2, 3	Pinjarra	Pasture	8.0	0.59	21	4	44	1.7	0.023	5.6	4	95	1	21 464	6
W	2	Pingelly	Pasture	25	2.11	51	10	188	8.6	0.052	4.7	9	84	7	58 166	3
I	2	Pingelly	Pasture	33	2.60	47	12	293	4.8	0.065	4.9	8	85	7	71 807	6
T	2	Pingelly	Crop	37	0.48	26	1	55	1.9	<0.010	4.6	6	89	5	142 720	5
R	2	Pingelly	Pasture	47	1.86	39	6	75	3.6	0.037	4.8	10	86	4	41 054	4
2T	2	Pingelly	Pasture	56	1.26	26	10	105	10.8	0.108	4.8	6	92	2	5 712	2
HD	2	Pingelly	Crop	75	2.28	52	8	138	4.7	0.039	5.2	9	85	6	100 788	2
NL	2	Pingelly	Crop	110	2.15	75	4	129	5.5	0.031	5.0	9	83	8	125 544	4
30D	2	Pingelly	Pasture	131	1.93	64	5	37	11.3	0.113	4.6	10	84	6	89 842	3

† PBI = phosphorus-buffering index
* Total copies (per g of soil)

12.2.1.4 Statistical analysis

Data from the cultivar lines and core lines were analysed in Genstat version 14.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK) using a one-way ANOVA to assess the effect of line on the percentage of root length colonised by AMF; the effect of block was included in the analysis. Normality was checked and transformations were not required. Simple regressions were undertaken on the mean for each core and cultivar line (i.e. n=139) in Sigmaplot version 12 (Systat Software Inc).

12.2.2 Experiment 2. Colonisation by AMF of two cultivars in 11 soil types

12.2.2.1 Soils

The top 10 cm from eight field soils was collected from the UWA Future Farm at Pingelly, three from cropping paddocks and five from pasture paddocks (Table 12.1); a remnant bushland soil from 0–40 cm depth (used in Experiment 1, "FH" in Table 12.1) was also included. In addition, a sandy red loam was collected from 0–30 cm depth from a pasture paddock at Mukinbudin (30º 56' S, 118º 15' E) in the eastern wheatbelt and a texture-contrast sand was collected from a permanent pasture paddock at 10–30 cm depth from Pinjarra (32º 62' S, 115º 87' E) on the coastal plain south of Perth. Soils were dried at 40ºC for one week, thoroughly mixed, and a representative subsample taken for analysis as described for Experiment 1. The extractable P of the 11 soils varied greatly from 8.5–131 mg kg^{-1} (Table 12.1). All soils were acidic (pH_{CaCl2} 4.6–5.6), low in mineral N (1–12 mg kg^{-1}) and low in organic matter (<2.5%) (Table 12.1). Texture varied only a little, with clay ranging from 4–11%, sand from 82–95% and silt from 1–10% (Table 12.1).

A subsample of soil was also analysed for AMF using a commercial assay by SARDI (South Australian Research and Development Institute) Plant and Soil Health (Adelaide, South Australia) using six qPCR assays (AMFa, a2, b, c2, d and e). Each assay detects several species. Methods are presented by Simpson *et al*. (2011). The total copies per g of soil varied more than 10-fold and were highest in the three cropping paddocks (Table 12.1). The number of assays which detected AMF varied from 2–6 among the 11 soils (Table 12.1). The most prominent group was generally AMFa, which contains *Glomus mosseae*.

12.2.2.2 Experimental design and plant growthl

The experiment was a fully-crossed design of cultivar (Riverina, Woogenellup) × soil type (11 field soils) with four replicates. It was located in a naturally-lit glasshouse at UWA which reached an average daily maximum light intensity of 1637 µmol m^2 s^{-1} and had an average diurnal temperature range of 15-24ºC. Riverina and Woogenellup were chosen as representatives of ssp. *yanninicum* and ssp. *subterraneum*, respectively. Free-draining pots (90 × 90 × 180 mm) were filled with 1 kg of unsieved soil and wet to 70% field capacity two days prior to sowing. Ten mL of nutrient solution was applied to all soils three times during the experiment to ensure nutrients other than P were not limiting (150 KCl, 236 $Ca(NO_3)_2$, 493 $MgSO_4$, 160 NH_4NO_3, 55 Fe-EDTA, 2.9 H_3BO_3, 1.8 $MnCl_2$, 0.22 $ZnSO_4$, 0.051 $CuSO_4$, and 0.12 Na_2MnO_4 (g L^{-1}); 136 g L^{-1} of KH_2PO_4 was included once. Seed were germinated as described for Experiment 1. Seed weight was 1.02 and 0.85 g 100 $seeds^{-1}$ and seed P concentration was 6.73 and 8.46 g kg^{-1} for Woogenellup and Riverina, respectively. Once a radicle had emerged, five seeds were planted into each pot on 17 February 2014 (late-summer) at a depth of 1 cm. Rhizobia were added as described for Experiment 1 and a

shallow layer of alkathene beads applied to the soil surface. Pots were maintained at 70% field capacity by watering to weight every 2-3 days; watering did not result in loss of water through pot drainage holes. The experiment was fully randomised and then re-randomised weekly.

12.2.2.3 Harvestl

The experiment was harvested after six weeks. Shoot DM and colonisation by AMF were assessed as described for Experiment 1.

12.2.2.4 Statistical analysis

Data were analysed in Genstat version 14.1 using a two-way ANOVA to assess the effect of cultivar (Riverina, Woogenellup) and soil type (11 soils) on response variables. As the experiment was fully randomised, a block effect was not included. All interactions were examined. Normality was checked and transformations were not required.

12.2.3 Experiment 3. Effect of AMF on root traits important for P uptake

12.2.3.1 Soil

This experiment used two soils: the sand collected from Pinjarra and the red sandy loam from Mukinbudin used in Experiment 2 (soils “P” and “M”, respectively, in Table 12.1). Both soils had low extractable P (<10 mg P kg^{-1}) and very low organic carbon (<1%). The pH_{CaCl2} was 5.6 for the Pinjarra soil and 4.7 for the Mukinbudin soil (Table 12.1). The Pinjarra soil had more sand (95%) and less silt and clay than the Mukinbudin soil. Three soil treatments were prepared and then air-dried: 1) unpasteurised soil with indigenous AMF; 2) soil pasteurised at 63ºC for 1 hour (P1); 3) soil pasteurised at 63ºC for 1 hour, followed by two successive treatments at 80ºC for 1 hour, with 24 hours between each pasteurisation (P3). The P3 treatment was intended as a backup in case P1 did not fully remove indigenous AMF. In the end, neither treatment resulted in colonisation when soils were not inoculated. Results of both treatments are presented, but their effects were generally similar.

Experimental design and plant growth

The experiment was a fully-crossed design of cultivar (Riverina, Woogenellup) × low-P field soil (sand, sandy loam) × soil treatment (unpasteurised with indigenous AMF, P1, P3) × inoculation (no inoculum, plus inoculum of two species of AMF) with five replicates. Soil from all treatments was dried at 40ºC and weighed into 1.5 L black pots lined with plastic bags. Pots were half-filled with their respective soil type. The top half of the pot was then filled with a homogenous 50/50 mixture of soil and pasteurised river sand, or soil and inoculum of AMF in the form of sand, roots and fungal material from pot cultures that had been grown in river sand at UWA (see Ryan *et al*. (2007) for inoculum production methods). The inoculum consisted of mixture of *Scutellospora calospora* and *Glomus mosseae*.

Seeds of Woogenellup and Riverina were germinated as described for Experiment 1. After germination, rhizobia were added as described for Experiment 1 and a shallow layer of alkathene beads applied to the soil surface. Pots were watered to 80% field capacity and maintained at that weight for the duration of the experiment by watering to weight every 2-3 days. No additional nutrients were applied. On 9 July 2012 (mid-winter), pots were placed into root cooling tanks set at 17ºC, to mimic the soil temperature in the field in spring, within

a naturally-lit glasshouse at UWA which reached an average daily maximum light intensity of 1549 µmol m^2 s^{-1} and had an average diurnal temperature range 8–24ºC. We chose to use a root cooling tank in this experiment only as the focus was the effect of AMF on plant P uptake and growth, and it has been suggested that soil temperature may play a large role in determining the impact of AMF on crop growth in southern Australia (Ryan and Kirkegaard, 2012). The experiment was fully randomised and re-randomised weekly and harvested after six weeks of growth. Plant-free pots were also maintained for the non-inoculated unpasteurised, P1 and P3 treatments (three replicates).

12.2.3.2 Harvest

After six weeks of growth, shoots were harvested, dried, ground and digested as described for Experiment 1. The concentration of P and other elements was measured using inductively coupled plasma (ICP) atomic absorption with a Perkin Elmer Optima 5300 DV optical emission spectrometer (OES; Shelton, CT, USA). Roots were washed in deionised water and then assessed for disease using a 0–5 rating system adapted from Wong *et al*. (1984): 0, all roots healthy; 1.0, <25% of root system brown; 2.0, 25–50% of root system brown; 3.0, 50–75% of root system brown; 4.0, >75% of root system brown; 5.0, plant dead. Roots were also assessed for nodulation, based on a rating modified from Corbin *et al*. (1977) as follows: 0, no nodules; 1, <10 nodules on lateral roots; 2, 10 or more nodules on lateral roots; 3, nodulation on crown and <20 nodules on lateral roots; 4, nodulation on crown and >20 nodules on lateral roots. Root length and average root diameter and root system fresh weight were measured as described for Experiment 1. Representative subsamples of roots were then taken, weighed, and stored for later analysis of root hairs (25% (v/v) ethanol) and colonisation by AMF (50% (v/v) ethanol). Total root DM was later determined.

Subsamples for root hair examination were placed in 5% (v/v) Schaeffer blue ink/95% vinegar (v/v) and examined at 40× magnification on an Olympus BX51. Images were taken on an Olympus DP72 camera and root hair length measured from three sections of roots at least 20 mm from the growing tip and analysed using the Olympus DP2-BSW software package. Colonisation by AMF was determined as described for Experiment 1.

12.2.3.3 Statistical analysis

Data were analysed in Genstat version 14.1. For the Pinjarra soil, a three-way ANOVA was used to assess the effect on response variables of soil treatment (unpasteurised soil with indigenous AMF, P1, P3), inoculation (with and without inoculum of AMF) and cultivar (Riverina, Woogenellup). As pasteurisation of the Mukinbudin soil caused toxicity that greatly reduced plant growth, data for the unpasteurised soil only were analysed using a two-way ANOVA to assess the effect of inoculation and cultivar on response variables. All interactions were examined. Normality was checked visually using the residual plots generated in Genstat: to achieve normality, two outliers were removed from each of shoot P concentration and shoot P content for the Pinjarra soil. Transformation of the data was therefore not required.

12.3 Results

12.3.1 Experiment 1. Colonisation by AMF of core lines and cultivars lines of subterranean clover

The aim of this experiment was to determine the variation in the percentage of root length colonised by AMF in the 97 core lines and the 42 cultivar lines. The percentage of root length colonised by AMF varied significantly among the core lines, from 14–55% (Fig. 12.1; P<0.001). Colonisation of the cultivar lines also varied significantly (P<0.001) and covered a similar range, although one cultivar, Izmir, had 68% of root length colonised. The core lines had a higher mean colonisation level than the cultivar lines (core lines, 36.8% ± 1.0 s.e.; cultivar lines 32.8% ± 1.7 s.e; two-tailed t-test *P*=0.037). In addition, 41% of the core lines were highly colonised (>40% of root length colonised) compared with 24% of the cultivar lines.

Among the three subspecies, the average percentage of root length colonised did not differ (two-tailed t-test *P*>0.05) between ssp. *brachycalycinum* (28.5% ± 1.5 s.e.) and ssp. *yanninicum* (24.5% ± 3.0), but colonisation of ssp. *subterraneum* (39.2% ± 0.9) was significantly higher than for both ssp. *brachycalycinum* and ssp. *yanninicum* (two-tailed t-test *P*<0.0001) (Fig. 12.1). The percentage of lines that were highly colonised (>40% of root length) was 0% for ssp. *yanninicum*, 14% for ssp. *brachycalycinum* and 46% for ssp. *subterraneum* (Fig. 12.1). Correlations between colonisation and other traits were investigated using the mean for each core line and cultivar line (i.e. n=139). The strongest correlations involved shoot P concentration ([P]), which ranged from 0.45–2.00 g kg^{-1}. There was a positive linear correlation between the percentage of root length colonised by AMF and shoot [P] (Fig. 12.2, r^2=0.36, *P*<0.0001) and between the total length of colonised root and shoot [P] (r^2=0.35, *P*<0.0001). There was a weak positive linear correlation between the percentage of root length colonised by AMF and shoot P content (r^2=0.10, *P*<0.001).

Relatively weak correlations with colonisation were present for other parameters. Root mass ratio ranged from 0.32–0.59 and had a negative linear correlation with the percentage of root length colonised by AMF (r^2=0.06, *P*<0.01). Specific root length ranged from 85–175 m g^{-1} and had a positive correlation with the percentage of root length colonised by AMF (r^2=0.14, *P*<0.0001). Average root diameter ranged from 0.295–0.424 mm and had negative linear correlations with the percentage of root length colonised by AMF (r^2=0.13, *P*<0.0001) and specific root length (r^2=0.90, *P*<0.0001). There was no correlation between shoot DM and the percentage of root length colonised by AMF.

12.3.2 Experiment 2. Colonisation by AMF of two cultivars in 11 soil types

The aim of this experiment was to determine whether the ranking of lines by the percentage of root length colonised, as done in Experiment 1, was consistent across 11 soils that varied greatly in extractable P for two cultivars. The two cultivars used, Riverina and Woogenellup, had 20% and 28%, respectively, of root length colonised in Experiment 1 (Fig. 12.1). In this experiment, the effect of cultivar on colonisation differed with soil type (P<0.001) (Fig. 12.3a). However, Woogenellup remained the most highly colonised of the two cultivars in eight soils and did not differ from Riverina in two soils (Fig. 12.3a). Colonisation was absent in one soil. There was no relationship between extractable soil P and the percentage of root length colonised for either cultivar (Fig. 12.3a).

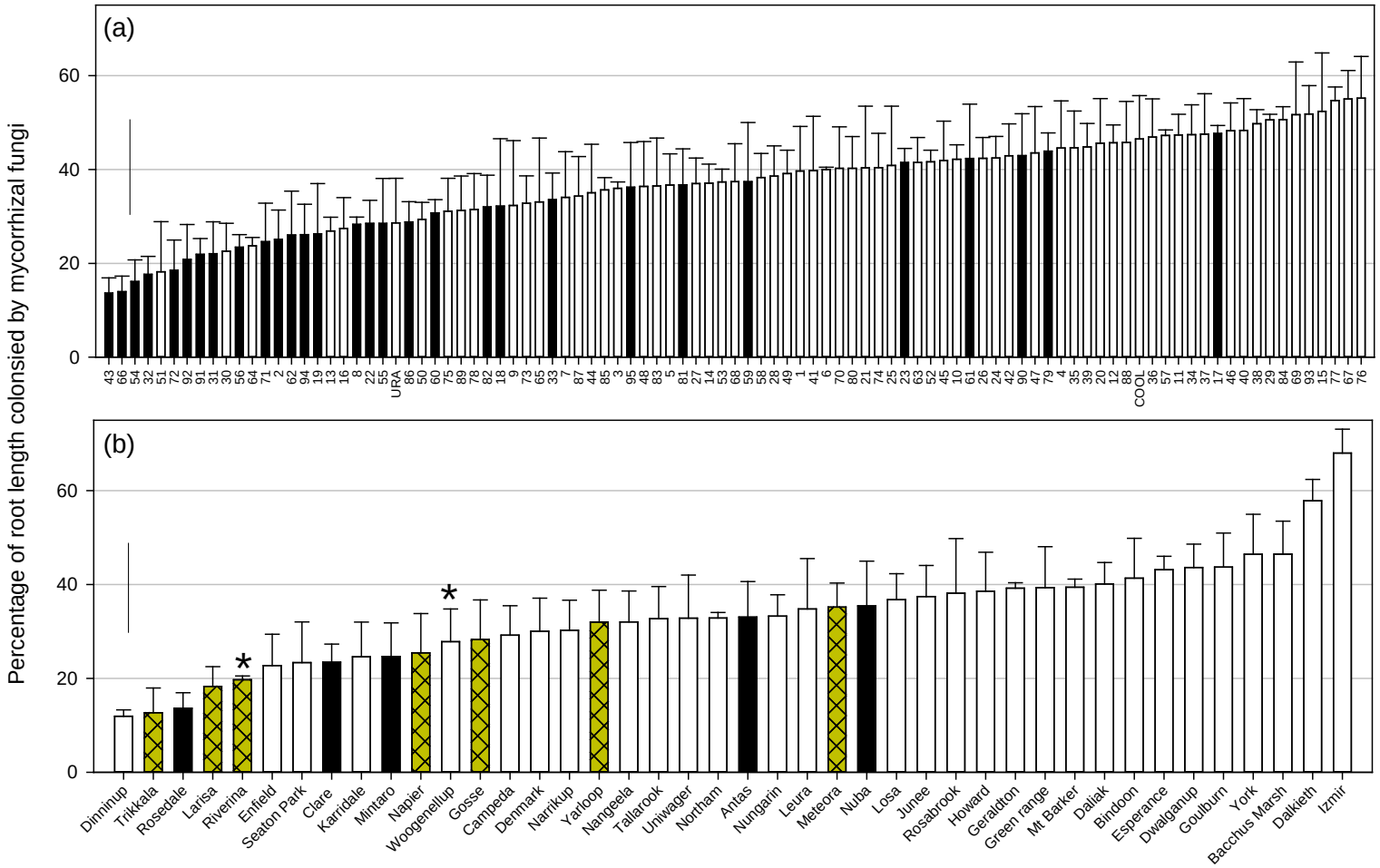


Figure 12.1 The percentage of root length colonised by indigenous arbuscular mycorrhizal fungi in (a) the core collection (lines L01-L95 and cultivars Urana (URA) and Coolamon (COOL)) (core lines) and (b) 42 cultivar lines of subterranean clover grown for six weeks in the glasshouse (Experiment 1) (mean+s.e., n=4) (white, ssp. *subterraneum*; black, ssp. *brachycalycinum*; hatched, ssp. *yanninicum*). The cultivars used in Experiments 2 and 3 are indicated with a star. The LSD at *P*=0.05 is provided.

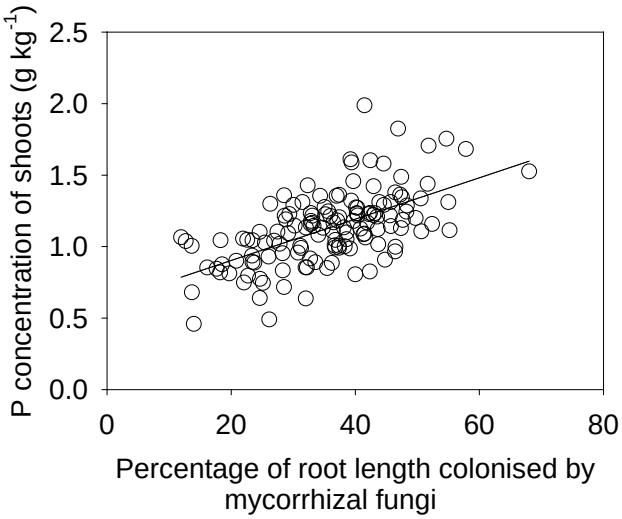


Figure 12.2 The relationship between shoot phosphorus (P) concentration and percentage of root length colonised by indigenous arbuscular mycorrhizal fungi for the 97 lines in the core collection and 42 cultivar lines grown for six weeks in the glasshouse (R^2=0.36, *P*<0.0001, n=139) (Experiment 1).

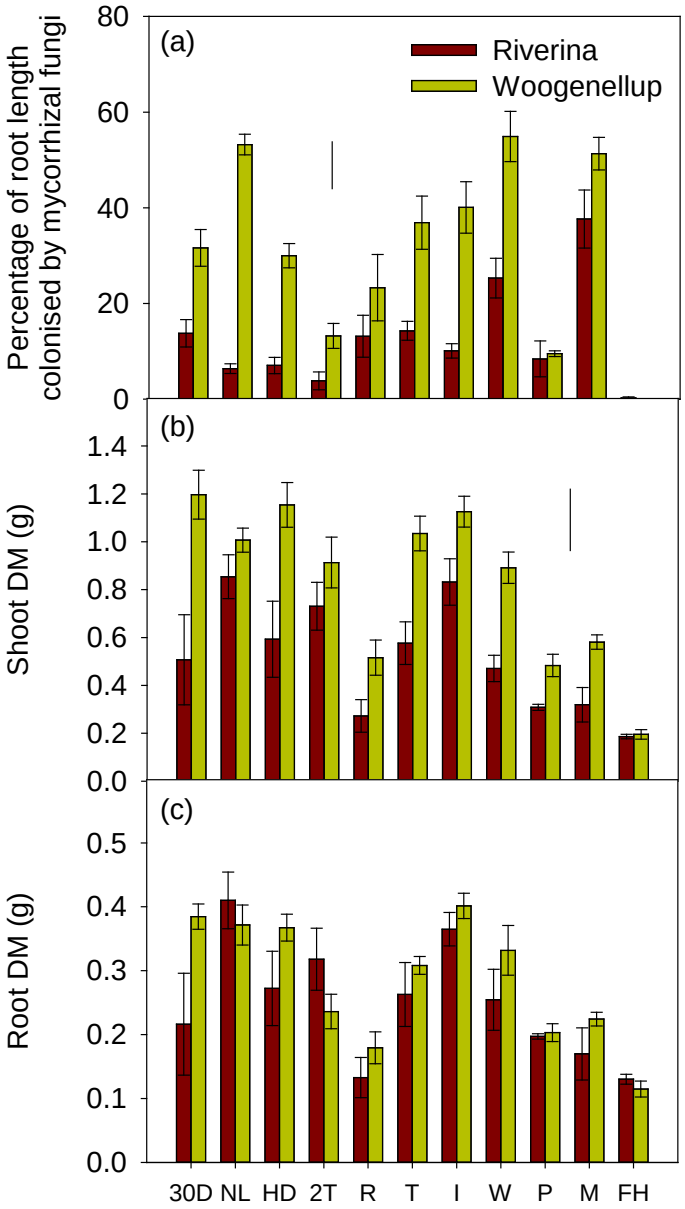


Figure 12.3 (a) The percentage of root length colonised by indigenous arbuscular mycorrhizal fungi (AMF), (b) shoot dry mass (DM) and (c) root DM for subterranean clover cultivars Riverina (ssp. *yanninicum*) and Woogenellup (ssp. *subterraneum*) grown for six weeks in the glasshouse in 11 field soils (Experiment 2) (mean±s.e., n=4). Soils are ordered from highest (30D) to lowest (FH) in extractable P (see Table 12.1). There was a significant interaction between soil and cultivar for AMF (*P*<0.001) and shoot DM (*P*<0.009) and the LSD at *P*=0.05 is provided. For root DM, there was a significant effect of cultivar (*P*=0.021) and soil (P<0.001) (no LSD is provided as no interaction; marginal means for cultivar were: Riverina 0.248 and Woogenellup 0.284, LSD at *P*=0.05 is 0.030).

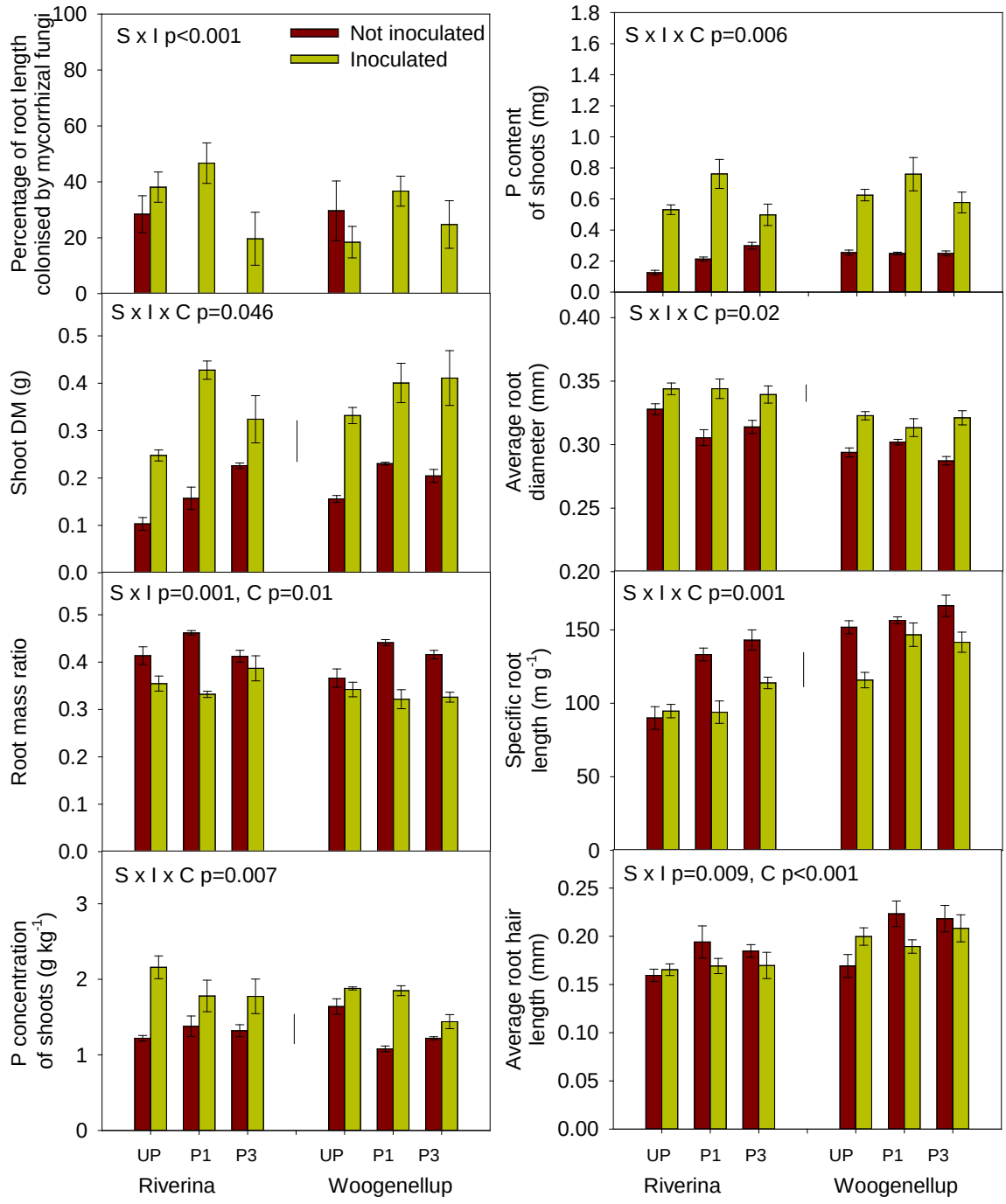


Figure 12.4 The effect of three experimental factors on subterranean clover grown in Pinjarra soil: Soil treatment (S) [unpasteurised, UP; pasteurised once, P1; pasteurised three times, P3]; Inoculation (I) [with and without inoculum of AMF]; Cultivar (C) [Riverina, Woogenellup] (Experiment 3) (mean±s.e., n=5). DM=dry mass. *P*-values for significant interactions and main effects (if no significant interaction included a factor) are shown on the graphs. The LSD at *P*=0.05 is provided if the three-way interaction of S × I × C was significant at *P*<0.05, except for shoot P content where data were logged prior to analysis. Note that y-axis scales are the same as in Figure 10.6.

For shoot DM, the effect of cultivar differed with soil type (P<0.001), with Woogenellup producing either more DM than Riverina or a similar amount (Fig. 12.3b). For root DM, there was a significant effect of cultivar (P=0.021) and soil (P<0.001), with Woogenellup having slightly more root DM than Riverina (Fig. 12.3c).

12.3.3 Experiment 3. Effect of AMF on root traits important for P uptake

The aim of this experiment was to determine if root traits associated with P uptake changed in the presence of AMF. The experiment was conducted using two soils; Pinjarra (sand) and Mukinbudin (sandy loam). At the end of the experiment, the unpasteurised and pasteurised plant-free control pots for each soil did not differ in extractable P (5–6 mg kg^{-1}) or mineral N (22–26 mg kg^{-1} Pinjarra, 27–32 mg kg^{-1} Mukinbudin) suggesting no nutrient flush as a result of the pasteurisation. However, pasteurisation had a large negative impact on plant growth in the Mukinbudin soil, the cause of which was not able to be identified. As a consequence, only the unpasteurised soil treatment is presented for the Mukinbudin soil.

Plants in the unpasteurised Pinjarra sand had ~30% of root length colonised by AMF (Fig. 12.4). Pasteurisation resulted in no colonisation (Fig. 12.4). Inoculation into the pasteurised soil with AMF (*S. calospora* and *G. mosseae*) returned colonisation to that of plants in the unpasteurised soil. Inoculation into the unpasteurised soil, which contained a population of indigenous AMF (see Table 12.1), did not change the percentage of root length colonised. The effect of inoculation on shoot DM varied with soil treatment and cultivar, but was always positive (often >100%), even in the unpasteurised soil. Pasteurisation generally resulted in increased shoot DM, with the biggest increase being ~100% for Riverina that was not inoculated.

Inoculation had a large effect on the concentrations of only two shoot nutrients, P and molybdenum. The effect of inoculation on shoot [P] varied with cultivar and soil treatment, but was always positive (Fig. 12.4). The largest increase occurred for Riverina in the unpasteurised soil; from 1.22 g kg^{-1} to 2.16 g kg^{-1}. Inoculation increased shoot P content by ~50% to ~400% (Fig. 12.4). Shoot molybdenum concentration ([Mo]) decreased with inoculation by 40–74% (Fig. 12.5).

The effect of inoculation on root traits varied (Fig. 12.4). Root mass ratio changed slightly with inoculation, most often decreasing from ~0.4 to ~0.3 g g^{-1}. Inoculation increased the average root diameter in a manner that varied with cultivar and soil treatment; the largest increase being ~0.04 mm. The effect of inoculation on specific root length also varied with cultivar and soil treatment, but was generally negative, with the decrease being up to 30%; a notable exception was for Riverina in the unpasteurised soil where there was no effect of inoculation. The effect of inoculation on average root hair length was generally small and tended to be positive in the unpasteurised soil and negative in the pasteurised soil. Riverina generally had a higher average root diameter, lower specific root length and shorter average root hair length than Woogenellup.

In the unpasteurised soil, root disease scores were <1 (on the 0–5 scale) for Riverina and 1–2 for Woogenellup. Root disease was mostly absent in the pasteurised treatments. Nodulation was rated as a 1 or 2 for all pots (i.e. plants in all pots were nodulated, with up to a total of 19 nodules on lateral roots).

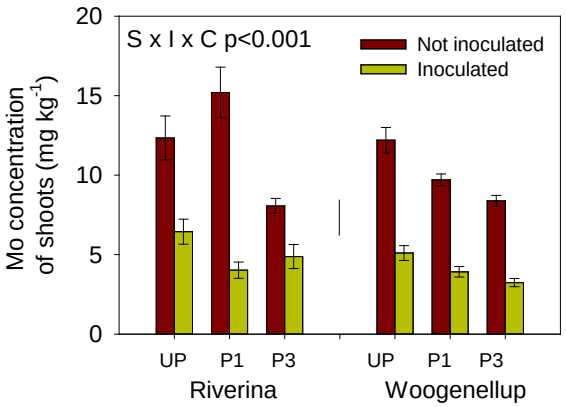


Figure 12.5 The effect of three experimental factors on shoot molybdenum (Mo) concentration of subterranean clover grown in Pinjarra soil: Soil treatment (S) [unpasteurised, UP; pasteurised once, P1; pasteurised three times, P3]; Inoculation (I) [with and without inoculum of AMF]; Cultivar (C) [Riverina, Woogenellup] (Experiment 3) (mean±s.e., n=5). The LSD at *P*=0.05 is provided for the three-way interaction of S × I × C.

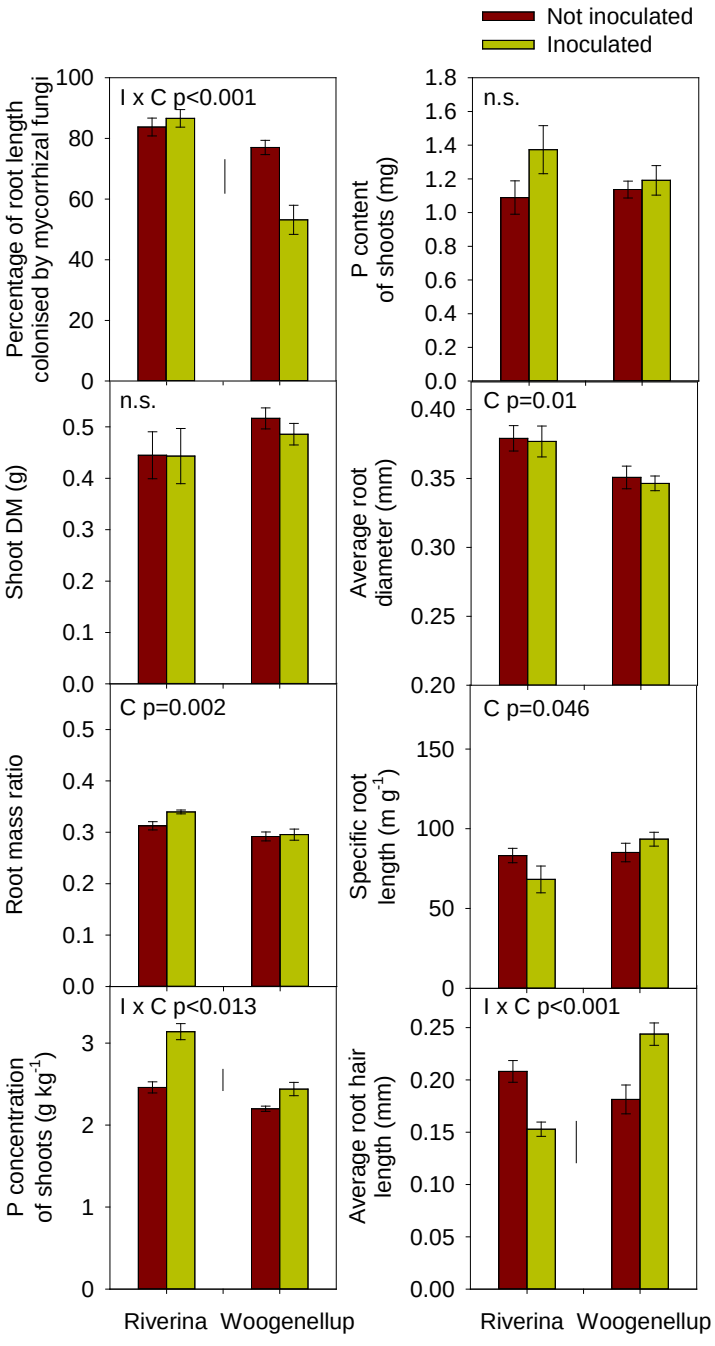


Figure 12.6 The effect of two experimental factors on subterranean clover grown in Mukinbudin soil: Inoculation (I) [with and without inoculum of AMF]; 2. Cultivar (C) [Riverina, Woogenellup] (Experiment 3) (mean±s.e., n=5). DM=dry mass. *P*-values for significant interactions and main effects (if no significant interaction included a factor) are provided. LSD at *P*=0.05 is provided if the two-way interaction of I × C was significant at *P*<0.05; n.s.= not significant. Note that y-axis scales are the same as in Figure 12.4.

For the Mukinbudin soil, results are presented only for the unpasteurised treatment. In this soil, the percentage of root length colonised by indigenous AMF was high, ~80%, and inoculation had no effect for Riverina, but decreased colonisation for Woogenellup (Fig. 12.6). Shoot DM and root mass ratio were not affected by inoculation. Shoot [P] and P content were much greater than in the Pinjarra soil; only shoot [P] increased with inoculation, and the increase was smaller than that in the Pinjarra soil. Specific root length and average root diameter were not affected by inoculation, but differed slightly with cultivar. Average root hair length was similar to that in the Pinjarra soil, and was clearly affected by inoculation; decreasing for Riverina (which was unaffected in the Pinjarra soil) and increasing for Woogenellup (as also occurred in the Pinjarra soil).

Root disease scores were always <1. Nodulation was rated as a 2 for all pots (i.e. there was a total of 10-19 nodules on lateral roots). Shoot [Mo] was below detection limits for all treatments (data not shown). Shoot nutrients other than P and Mo were not affected by inoculation (data not shown).

12.4 Discussion

The main findings of this paper were: 1) large variation in the percentage of root length colonised by AMF in the 97 core lines and in the 42 cultivar lines, but with a greater proportion of the core lines being highly colonised; 2) a reasonably robust relative ranking of cultivars Riverina and Woogenellup for colonisation over 11 soils varying greatly in extractable P; 3) inoculating field soil with AMF had relatively little effect on root traits relevant to P uptake; and 4) inoculating an unpasteurised field soil which contained indigenous AMF with AMF greatly enhanced plant P uptake and growth.

12.4.1 Large differences among lines in the percentage of root length colonised

The percentage of root length colonised for the 139 lines (core lines plus cultivar lines) in Experiment 1 varied greatly. In other glasshouse studies, colonisation of subterranean clover reached close to 90% (Abbott and Robson 1977). The lower maximum colonisation in our study (68%) probably reflects lower inoculum density (Table 12.1); this may have had the unintended favourable effect of allowing differences in hosting ability to be well expressed.

Subspecies *subterraneum* had higher levels of colonisation than the other two subspecies. This result may reflect better adaptation of it, and ssp. *yanninicum*, to the moderately acidic soil in the screening (pH_{CaCl2} = 4.7) than ssp. *brachycalycinum*, the germplasm of which had been mostly collected from alkaline sites (Nichols *et al*. 1996), combined with a preference of ssp. *yanninicum* for a soil with a relatively high water-holding capacity (Nichols *et al*. 2013). Thus, it is possible that the higher colonisation of ssp. *subterraneum* may reflect better adaptation to the soil in the screening, although the large range of colonisation within this subspecies suggests other factors are also at play.

The average level of colonisation for the core lines was higher than for the cultivar lines at 37% and 33% of root length colonised, respectively. This is consistent with a recent meta-analysis of 39 publications, where Lehmann *et al*. (2012) found mean colonisation of 41%, 30% and 32% for ancestors, old cultivars and new cultivars, respectively, of annual crop plants. However, some large studies are not consistent with these findings. For instance, An

et al. (2010) found large variation in the percentage of root length colonised among more than 200 lines of maize examined in the field in Japan, but that colonisation was similar, perhaps even higher, for modern hybrids compared with older landraces. Similarly, Leiser *et al*. (2015) found large variation in colonisation level, but no effect of origin (landrace or researcher bred), for 187 sorghum (*Sorghum bicolor* L. Moench) lines from west and central Africa. Whether the variation in colonisation we found among subterranean clover can be exploited by breeders will depend on the heritability of the trait. The percentage of root length colonised in African sorghum lines had low heritability and hence it was concluded that the potential for genotypic selection was low (Leiser *et al*. 2015).

12.4.2 The relative ranking of two cultivars for percentage of root length colonised was reasonably robust over 11 soils

In comparisons of Riverina and Woogenellup in Experiment 2, Riverina was the least colonised in eight soils, with colonisation levels similar in the remaining three soils. The percentage of root length colonised did not appear related to extractable P concentrations, which varied greatly, perhaps instead reflecting the inoculum level in each soil (Table 12.1). Cultivar preference for the species of AMF present in each soil (see Table 12.1) may also have influenced colonisation (Mao *et al*. 2014). Overall, we conclude that the difference between Riverina and Woogenellup in percentage of root length colonised was robust enough to support the screening of root colonisation in a single soil type. However, it cannot be discounted that for the core lines and cultivars lines, rankings could vary if a different soil was used. Whether the rankings would be consistent under variable field conditions also remains to be determined. When An *et al*. (2010) evaluated >200 lines of maize in the field in two consecutive years, correlation in colonisation between the years was poor.

12.4.3 Should colonisation by AMF be considered when breeding subterranean clover?

Overall, the high degree of variation in the core lines and cultivar lines, especially in ssp. *subterraneum*, suggests that it may be feasible to select for higher or lower colonisation. It is convenient that cultivar lines had a similar, perhaps even slightly greater, variation in the percentage of root length colonised than was present in the core lines, as selecting from existing cultivars would encapsulate other agronomically-desirable characteristics such as disease resistance.

Breeding subterranean clover to achieve a higher percentage of root length colonised than current cultivars should be approached with caution. For instance, it should not be assumed that there is a positive relationship between the percentage of root length colonised and total plant P-uptake and growth (Linderman and Davis 2004; Smith and Smith 2011; Tawaraya *et al*. 2001). While there was a positive correlation in Experiment 1 between colonisation level and shoot [P] (Fig. 12.2), there was no relationship with shoot DM. In addition, the positive P balance of southern Australian agricultural systems (Vu *et al*. 2011) has resulted in large increases in extractable soil P (e.g., Weaver and Reed (1998)) and AMF have little impact on P uptake and growth of subterranean clover when extractable (Colwell) P is greater than ~20 mg kg^{-1} (Abbott and Robson 1977; Nazeri *et al*. 2013; Schweiger *et al*. 1995). Finally, the role of AMF in P nutrition and growth of autumn-sown crops and pastures in temperate southern Australia is not clear. For both wheat and linseed (*Linum usitatissimum* L.), which is generally considered to be highly dependent on AMF, field experiments where colonisation

levels were manipulated through pre-crops showed no net benefit for P uptake from higher colonisation, even under high P limitation (Kirkegaard and Ryan 2014; Ryan and Kirkegaard, 2012). It therefore seems that investigation into the contribution of AMF to plant P uptake and growth under field conditions is required before it can be determined if the level of colonisation by AMF should be considered in subterranean clover breeding programs.

12.4.4 Inoculating a pasteurised field soil with AMF had relatively little effect on root traits relevant to P uptake

In the pasteurised Pinjarra soil, inoculation with AMF changed key root traits associated with P uptake to result in a smaller, coarser root system. A reduction in root mass ratio for annual crop species is quite commonly reported with inoculation (Gavito *et al*. 2000; Guo *et al*., 2006; Sun and Tang 2013). Also, consistent with our study, for maize, Sheng *et al*. (2009) reported that inoculation with AMF increased average root diameter by around 0.05 mm and decreased specific root length from ~150 m g^{-1} to 100 m g^{-1} (see also Mendoza (2001)). However, not all studies are consistent with our results. For instance, Sun and Tang (2013) found inoculation of sorghum with AMF increased specific root length from ~150 m g^{-1} to 200–250 m g^{-1} and had little impact on the diameter of "thin roots" and caused average root hair length to decrease by ~30%. Moreover, for strawberries (*Fragaria* × *ananassa* Duch), Fan *et al*. (2011) reported that inoculation caused average root diameter to increase, similar to the present study, but in contrast, inoculation increased specific root length from 20 to 29 m g^{-1}.

Overall, in our study and in some, but not all, studies in the literature, colonised plants invested less biomass in roots (lower root mass ratio). This is consistent with plants reducing carbon losses through trade-offs among morphological and physiological traits (Lynch and Ho 2004; Ryan *et al*. 2012); that is, when carbon is allocated to the mycorrhizal symbiont, less carbon is allocated to root growth. However, lower root mass ratio, lower specific root length and increased root diameter are also common responses to increased P availability (Hill *et al*. 2006) and thus could reflect the increase in plant P uptake in the inoculated plants. Shorter root hairs can also be explained in this manner, but alternatively could result from reduced P uptake through root hairs when AMF are present (Smith *et al*. 2009). Variation among studies in the effect of AMF on these traits could reflect many factors including plant P status and the percentage of root length colonised (Mendoza 2001).

When assessing the relevance of the changes in root traits that occurred with inoculation with AMF in Experiment 3 to screenings of germplasm to identify lines with root traits favourable for improving P uptake, a number of factors require consideration. First, while the traits differed between the two cultivars, the effect of inoculation with AMF on these traits was generally quite similar. This suggests that the ranking of cultivar lines for a particular trait may not be greatly affected by the presence or absence of AMF. Secondly, the change in the traits with inoculation was minor compared with the variation among core lines and cultivar lines. For instance, specific root length had the greatest change with inoculation (up to 30%), but the variation among the core lines and cultivar lines was 78–167 m g^{-1}. Thus, for subterranean clover we conclude that it is not necessary to deliberately include or exclude AMF when screening for root traits associated with P uptake. It therefore follows that screening in field soil can be undertaken on pasteurised soil, removing the need to find soil free of the root pathogens of subterranean clover that are widespread in the southern Australian cropping zone (O'Rourke *et al*. 2009; Simpson *et al*., 2011b). However, it should

be noted that, functionally, AMF have been found to substitute for root hairs on mutant plants that lack root hairs (Jakobsen *et al*. 2005) and to possibly override the P-acquisition benefits of selecting for longer root hairs in white clover (*Trifolium repens* L.) (Caradus 1981). Thus, the benefits to plant P-acquisition of selection for desirable root traits should be undertaken in the presence of AMF.

12.4.5 Inoculation increased plant growth and P uptake when indigenous AMF were present

Shoot DM and shoot P content increased by more than 100% when inoculum was added to the unpasteurised Pinjarra field soil. Abbott and Robson (1977) reported similar results in a glasshouse experiment using a low-P field soil from Western Australia. In both studies, it is not known to what extent the introduced AMF colonised the roots. It is possible that the positive responses to inoculation reflected the introduction of other microorganisms present in the inoculum medium (Daniels Hetrick *et al*. 1998). However, we consider this unlikely because Nazeri *et al*. (2013) added "sievings" of the same batch of *S. calospora* inoculum used in this experiment to subterranean clover and observed no effect on plant growth or P uptake. Thus, it appears that the strains of AMF in the inocula we used are more effective at enhancing P uptake and growth of subterranean clover than the indigenous populations in the Pinjarra soil. Interestingly, the removal of the indigenous AMF resulted in a large increase in shoot DM in the Pinjarra field soil. This could not be attributed to a "flush" of nutrient resulting from pasteurisation of the soil. It is possible that the indigenous AMF were reducing plant growth (Ryan *et al*. 2005), but the result is also consistent with the removal of root pathogens. In contrast to our results, Sainz and Arines (1988) found the effect of indigenous AMF on P uptake and growth of red clover (*T. pratense* L.) to be much greater than when single species inocula were used.

The ability of inocula of AMF to enhance growth of subterranean clover in the field was investigated by Abbott *et al*. (1983) who sowed subterranean clover with two pure cultures of AMF at four field sites in Western Australia. Colonisation by the introduced AMF only occurred when the indigenous AMF had low infectivity (Abbott *et al*. 1983). Shoot DM increased significantly with the introduced AMF at only one site (lowest extractable soil P of 6 mg kg^{-1}): by up to 75% at week 6 and up to 40% at week 8 (Abbott *et al*., 1983). However, at week 18 there was no effect of the introduced AMF (Abbott *et al*. 1983). The long-term persistence of the strains used for inoculation is not known, and thus the lack of a long-term effect could reflect either poor persistence of the inoculated fungi or an inability of the inoculated fungi to enhance P uptake and shoot growth as the season progressed through winter into spring. Further investigation is required of the ability of indigenous AMF to enhance P uptake and growth in subterranean clover under laboratory and field conditions.

12.4.6 Inoculation with AMF reduced shoot Mo concentration

While only required in minute amounts, Mo can become limiting for legume growth in acid soils (Zimmer and Mendel 1999); the necessity of adding Mo to subterranean clover on acid soils in Western Australia to ensure adequate rhizobia nodulation has long been known (Anderson and Moye 1952). This is the first report of a reduction in shoot [Mo] in response to inoculation with AMF, albeit only in one of the two soils tested. The mechanism behind this effect is unclear. Plant uptake of Mo declines with increasing soil acidity (Mortvedt, 1981). However, inoculation of subterranean clover with AMF was reported by Nazeri *et al*. (2014)

to raise the pH of the rhizosphere. The [Mo] of 10 mg kg^{-1} shoot DM in Experiment 3 is at the high end of “adequate”, which is considered to be 0.5–10 mg kg^{-1} for subterranean clover (Weir and Cresswell 1994).

12.5 Conclusions

While there was a wide range of colonisation levels among the core lines and cultivar lines of subterranean clover, a greater proportion of the core lines had more than 40% of root length colonised than did the cultivar lines. It remains to be determined if this variation in colonisation level has a heritability sufficiently high to enable it to be usefully exploited by breeders. Colonisation was lower for ssp. *brachycalycinum* and ssp. *yanninicum* than ssp. *subterraneum*, suggesting the former two subspecies as priorities for selection for higher colonisation. However, there is not yet sufficient evidence for a benefit from AMF under field conditions to justify recommendation of this strategy. In this context, our finding that indigenous AMF may be less effective at enhancing plant P uptake and growth than the inoculants used in our study requires further investigation.

While key root traits for enhancing plant P uptake changed in the presence of AMF, the changes were small relative to the variation present within core lines and cultivar lines, and may simply be a result of the improved plant P status that resulted from inoculation with AMF. Thus, we suggest that there is no need for AMF to be deliberately included or excluded when screening germplasm of subterranean clover for root morphological traits associated with P uptake.

Acknowledgement

The authors gratefully acknowledge the input to Experiment 3 of Jiayin Pang and Martin Barbetti.

13 Rhizosphere carboxylates and morphological root traits in pasture legumes and grasses.

Daniel R. Kidd, Megan H. Ryan, Rebecca E. Haling, Hans Lambers, Graeme A. Sandral, Zongjian Yang, Richard A. Culvenor, Gregory R. Cawthray, Adam Stefanski, Richard J. Simpson

Published 2016. Plant Soil 402: 77-89.

13.1 Introduction

The importance of phosphorus (P) for global food security, coupled with the increasing cost of P fertilisers, their non-renewable nature and the negative environmental effects from their overuse have recently motivated renewed interest and research into more P-efficient agricultural systems (Cordell and White 2015; Richardson *et al*. 2011; Simpson *et al*. 2011). A universally accepted goal is development of agricultural cultivars that yield well when grown in soils with lower concentrations of extractable P relative to those currently needed for high yields (Lynch 2007). Over time, soils managed at a reduced extractable P concentration are predicted to require less P fertiliser. In soils that have a high P sorption capacity P accumulation will be reduced (Simpson *et al*. 2015) and in soils where the P sorption capacity is saturated the potential for losses in runoff and leaching will also be reduced. The legumes most commonly used in Australian temperate pastures (e.g. *Trifolium* and *Medicago* spp.) are an obvious target for improvement because they have relatively high critical P (the soil P concentration for near maximum yield) requirements. In pastures that rely on these legumes for nitrogen (N) fixation, it is their high P requirements that determine the extractable P concentrations to which soils are fertilised (Ozanne *et al*. 1976; Simpson *et al*. 2014). Typically, the legumes only achieve maximum yield when provided with up to twice the amount of P required by the temperate grasses with which they are grown (Ozanne *et al*. 1976).

The ability to yield well with lower extractable soil P concentrations is primarily associated with morphological traits such as long fine roots and long root hairs that enable foraging for “plant-available” P and its uptake from the soil solution. Together these traits confer a large root hair cylinder volume (RHCV) which is strongly correlated with P uptake (Evans 1977; Hill *et al*. 2010; Yang *et al*. 2015). Consistent with their low critical P requirement, temperate grasses are generally reported to have thin roots, high specific root length, long root hairs and a relatively large RHCV (Yang *et al*. 2015). In contrast, legumes such as the *Trifolium* spp. generally have thicker roots, reduced specific root length, shorter root hairs and a relatively small RHCV (Evans 1977; Yang *et al*. 2015). In addition to RHCV, root system architecture or plasticity that deploys roots in nutrient-rich surface soil layers or in nutrient patches can also facilitate access to plant available P (Hodge 2006; Lynch 2011; Richardson *et al*. 2011). However, there are sources of P in soil other than those termed plant available.

Many fertilised soils have accumulated large amounts of P as a result of continued fertiliser use (*e.g.* Simpson *et al*. 2015). Much of this P is in “sparingly-available” forms that must be returned to a plant available form before it can be accessed by roots. Physiological root traits, such as exudation of carboxylates (low molecular weight organic anions) and

phosphatases into the rhizosphere, can potentially enable plants to “mine” these sources of soil P (Richardson *et al*. 2011). Some crop species contain high amounts of carboxylates in their rhizosphere, for instance *Lupinus albus* (white lupin, a cluster-root forming species), *Cicer arietinum* (chickpea) and, to a lesser extent, *L. angustifolius* (narrow-leafed lupin) (Pearse *et al*. 2006). The contribution of the P released by carboxylates to plant growth is difficult to quantify, but a positive correlation between plant P content and amount of rhizosphere carboxylates has been reported by Veneklaas *et al*. (2003) for *C. arietinum* and Ryan *et al*. (2012) for *Kennedia* spp.

There has been little investigation into rhizosphere carboxylates of the temperate pasture legumes commonly grown in Australia. A single report suggests low amounts for *T. subterraneum* (subterranean clover) grown under P-sufficient conditions (Nazeri *et al*. 2014), high amounts in some native perennial legumes and moderate amounts in *Medicago sativa* (lucerne) (Pang *et al*. 2010a; 2010b). Thus, it remains to be determined if there are pasture legumes which can replicate the high amounts of rhizosphere carboxylates found in *L. albus* or *C. arietinum*.

Trifolium subterraneum is the most important annual pasture legume in southern Australian pasture systems (Nichols *et al*. 2007, 2013). Several species of annual medic (*Medicago* spp.) and the perennial *M. sativa* are also commonly grown (Nichols *et al*. 2012). However, in recent decades other annual and perennial legume species have been developed to provide farmers with options for situations where *T. subterraneum* and *Medicago* spp. are not well-adapted (Loi *et al*. 2005; Nichols *et al*. 2012). Some of these novel pasture legumes such as *Ornithopus sativus* (French serradella) are now widely used in pasture systems, while others such as *Biserrula pelecinus* (biserrula) and *O. compressus* (yellow serradella) are moderately popular with farmers (Nichols *et al*. 2012). Interestingly, *Ornithopus* spp. reportedly have lower critical P requirements than *T. subterraneum* (Bolland 1985; Bolland and Paynter 1992; Paynter 1990, 1992). A large specific RHCV in *Ornithopus* species, relative to *T. subterraneum*, likely contributes to their lower critical P requirement (Yang *et al*. 2015).

The aim of this study was to compare the amount and composition of rhizosphere carboxylates and morphological root traits related to the acquisition of P from soil, among 13 species of pasture legumes including *T. subterraneum* (8 lines), *O. compressus* and *O. sativus*. Two pasture grasses were also included (*Dactylis glomerata, Phalaris aquatica*). Rhizosphere carboxylates were the major focus because little is known about them in these species. The significance of rhizosphere carboxylates for the pasture species was gauged by comparing them with three crops known to produce large amounts of rhizosphere carboxylates: *L. albus*, *L. angustifolius* and *C. arietinum*.

13.2 Materials and Methods

13.2.1 Plant material and growth conditions

Eighteen species were included in the experiment: six cultivars and two lines from the core collection (see Nichols *et al*. 2013) of *T. subterraneum*, twelve other pasture legume species including *O. sativus* and *O. compressus*, two pasture grasses (*D. glomerata* and *P. aquatica*) and three crop species reported to have large amounts of rhizosphere carboxylates (*L. albus*, *L. angustifolius, C. arietinum*) (Table 13.1). The experiment was undertaken in a

glasshouse at The University of Western Australia (31°98' S, 115°81' E) commencing on 31 March 2014. Glasshouse temperatures were maintained between 16 °C and 22 °C and average relative humidity was 62 %. There were five replicates and all pots were randomised weekly.

To facilitate collection of rhizosphere carboxylates, plants were grown in washed coarse river sand; this substrate has a low adsorption capacity and little loss of carboxylates due to microbial breakdown (Ryan *et al*. 2012). Soil analyses were carried out by CSBP laboratories Bibra Lake, Western Australia. Nutrient composition was: ammonium-N 2 mg kg^{-1}; nitrate-N <1 mg kg^{-1} (Searle 1984); bicarbonate-extractable P 2 mg kg^{-1}; bicarbonate-extractable potassium 20 mg kg^{-1} (Colwell 1963); and sulphur 7.4 mg kg^{-1} (Blair *et al*. 1991). The pH in $CaCl_2$ was 7.8 (Rayment and Higginson 1992).

The sand was pasteurised at 60 °C for 1 hour, oven-dried at 40 °C, weighed (1.2 kg) into open rectangular pots (90 × 90 × 180 mm) and initially wet to field capacity by adding 200 ml of deionised water; pots were then maintained for the duration of the experiment at 75% of field capacity. Twenty millilitres of nutrient solution were added to each pot containing the following (mg): KH_2PO_4 21.7; KNO_3 80.8; $Ca(NO_3)_2$ 94.4; $MgSO_4$ 39.3; NH_4NO_3 105.6; Fe EDTA 73.4; H_3BO_3 0.3; $MnCl_2$ 0.2; $ZnSO_4$ 2.2; $CuSO_4$ 0.5; and Na_2MoO_4 0.01. Following this initial application, nutrients other than P were supplemented weekly (mg): K_2SO_4 60.0; $Ca(NO_3)_2$ 47.2; $MgSO_4$ 49.3; NH_4NO_3 32.0; Fe EDTA 1.1; H_3BO_3 0.3; $MnSO_4$ 0.4; $ZnSO_4$ 0.4 $CuSO_4$ 0.5; Na_2MoO_4 0.2. The two grass species received double the concentration of NH_4NO_3 provided to the legumes. The experiment was inoculated with a commercial inoculant containing four species of arbuscular mycorrhizal fungi (AMF) (Microbesmart Pty Ltd., South Australia).

All seeds were surface sterilised with 50 % (v/v) ethanol. Seeds were germinated on damp Whatman No.5 (90 mm) filter paper. Once a radicle had emerged, three seeds were planted in each pot at 1 cm depth. Legumes were inoculated with appropriate rhizobia at the recommended rate at sowing (Table 13.1). The surface of the sand in each pot was covered with a thin layer of white alkathene beads to reduce evaporation. Five plant-free pots were also maintained.

13.2.2 Plant analyses

Plants were harvested after six weeks in order of replicate. The three plants in each pot were removed and placed into a tray where their roots and adhered sand were carefully teased apart from the bulk sand, taking care not to break roots. Once the root systems could be lifted from the tray without roots breaking, they were held by the base of the shoots above the tray and gently shaken; the sand remaining adhered to the roots was classified as the rhizosheath. The root systems from each pot were then placed into a 500 ml beaker and rinsed with 50 ml of 0.2 mM $CaCl_2$ solution using a large syringe until all visible sand had been washed into the solution. One millilitre of the solution was then filtered through a 0.22 µm Acrodisc syringe filter into a 1 ml Waters HPLC vial containing 25 µl of orthophosphoric acid. Vials were immediately capped and placed on ice before being transferred to a –20 °C freezer at regular intervals and stored until analysis for rhizosphere carboxylates by liquid chromatography. Roots were further rinsed in deionised water before shoots were separated.

Shoots were oven-dried at 70°C for 5 days, weighed and finely ground. A 0.1 g subsample was digested in a 3:1 HNO_3:$HClO_4$ solution and total P measured using a UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan) by the malachite green method (Motomizu *et al*. 1983).

Roots were examined for symptoms of disease and nodulation. No disease was evident and all legumes were nodulated. Roots were then partially dried using paper towel and stored in plastic bags at 4 °C. Once harvest was complete, total root length and average root diameter were determined using an Epson scanner and WinRHIZO 4.1 computer software (Regent Instruments Inc, Quebec, Canada). Two subsamples were taken: one for examination of root hairs and one for determination of colonisation by AMF. Fresh weights of the total roots and subsamples were recorded. Subsamples were stored in 50% (v/v) ethanol and the remaining roots oven-dried at 70 °C for five days and weighed. Total root dry mass (DM) was then calculated.

For determination of root hair lengths, subsamples were stained briefly in a 5 % (v/v) Schaeffer blue ink and vinegar solution and viewed at 40 × magnification on an Olympus BX51 microscope. Photographs were taken on an Olympus DP72 camera and root hair length measured from 10 sections of root of ~10 mm length which were at least 20 mm from the root tip. The length of 10 root hairs (when available) from each section was determined using the Olympus DP2-BSW software package. For determination of colonisation by AMF, subsamples were cleared and stained following the method of Vierheilig *et al*. (1998).

Root mass fraction was calculated as follows:

Root mass fraction (g g^{-1}) = root DM / (root DM + shoot DM)

Average root diameter (D), average root hair length (RHL) and specific root length (SRL) for each species/line was used to calculate the root hair cylinder volume (RHCV and specific RHCV, that is, the volume of sand that encompasses the root and root hair zone per unit root mass.

RHCV (cm^3 m^{-1}) = π $(0.5D+RHL)^2$ × 100

Specific RHCV (cm^3 g^{-1} root DM) = SRL × π $(0.5D+RHL)^2$

Rhizosphere carboxylates were analysed by reverse-phase liquid chromatography, as described by Cawthray (2003), except for oxalate where the method detailed in Uloth *et al*. (2015) was followed. Carboxylates examined in this study and their relevant limits of detection (µM) were citrate 5.0; malate 7.0; malonate 8.0; oxalate 5.0; fumarate 0.06; lactate 13.0; acetate 24.0; maleate 0.05; shikimate 0.015; succinate 15.0; citramalate 4.0; *cis*-aconitate 0.1; and *trans*-aconitate 0.1. Samples of 5 g were also collected from the plant-free controls. The amount of rhizosphere carboxylate was calculated relative to root DM, root length and RHCV.

Table 13.1 The pasture and crop species grown in this study and the strain of rhizobia used for inoculation.

Growth form	Species and authority	Common name	Cultivar/line	Rhizobial strain
Annual pasture legumes	*Biserrula pelecinus* L.	Biserrula	cv. Mauro	WSM1497
	Ornithopus compressus L.	Yellow serradella	cv. Santorini	WSM471
	O. sativus Brot.	French serradella	cv. Margurita	WSM471
	Trifolium hirtum All.	Rose clover	cv. Hykon	WSM1325
	T. incarnatum L.	Crimson clover	cv. Dixie	WSM1325
	T. purpureum Lois.	Purple clover	cv. Electra	WSM1325
	T. spumosum L.	Bladder clover	cv. Bartolo	WSM1325
	T. subterraneum L. ssp. *brachycalycinum*	Subterranean clover	line L66	WSM1325
	T. subterraneum L. ssp. *brachycalycinum*	Subterranean clover	line L67	WSM1325
	T. subterraneum L. ssp. *subterraneum*	Subterranean clover	cv. Dalkeith	WSM1325
	T. subterraneum L. ssp. *subterraneum*	Subterranean clover	cv. Dinninup	WSM1325
	T. subterraneum L. ssp. *subterraneum*	Subterranean clover	cv. Izmir	WSM1325
	T. subterraneum L. ssp. *subterraneum*	Subterranean clover	cv. Woogenellup	WSM1325
	T. subterraneum L. ssp. *yanninicum*	Subterranean clover	cv. Riverina	WSM1325
	T. subterraneum L. ssp. *yanninicum*	Subterranean clover	cv. Trikkala	WSM1325
Biennial pasture legume	*Hedysarum coronarium* L.	Sulla	cv. Flamenco	WSM1592
Perennial pasture legumes	*Bituminaria bituminosa* (L.) C.H.Stirt var. *albomarginata*	Tedera	Unknown	WSM4083
	Lotus corniculatus L.	Birdsfoot trefoil	LC07AUYF	SU343
	Medicago sativa L.	Lucerne	cv. SARDI 10	RRI128
	T. ambiguum M.Bieb.	Caucasian clover	cv. Kuratas	CC283b
Perennial pasture grasses	*Dactylis glomerata* L.	Cocksfoot	cv. Porto	Not applicable
	Phalaris aquatica L.	Phalaris	cv. Advanced AT	Not applicable
Annual crop legumes	*Cicer arietinum* L.	Chickpea	Unknown	CC1192
	Lupinus albus L.	White lupin	Unknown	WSM471
	L. angustifolius L.	Narrow leaf lupin	cv. Mandelup	WSM471

13.2.3 Data analyses

Rhizosphere carboxylates that were below the detection limit were given a value of zero. Lactate and acetate were present in a small number of pots and did not vary consistently with plant species; data are therefore not presented or included in calculations of total carboxylates.

For each measured variable, data were examined for outliers and normality, and transformations undertaken if required. Data were then analysed in Genstat version 14.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK) using a one-way ANOVA to assess the effect of the 25 species/lines on each variable; the effect of replicate was included in the analysis. The means for each species/line were graphed with the standard error of the mean using SigmaPlot version 12 (Systat Software Inc.). Note that *C. arietinum* was absent from three replicates and *T. subterraneum* cv. Riverina from two replicates.

13.3 Results

13.3.1 Rhizosphere carboxylate amount

The total amount of rhizosphere carboxylates relative to root DM differed greatly among species/lines (P<0.001; Fig. 13.1a) ranging from zero for *T. ambiguum* to 58 µmol g^{-1} root DM for *L. albus*, which did not differ from *L. angustifolius* and *O. sativus*. *Ornithopus compressus*, *M. sativa* and *T. hirtum* ranged from 28–42 µmol g^{-1} root DM. All other species exhibited less than 18 µmol g^{-1} root DM. Carboxylates did not differ among lines of *T. subterraneum,* all of which had less than 11 µmol g^{-1} root DM (Fig. 13.1a).

The total amount of rhizosphere carboxylates relative to root length also varied greatly among species/lines (P<0.001 for $\log_{10}$ transformed data; Fig. 13.1b). The most notable trend was that carboxylate amounts for the *Lupinus* spp. were at least five-fold greater than those of the remaining species. Of those species, *L. corniculatus*, *M. sativa* and *C. arietinum* had the greatest amounts. The *Ornithopus* spp. had a larger amount of carboxylate than any of the *T. subterraneum* lines.

The total amount of rhizosphere carboxylates relative to RHCV showed a broadly similar trend to the amount relative to root length, being by far greatest for *L. albus*, followed by *L. angustifolius*, *M. sativa* and *C. arietinum* (Table 13.2). However, the amount of carboxylate for the *Ornithopus* spp. was less than that of many of the *T. subterraneum* lines.

13.3.2 Rhizosphere carboxylate composition

Citrate was the dominant carboxylate in the rhizosphere of most species including *L. albus, L. angustifolius*, *M. sativa*, *T. hirtum*, *B. pelecinus* and *T. purpureum* (Fig. 13.2). Malonate was the dominant carboxylate for *C. arietinum*, while a mixture of citrate (~40 %) and malonate (~60 %) was measured for *O. compressus* and *O. sativus*. The two grasses, *P. aquatica* and *D. glomerata*, had a mixture of malate (~40 %) and citrate (~45–55 %). Malate comprised ~20 % of the carboxylates in *L. angustifolius*. Citramalate was present only in *L. corniculatus*, where it constituted ~70 % of all carboxylates. Oxalate comprised more than 20 % of carboxylates for the biennial *H. coronarium* and perennial *B. bituminosa*. Low amounts of fumarate were found for all species, except *T. spumosum* and *T. ambiguum*. The

carboxylate *trans*–aconitate was present only in *P. aquatica*, *L. albus* and *L. angustifolius* (1.16, 0.05 and 0.02 µmol g^{-1} root DM, respectively): *cis*–aconitate was not consistently present for any species. The sand from the plant-free pots contained only fumarate and maleate

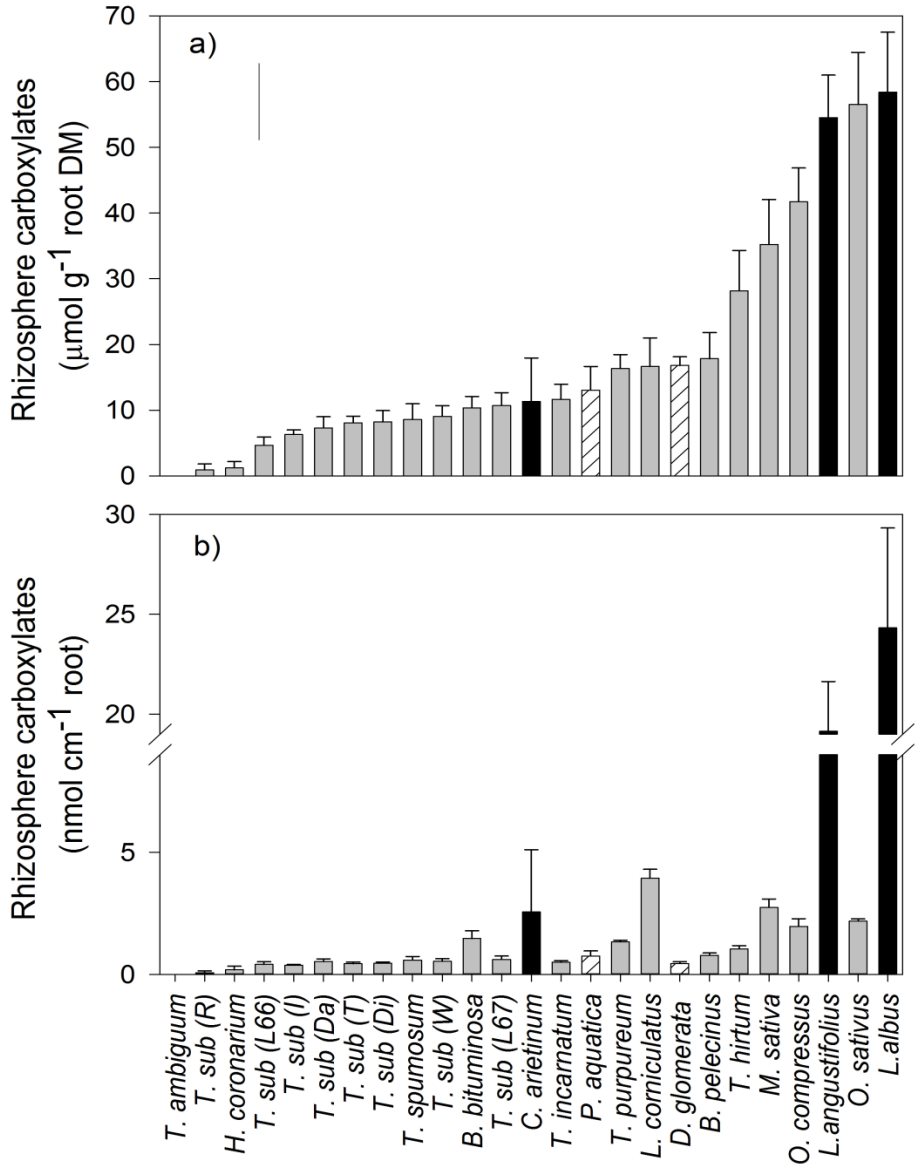


Figure 13.1 Total rhizosphere carboxylates (citrate, malonate, malate, fumarate, oxalate, citramalate) relative to a) root dry mass (DM) and b) root length for 13 pasture legumes (grey fill), two pasture grasses (hatch) and three species of grain legumes (black fill) (mean + s.e., l.s.d. at *P*=0.05; note, no l.s.d. for b) as data required log transformation prior to analysis). For *T. subterraneum*: R indicates cv. Riverina; W, cv. Woogenellup; Da, cv. Dalkeith; L66, Line 66; I, Izmir; L67, Line 67; Di, Dinninup; T, Trikkala. There were no detectable carboxylates found for *T. ambiguum*.

Table 13.2 Shoot dry mass, root mass fraction, shoot phosphorus concentration, root length, root hair cylinder volume (RHCV), specific RHCV and carboxylates per unit RHCV for 18 species.

Species	Shoot dry mass (mg)	Root mass fraction ($g\ g^{-1}$)	Shoot P ($mg\ g^{-1}$)	Root length (m)	RHCV ($cm^3\ m^{-1}$)	Specific RHCV ($cm^3\ g^{-1}$)	Carboxylates (µmol per unit RHCV)
Biserrula pelecinus	56	0.36	3.91	6.54	0.97	203	55
Ornithopus compressus	100	0.33	2.79	10.6	1.22	266	170
O. sativus	59	0.32	3.28	6.65	1.25	330	116
Trifolium hirtum	64	0.32	1.67	7.13	0.52	127	141
T. incarnatum	137	0.33	1.53	13.2	0.32	63	214
T. purpureum	37	0.40	1.08	3.00	0.37	45	108
T. spumosum	41	0.43	1.21	4.46	0.27	38	111
T. subterraneum							
line L66	127	0.41	1.93	9.91	0.40	44	107
line L67	178	0.36	1.80	14.7	0.26	41	363
cv. Dalkeith	161	0.41	2.00	15.2	0.31	43	270
cv. Dinninup	135	0.36	2.20	12.1	0.26	42	208
cv. Izmir	93	0.42	2.38	10.9	0.34	56	119
cv. Woogenellup	265	0.38	1.40	23.1	0.39	56	323
cv. Riverina	103	0.36	3.30	6.21	0.32	38	31
cv. Trikkala	160	0.34	1.87	11.5	0.29	41	167
Hedysarum coronarium	132	0.28	1.87	4.01	0.84	63	9
Bituminaria bituminosa	222	0.27	1.92	6.04	0.33	25	269
Lotus corniculatus	44	0.49	1.52	4.79	0.54	59	93
Medicago sativa	88	0.42	1.91	7.14	0.42	50	449
T. ambiguum	52	0.45	1.49	3.04	0.29	22	0
Dactylis glomerata	148	0.43	2.38	26.0	1.95	455	95
Phalaris aquatica	90	0.39	2.78	9.92	2.17	396	36
Cicer arietinum	200	0.40	4.02	7.33	0.69	30	468
Lupinus albus	1466	0.23	1.67	10.6	2.24	55	1102
L. angustifolius	916	0.31	1.76	11.7	2.83	81	794
P-value	<0.001	<0.001	<0.001	<0.001	n.a.[1]	n.a.[1]	n.a.[1]
LSD at *P*=0.05	60	0.07	0.77	3.97	n.a.	n.a.	n.a.

[1] No *P*-value; Values calculated using mean data for each species

.

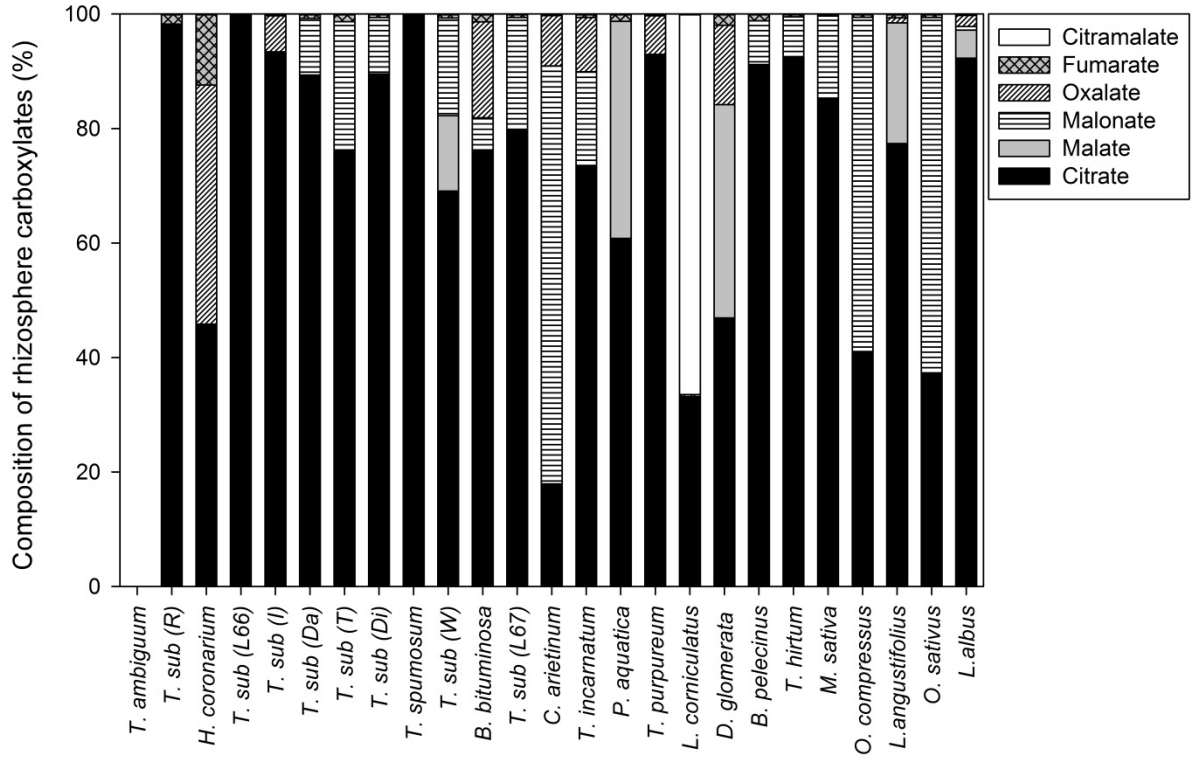


Figure 13.2 Composition of rhizosphere carboxylates for 13 species of pasture legumes, two pasture grasses and three species of grain legumes. For *T. subterraneum*: R indicates cv. Riverina; W, cv. Woogenellup; Da, cv. Dalkeith; L66, Line 66; I, Izmir; L67, Line 67; Di, Dinninup; T, Trikkala. There were no detectable carboxylates found for *T. ambiguum*.

13.3.3 Shoot dry mass and P concentration

Shoot DM and shoot P concentration both differed among species/lines (P<0.001, Table 13.2). Shoot DM ranged from 37–1466 mg and was greatest for the large-seeded *Lupinus* spp. Shoot P concentration ranged from 1.08–4.02 mg g^{-1}. Apart from *T. subterraneum* cv. Riverina which exhibited poor and inconsistent growth in this experiment, the two grass species, *B. pelecinus* and *Ornithopus* spp. had the greatest shoot P concentration.

13.3.4 Root morphology

Colonisation by AMF was either absent or <1 % of root length at harvest (results not presented). *Lupinus albus* formed cluster-like roots. Root mass fraction and root length varied among species/lines (P<0.001; Table 13.2). Root mass fraction ranged from 0.23–0.49 g g^{-1}. The *Lupinus* spp. were low (23 and 31 g g^{-1}) while the *Ornithopus* spp. were slightly greater (0.32 and 0.33 g g^{-1}). The *T. subterraneum* lines varied from 0.34 to 0.42 g g^{-1}. Root length ranged from 3–26 m and was greatest for *D. glomerata* (26 m) followed by four lines of *T. subterraneum* (15–23 m). The *Ornithopus* spp. showed intermediate values (11 and 7 m), as did the remaining lines of *T. subterraneum* (6–12 m) and the *Lupinus* spp. (11 and 12 m).

Average root diameter and specific root length differed among species/lines (*P*<0.001; Fig. 13.3). Average root diameter was greatest for the *Lupinus* spp. (0.89 and 0.68 mm), followed by *C. arietinum*, *H. coronarium* and *B. bituminosa* (Fig. 13.3a). Average root diameter then differed relatively little, but still significantly, among remaining species/lines (0.26–0.37 mm, LSD=0.03). Specific root length varied 10.8-fold among the species/lines (Fig. 13.3b). The *Lupinus* spp. and *C. arietinum* had reduced specific root length relative to all other species (<42 m g^{-1}), while the *Ornithopus* spp. and grasses were among the species with the greatest specific root length (>180 m g^{-1}). There was variation in specific root length among lines of *T. subterraneum* (111–167 m g^{-1}). Specific root length showed a negative correlation with average root diameter (R^2=0.73; *P*<0.001).

Root hair length varied among the species/lines by 6.1-fold (*P*<0.001; Fig. 13.4). The grasses had the longest root hairs (>0.65 mm). The *Lupinus* spp., *Ornithopus* spp. and *B. pelecinus* also had relatively long root hairs (>0.40 mm). All *Trifolium* spp., except *T. hirtum*, had root hairs shorter than 0.2 mm.

The RHCV and specific RHCV varied 11-fold and 21-fold, respectively, among the species/lines (Table 13.2). The *Lupinus* spp. had the largest RHCV, but a relatively small specific RHCV. The grasses had a large RHCV and the greatest specific RHCV. The *Ornithopus* spp. and *B. pelecinus* had a RHCV and specific RHCV that was less than that of the grasses, but greater than the majority of other species.

There were no significant correlations between root morphological traits and the amount of rhizosphere carboxylates when expressed on the basis of root DM or length.

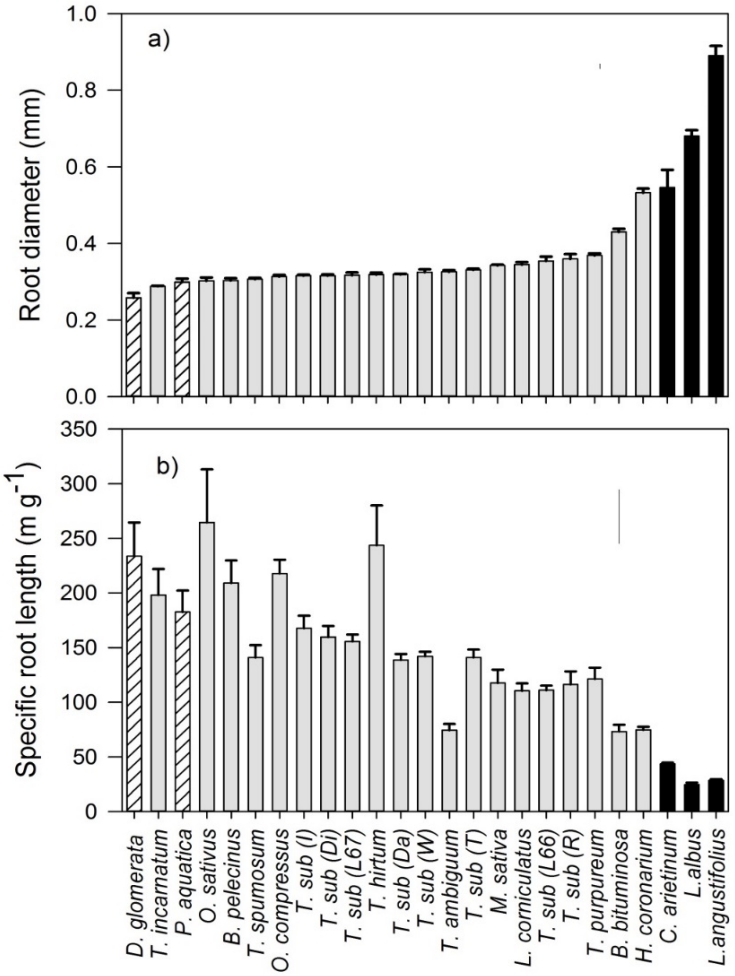


Figure 13.3 (a) Root diameter and (b) specific root length for 13 pasture legumes (grey fill), two pasture grasses (hatch) and three species of grain legumes (black fill) (mean + s.e., l.s.d. at *P*=0.05). For *T. subterraneum*: R indicates cv. Riverina; W, cv. Woogenellup; Da, cv. Dalkeith; L66, Line 66; I, Izmir; L67, Line 67; Di, Dinninup; T, Trikkala.

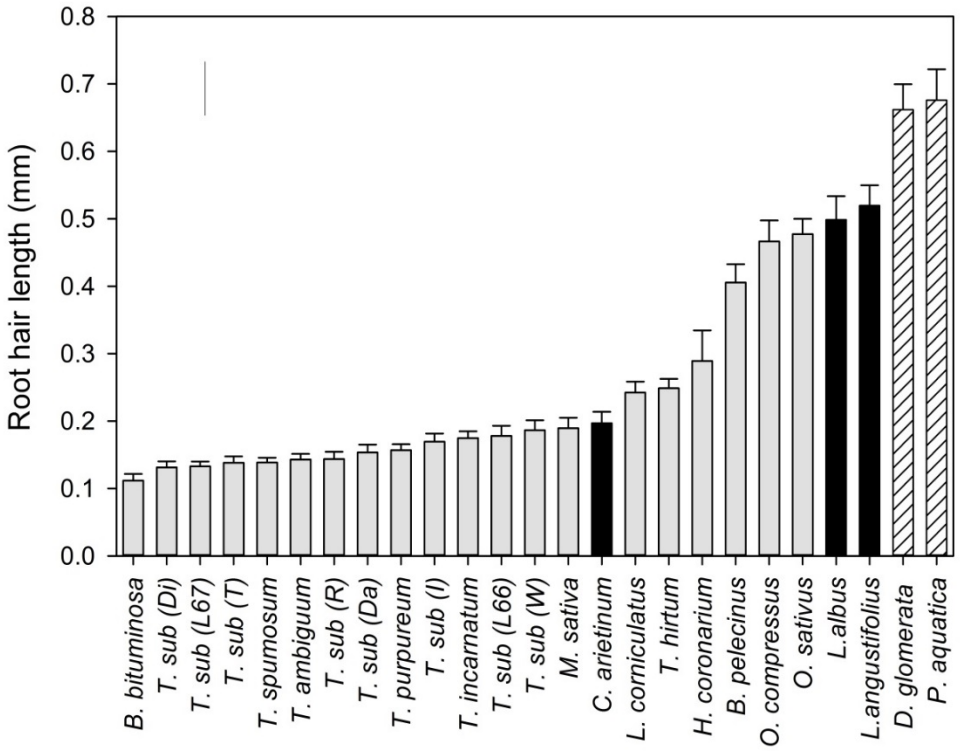


Figure 13.4 Root hair length for 13 pasture legumes (grey fill), two pasture grasses (hatch), three species of grain legumes (black fill)(mean + s.e., l.s.d. at *P*=0.05). For *T. subterraneum*: R indicates cv. Riverina; W, cv. Woogenellup; Da, cv. Dalkeith; L66, Line 66; I, Izmir; L67, Line 67; Di, Dinninup; T, Trikkala.

13.4 Discussion

13.4.1 Amount of rhizosphere carboxylates

Ornithopus sativus accumulated an amount of rhizosphere carboxylates relative to root DM similar to that of *L. albus* and *L. angustifolius*. Other species with relatively high carboxylates were, in decreasing order, *O. compressus*, *M. sativa* and *T. hirtum*. Comparison with other studies is problematic as rhizosphere carboxylate amount will differ with soil type (Veneklaas *et al*. 2003) and plant age (Suriyagoda *et al*. 2012), and may decrease with increasing P availability (Pang *et al*. 2010a; Suriyagoda *et al*. 2012). Even so, confidence in the magnitude of the carboxylate amounts measured in our study is provided by reports of reasonably similar amounts relative to root DM for *L. albus, L. angustifolius*, *M. sativa* and *T. subterraneum* grown in a range of media (Lipton *et al*. 1987; Nazeri *et al*. 2014; Pang *et al*. 2010a; Pearse *et al*. 2006). For *C. arietinum* only, the rhizosphere carboxylate amount in this study was low compared with other studies (Veneklaas *et al*. 2003; Pearse *et al*. 2006); poor plant growth and high shoot P concentration (Table 13.2) may have limited carboxylate production.

Both the *Ornithopus* spp. and *Lupinus* spp. showed high rhizosphere carboxylates relative to root DM and had a similar total root length. However, the specific root lengths were much less in the *Lupinus* spp. Thus, when the amount of carboxylates was expressed on a root

length basis, it was up to10-fold greater for the *Lupinus* spp. than the *Ornithopus* spp. This suggests that the *Lupinus* spp. maintain high amounts of carboxylate in their root cylinder, which is consistent with their high values for carboxylates per unit of RHCV (Table 13.2). The greater carboxylate amount in the RHCV of the *Lupinus* spp. presumably assists release of sparingly available forms of P (Gardner *et al*. 1983; Richardson *et al*. 2011; Ryan *et al*. 2014). For instance, Hocking *et al*. (1997) showed that *L. albus* and, to a lesser extent, *L. angustifolius*, can access pools of soil P largely unavailable to crops such as canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). For the *Ornithopus* spp., the relatively low amounts of rhizosphere carboxylates (particularly citrate) on a root length and RHCV basis suggest that they may not access sparingly soluble P as well as *Lupinus* spp.

Interestingly, *O. sativus* has also been reported to have high concentrations of acid phosphatase in surrounding soil relative to a range of other crop species, including *L. albus* (Eichler *et al*. 2004); this suggests an ability to access P from organic sources (Richardson *et al*. 2011). High amounts of both acid phosphatase and carboxylates in the rhizosphere of *Kennedia prostrata*, a low-P adapted Australian native, corresponded to a superior ability to use sparingly soluble soil P sources compared with that of *M. sativa* (Pang *et al*. 2015).

Despite large differences in root diameter and specific root length, both the *Lupinus* spp. and *Ornithopus* spp. had root hair lengths approaching those of the grasses. Long root hairs are a relatively low-carbon-cost means to ensure that capture of the P released by the high-carbon-cost carboxylates is maximised (Brown *et al*. 2013), assuming that the root hairs and carboxylate exudation occur in the same root zone (Ryan *et al*. 2014). The long root hairs of both the *Lupinus* spp. and *Ornithopus* spp. would also aid P uptake in the absence of carboxylates, as is often reported for grasses (Barrow 1975). Further investigation of the importance of root hairs in species with high rhizosphere carboxylates is warranted.

13.4.2 Composition of rhizosphere carboxylates

While the rhizosphere carboxylates of *Lupinus* spp. consisted predominantly of citrate (~80 %), the *Ornithopus* spp. had a mixture of citrate (~40 %) and malonate (~60 %). Malonate is considered uncommon both in species outside of the *Proteaceae* other than *C. arietinum*, *Sorghum bicolor*, *Glycine max* and *Cajanus sp.* (Roelofs *et al*. 2001) and as a major proportion of total carboxylate exudates. Aluminium toxicity was not present in this study, however it is interesting to point out that *Ornithopus* spp. are recognised for their aluminium tolerance (Mackay *et al*. 1991) and malonate is associated with the mobilisation of P from aluminium phosphate (Otani *et al*. 1996). The impact on plant P uptake from the mixture of citrate and malonate in *Ornithopus* spp. is unknown. Li and Copeland (2000) suggested malonate has a role in microbial inhibition, perhaps protecting other carboxylates from microbial degradation. However, when Oburger *et al*. (2009) added a mixture of malonate and citrate to five agricultural soils, P release was greater than when citrate or malonate were added alone, but the effect was additive, not synergistic.

The large proportion of rhizosphere carboxylates which consisted of citramalate for *L. corniculatus* was unexpected as there are no previous reports of citramalate in the root exudates of *L. corniculatus,* although it has been reported within the roots themselves (Navascues *et al*. 2012). Citramalate was recently studied in the root exudates of sugar beet by Khorassani *et al*. (2011) and was found to increase the concentration of P in solution.

13.4.3 Interaction of carboxylates with arbuscular mycorrhizal fungi (AMF)

AMF did not colonise the plants in this study to any significant degree, suggesting that the commercial inoculum was ineffective. However, pasture legumes and grasses, including *O. compressus*, are generally colonised by AMF (Hill *et al*. 2010; Schweiger *et al*. 1995; Thompson and Wildermuth 1989). As presence of AMF can reduce the amount of rhizosphere carboxylates by 50 % or more (Nazeri *et al*. 2014; Ryan *et al*. 2012), comparisons of rhizosphere carboxylates between *Lupinus* spp., which are generally considered non-hosts (Lambers *et al*. 2013; Thompson and Wildermuth 1989), and *Ornithopus* spp. should now be undertaken in the presence of AMF.

13.4.4 Phosphate foraging traits among legumes

Trifolium subterraneum, the most widely grown pasture legume in southern Australia, had larger average root diameter, reduced specific root length and a much reduced average root hair length compared with the *Ornithopus* spp. and grasses: a result consistent with that of Yang *et al*. (2015). This resulted in the specific RHCV of *T. subterraneum* being only 14–20% of the grasses which is consistent with an inferior ability to forage for P (Evans 1977; Hill *et al*. 2010; Tinker 1975). In addition, the amount of rhizosphere carboxylates for *T. subterraneum* was low when expressed relative to both root DM and root length. However, further research into the feasibility of selecting for improved P foraging is merited for *T. subterraneum* due to its broad adaptation and adoption (Nichols *et al*. 2012). It is possible that P-acquisition traits not measured in this study could vary among lines of *T. subterraneum. Ornithopus sativus*, *O. compressus*, *B. pelecinus* and *T. hirtum* had a specific RHCV comparable to the grasses and of these species, *O. sativus*, *O. compressus* and *T. hirtum* had greater quantities of rhizosphere carboxylates than *T. subterraneum* and the grasses. *Ornithopus compressus* and *O. sativus* are of particular interest as they are already adapted to, and grown in, large areas of southern Australia (Nichols *et al*. 2012). *Lotus corniculatus* may also merit further study due to the unusually large contribution of citramalate to its carboxylate composition.

13.4.5 Systems benefits from mixing P-acquisition strategies

Growing a mixture of species with contrasting nutrient foraging strategies increases overall soil exploration and thereby enhances yield (Zhang *et al*. 2014). Including a species with a large amount of rhizosphere carboxylates in a multi-species pasture could be particularly beneficial as it may allow neighbouring plants access to sorbed soil P (Li *et al*. 2013, 2014). Hence, we hypothesise that if rhizosphere carboxylates significantly aid P acquisition in *Ornithopus* spp. then adding them to pastures based on *T. subterraneum*, in situations where both species are well-adapted, may reduce the critical P requirement of the pastures without yield penalty. This hypothesis requires testing. However, it should be noted that *Ornithopus* spp. have small seeds and low seedling vigour compared with *T. subterraneum* and thus, on low-P soils, they might require more supplementary P at sowing/germination.

13.5 Conclusions

Of the 13 species of annual pasture legumes included in this study, *O. sativus* had the largest amounts of rhizosphere carboxylates relative to root DM and the greatest specific RHCV. This suggests there may be potential for *Ornithopus*-based pastures to be

maintained with lower P fertiliser inputs than are required for traditional *T. subterranean* pastures, presenting significant financial benefits to landholders as well as reducing P accumulation in soil and P runoff. The potential contribution of carboxylates to P acquisition by *Ornithopus* spp. merits further investigation.

14 An initial examination of inter-specific variation in root foraging traits in *Trifolium* species that are phylogenetically-allied to subterranean clover (*Trifolium subterraneum*).

Rowan Alden*, Rebecca E. Haling, Richard J. Simpson, Adam Stefanski, Graeme A. Sandral, Richard Snowball, Daniel R. Kidd, Hans Lambers, Megan H. Ryan

** research undertaken as part of a CSIRO summer student project awarded to RA.*

14.1 Introduction

Trifolium subterraneum is a key legume used in mixed pastures of southern Australia but it has a high external requirement for phosphorus (P) (Hill *et al*. 2005). The species has very short root hairs and thick roots which limits the potential for breeding more P efficient cultivars (Chapter 7). We assessed the variation in root traits of genetically-allied *Trifolium* spp. (Fig. 14.1) that could potentially be hybridised with *T. subterraneum*.

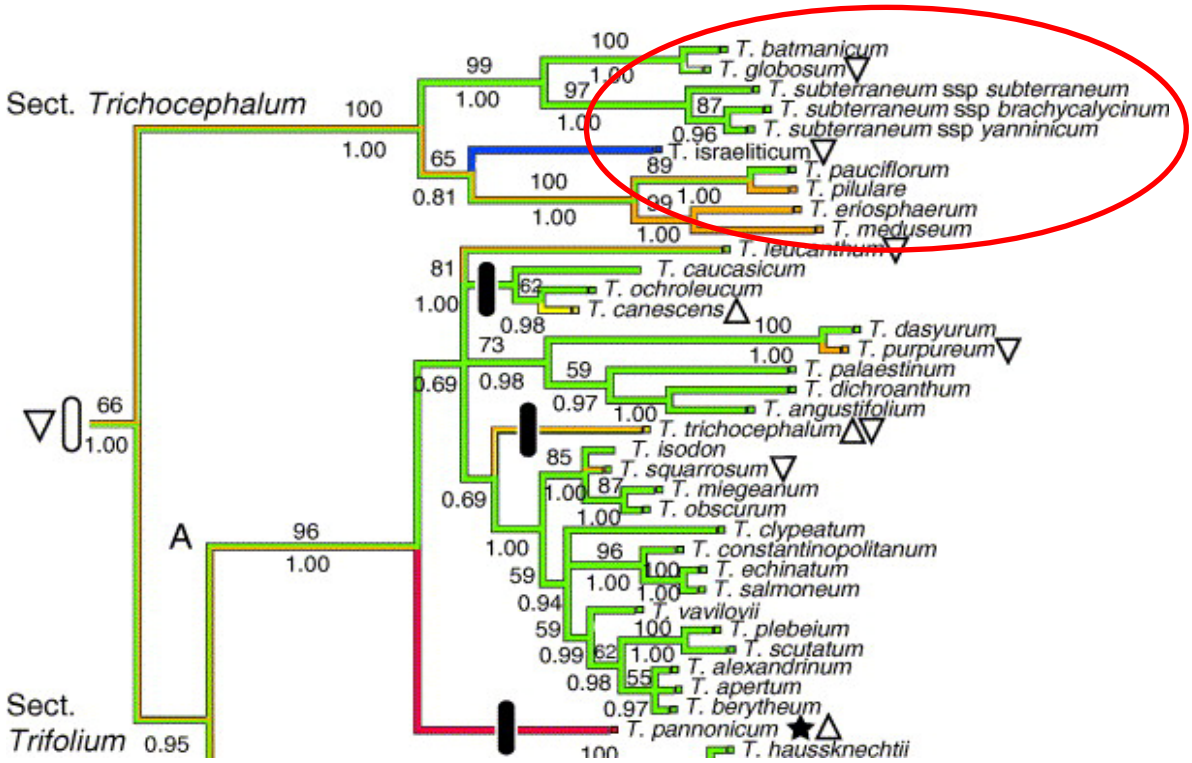


Figure 14.1 Phylogenetic relationships of *Trifolium* species (Ellison *et al*. 2006). The species most closely aligned to *T. subterraneum* occur in the Section *Trichocephalum*.

14.2 Materials and methods

14.2.1 Plant material

The shoot and root growth of a single genotype of each species in the *Trichocephalum* section (Ellison 2006) and *Ornithopus sativus* Brot. was assessed in response to application of P. Lines of *T. batmanicum* Katzn. (WCT45BAT), *T. eriosphaerum* Boiss. (29413), *T. globosum* L. (140662), *T. israeliticum* Zohary & Katzn. (24416), *T. meduseum* Blanche ex. Boiss (CG/1), *T. pauciflorum* (46340) and *T. pilulare* Boiss. (45040) were sourced from the Australian Trifolium Genetic Resource Centre. Cultivars of *T. subterraneum* L. (Seaton Park) and *O. sativus* (Margurita) were sourced from local suppliers. *Ornithopus sativus* was used as a benchmark of a legume with a low critical requirement for P.

14.2.2 Soil

Methods were based on that of Chapter 6 and 7. A sandy loam soil was collected from 2–10 cm depth of a Yellow Chromosol (Isbell 1996) at Ginninderra Experiment Station, Canberra, ACT, Australia (35°11'S, 149°3'E; elevation 597 m). The soil was steam pasteurised (60-65°C) to reduce levels of disease inoculums, sieved (< 5mm) and mixed with lime to raise pH (1:5 $CaCl_2$) to 5.5 (1.06 g $CaCO_3$ kg^{-1}). Nutrients were mixed through the soil at rates of 41.1 mg kg^{-1} $MgSO_4.7H_2O$, 43.0 mg kg^{-1} $CaSO_4.2H_2O$, 169 mg kg^{-1} KNO_3, 27.5 mg kg^{-1} $(NH_4)_2SO_4$, 16.7 mg kg^{-1} NH_4NO_3, 119 µg kg^{-1} H_3BO_3, 759 µg kg^{-1} $MnCl_2.4H_2O$, 359 µg kg^{-1} $ZnSO_4.7H_2O$, 33.3 µg kg^{-1} $CuSO_4.5H_2O$, 72.1 µg kg^{-1} $(NH_4)_2MoO_4$, 19.8 µg kg^{-1} $CoCl_2 \cdot 6H_2O$ and 1530 µg kg^{-1} Fe-EDTA. Three P treatments were established by mixing KH_2PO_4 to sub-samples of the amended soil at rates of 0, 15 and 200 mg P kg^{-1} (oven dry equivalent) i.e. 0, 5 and 60 mg P applied pot^{-1}. Pots (cylindrical PVC; 87 mm internal diameter, 190 mm soil height) were filled with 0.900 kg (oven dry equivalent) of un-fertilised soil and a top layer of 0.3 kg of P-fertilised soil. The boundary of the two layers was marked by placing small polyethylene beads around the edge.

14.2.3 Plant growth conditions

Fifty mg of viable seed was established per pot. Five replicates of each P rate were prepared for each species. Plants were grown in a controlled-environment cabinet with 12 h light (700 µmol $quanta^{-1}$ m^2 s^{-1}) and12 h dark at 25 and 15°C, respectively. Soil was inoculated with Group C and Group S rhizobium for *Trifolium* spp. and *Ornithopus sativus*, respectively. Pots were maintained at approximately 75% field capacity (17% moisture) by daily watering, and weighing of pots twice weekly. Pots were arranged in a randomised complete block design and rotated twice weekly. Reflective sheets were fitted to the rim of the pots and maintained at canopy height to mimic the light conditions that occurs in a sward.

14.2.4 Harvest and measurements

Plants were harvested six weeks after sowing. Shoots were cut at the soil surface. Soil was removed from the pot as an intact core and cut at the boundary of the un-fertilised bottom layer and P-fertilised top layer. Roots were washed from both layers. Roots from half of the P-fertilised top layer were scanned immediately using a flatbed scanner and root length and average root diameter analysed using WinRHIZO (Regent Instruments Inc., Quebec, Canada). A small sub-sample was stored in 50% ethanol for measurement of root hair

length. Roots and shoots were dried at 70°C for dry mass determination. Images of fully elongated root hairs were taken using a Leica MZFLIII Fluorescence microscope fitted with a Zeiss AxioCam camera. Root hair length of fifteen root hairs per pot were measured using ImageJ software (Rasband, 1997-2014).

Shoots and roots were milled to a powder and 25 to 50 mg samples ashed at 550°C for 4 hours. Samples were dissolved in HCl and P concentration determined colormetrically using malachite green (Irving and McLaughlin 1990).

14.2.5 Statistical analysis

The three P treatments were used to create a rapid means for assessing the relative critical external requirement for P for the nine species. For each species, a broken-stick model was fitted to the shoot dry matter data in response to P. It was assumed that the relationship between shoot dry matter and application of P between 0 and 5 mg P pot^{-1} was linear and represented the slope of the P response, and that shoot dry matter at 60 mg P pot^{-1} represented maximum growth. The break point was determined as the intersect between maximum growth and the linear response below this point; this was considered to represent the critical external requirement for P.

Total dry mass of roots in the P-fertilised layer was determined as the sum of the un-scanned and scanned samples. Total root length was determined by adjusting for the dry mass of roots that were scanned as a percent of total root dry mass. Specific root length was calculated by dividing length of the scanned roots by the mass of the scanned roots. Root hair cylinder was calculated as the cylinder of soil encompassing the root (i.e. root diameter and root length) and length of root hairs.

Parameters of root growth and P uptake were analysed in GenStat 16th Edition (VSN International, UK) using analysis of variance. Where necessary, data were log-transformed.

14.3 Results

14.3.1 Shoot dry matter response and critical external requirement for P

Shoot dry matter of all species increased in response to addition of P but the slope of the response and maximum yield differed between species (Fig. 14.2, Table 14.1). *Ornithopus sativus* achieved the highest shoot yield (3.78 g pot^{-1}) and lowest critical external requirement for P (24.7 mg P pot^{-1}). Amongst the *Trifolium* spp., *T. globosum* had the highest yield (3.46 g pot^{-1}) and lowest critical P requirement (36.7 mg P pot^{-1}) followed by *T. subterraneum* which had a lower critical P requirement (42.3 mg P pot^{-1}) relative to the other *Trifolium* spp. but yielded as well as, or better than these species. The critical external P requirement of some of the *Trifolium* species was very high (e.g. 144.2 mg P pot^{-1} for *T. eriosphaerum*). However, if the growth response of these species was part of a sigmoidal rather than linear response between 0 and 5 mg P pot^{-1}, these values may represent an over-estimation of the critical external P requirement.

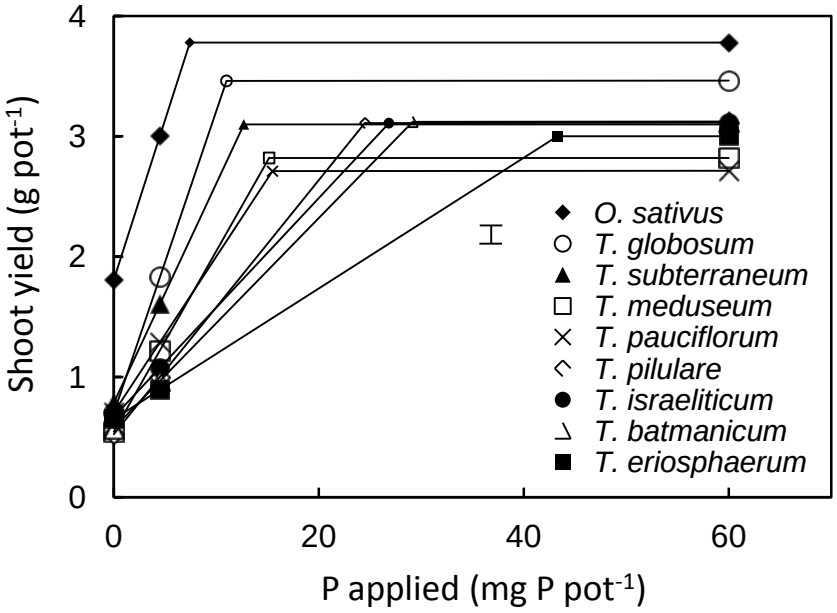


Figure 14.2 Shoot yield of *Ornithopus sativus* and eight *Trifolium* spp. in response to phosphorus (P) applied at 0, 5 and 60 mg P pot^{-1} to the top 0-5 cm of soil. Bar shows LSD for two-way interaction (*P*<0.05; n=5). Reduced symbols show estimate of the critical P requirement for each species calculated as the intersect (i.e. break-point) of the linear response between 0 and 5 mg P pot^{-1} and an assumed constant maximum response at 60 mg P pot^{-1}. NB: Estimates of the critical P requirement using this method may not be accurate if the growth response was sigmoidal.

Table 14.1 Estimate of the critical P requirement for each species calculated as the intersect (i.e. break-point) of the linear response between 0 and 5 mg P pot^{-1} and an assumed constant maximum response at 60 mg P pot^{-1} (Yield maximum). NB: Estimates of the critical P requirement using this method are approximations that assume a broken-stick model adequately fits to the available growth response data; they may not be accurate if the growth response was sigmoidal.

Species	Critical P (mg P kg^{-1})	Yield maximum (g pot^{-1})
O.sativus	24.7	3.78
T.globosum	36.7	3.46
T.subterraneum	42.3	3.10
T.meduseum	50.5	2.82
T.pauciflorum	51.6	2.71
T.pilulare	81.7	3.11
T.israeliticum	89.4	3.11
T.batmanicum	97.2	3.12
T.eriosphaerum	144.2	3.00
LSD P<0.05	n/a	0.07

14.3.2 Variation in nutrient foraging traits

Root length density, specific root length and root hair length at 60 mg P pot^{-1} was assumed to reflect the intrinsic nutrient foraging traits of the species (Fig.14.3). Intrinsic variation in the root length density of the *Trifolium* spp. ranged from 11 cm cm^{-3} (*T.eriosphaerum*) to 22 cm cm^{-3} (*T. meduseum*). Specific root length of the *Trifolium* spp. ranged from 129 m g^{-1} (*T. israeliticum*) to 206 m g^{-1} (*T. globosum*). Root hair length of the *Trifolium* spp. ranged from 0.20 mm (*T. israeliticum*) to 0.33 to 0.34 mm (*T. meduseum* and *T. pauciflorum*, respectively). *Ornithopus sativus* had significantly higher specific root length (264 m g^{-1}) and longer root hairs (0.58 mm) than the *Trifolium* species, and its root length density was at the upper end (20 cm cm^{-3}) of that measured among the *Trifolium* spp..

Some *Trifolium* spp. demonstrated marked changes in root morphology in response to low P supply. *Trifolium globosum and T. subterraneum* increased specific root length 1.2-fold, and root length density 1.5 to 2.4-fold, respectively (5 cf. 60 mg P pot^{-1}). *Trifolium pauciflorum*, *T. meduseum* and *T. pilulare* were the only *Trifolium* spp. to increase root hair length in response to low P supply. Most notably, *T. pauciflorum* and *T. meduseum* increased root hair length 1.2- to 1.3-fold (60 mg P pot^{-1} cf. 5 mg P pot^{-1}) and maintained longer root hairs than other *Trifolium* spp. at 0 mg P pot^{-1}. The remaining *Trifolium* spp. either showed no significant adjustment in root morphology, or nutrient foraging traits were not significantly greater than that of *T. subterraneum*.

Ornithopus sativus increased root hair length (1.3-fold), specific root length (1.3-fold) and root length density (1.6-fold) in response to low P supply (5 cf. 60 mg P pot^{-1}). At 0 mg P pot^{-1}, nutrient foraging traits of *O. sativus* were significantly greater than that of all of the *Trifolium* spp.: specific root length (1.5-fold higher), root hairs (1.7-fold long) and root length density (2-fold higher) .

14.3.3 Root cylinder volume and P uptake per unit surface area of root cylinder

Nutrient foraging traits were integrated to estimate their potential influence on P acquisition by calculating the root hair cylinder volume in the topsoil (Fig. 14.4a). Among the *Trifolium* spp., intrinsic root hair cylinder volumes (i.e. root hair cylinder volume at 60 mg P pot^{-1}), ranged from 15 cm^3 (*T. israeliticum*) to 43 cm^3 (*T. meduseum)*. *Trifolium pauciflorum* and *T. meduseum* had intrinsically larger root hair cylinder volumes than all other *Trifolium* spp. but values were till only 50%, or less, that measured for *O. sativus* (88 cm^3).

Among the *Trifolium* spp., maximum root hair cylinder volumes were measured at 5 or 60 mg P pot^{-1}, and corresponded to maximums in root length density and/ or root hair length evident in Fig. 14.4. At low P (0 and 5 mg P pot^{-1}), the root hair cylinder volume of *O. sativus* were substantially larger (3.2- to 5.6-fold) than that of the *Trifolium* spp. with more than 50% exploration of the fertilised volume of soil.

P uptake per unit surface area of the root hair cylinder among the *Trifolium* spp. and *O. sativus* were remarkably similar at both 0 and 5 mg P pot^{-1} (Fig. 14.4b). *Trifolium israeliticum* may be an exception in that it appeared to achieve twice the rate of P uptake per unit surface arera of the root hair cylinder than any of the other species. P uptake per unit surface area of the root hair cylinder was significantly higher at 60 mg P pot^{-1} relative to 0 and 5 mg P pot^{-1}.

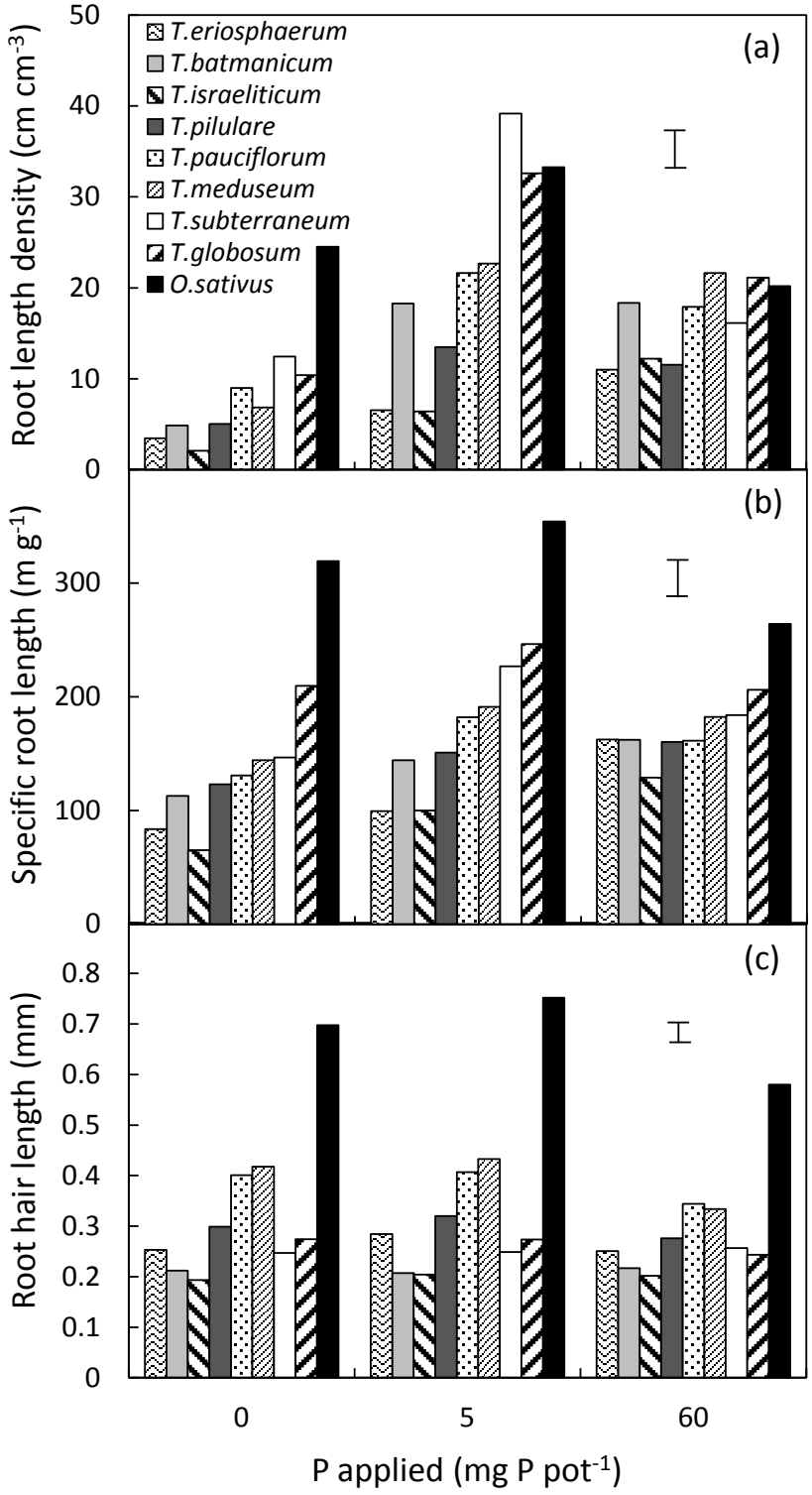


Figure 14.3 (a) Root length density (b) specific root length and (c) root hair length of eight *Trifolium* spp. and one *Ornithopus* spp. in response to P applied at 0, 5 and 60 mg P pot^{-1} to the top 0-5 cm of soil. Bars show LSD for two-way interactions (P<0.05; n=5).

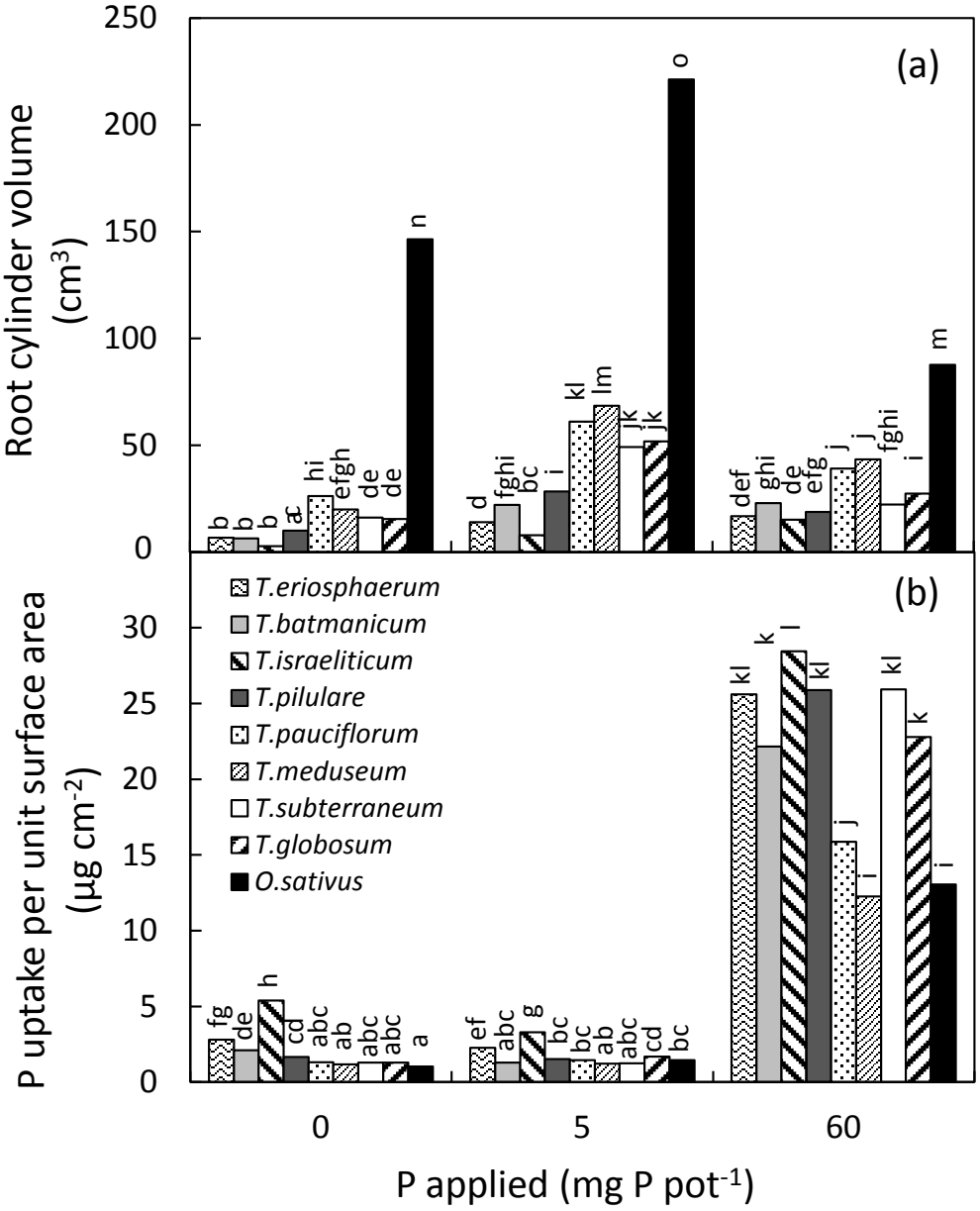


Figure 14.4 (a) Volume of root hair cylinder in topsoil and (b) P uptake per unit surface area of root hair cylinder of eight *Trifolium* spp. and one *Ornithopus* spp. in response to P applied at 0, 5 and 60 mg P pot^{-1} to the top 0-5 cm of soil. Different letters denote significant differences (P<0.05; n=5). Volume of topsoil in (a) was 280 cm^3.

14.4 Discussion

The legumes differed substantially in their critical P requirements and root foraging traits in the P-amended topsoil, but were often able to achieve similar maximum yields when grown with high P supply. However, there were substantial differences in the ways that the *Trifolium* spp. achieved a high potential for soil exploration. For example, *T. subterraneum* and *T. globosum* developed a relatively large root hair cylinder volume by achieving high root length density and high specific root length. In contrast, *T. meduseum* and *T. pauciflorum* achieved a relatively large root hair cylinder by deploying moderately long root hairs.

Similar rates of P uptake per unit surface area of the root hair cylinder at 0 and 5 mg P pot^{-1} (i.e. excluding luxury uptake that could occur when measured above the critical external requirement for P) provides further support that maximising soil exploration is key to achieving a low critical external requirement for P in legumes. One species (*T. israeliticaum*) may have achieved higher uptake per unit surface area of the root hair cylinder. This might be feasible for a species with an exceptional ability to exude organic anions or perhaps a special relationship with AMF. However, *T. israeliticaum* only achieved a very low root length density and low specific root length, both of which were counter-productive for total P uptake. Differences in yield potential (Table 14.1) and internal P use efficiency (not determined) may have also potentially influenced whether differences in soil exploration translated into differences in the critical P requirement of these species.

14.5 Conclusions

The results highlight significant and potentially useful variation in nutrient foraging traits among *Trifolium* spp. that are genetically allied with subterranean clover. This may indicate potential for introgression of root traits by inter-specific hybridisation. However, this was a preliminary investigation and further work is required to quantify the range in root phene variation within and among these species, whether the root traits are stable and characteristic of the species, and to determine whether successful interspecific crosses can be made. Of particular interest are *T. meduseum* and *T. pauciflorum* for their relatively long root hairs.

Acknowledgement

RA was the recipient of a CSIRO summer studentship.

15 Progress in the aligned PhD project: Root morphology and P-efficiency impacts of root disease organisms.

Research undertaken by Robert Jeffery

Supervisors: Megan H. Ryan, Martin Barbetti, Hans Lambers, Richard J. Simpson

15.1 Introduction and overview

UWA PhD student, Mr Rob Jeffery, commenced in September 2012. His research is conducted at UWA, Perth and he is supervised by Megan Ryan, Richard Simpson, Hans Lambers and Martin Barbetti. Mr Jeffery is currently completing his last experiment. The majority of samples from his other experiments have been processed. At the time this summary was prepared, a full draft of his first experimental chapter was close to completion andit was anticipated that the thesis would be submitted in June 2016.

Mr Jeffery first investigated the response of root morphology traits related to P uptake to application of six levels of P addition for six cultivars of subterranean clover grown in mini-swards. He then examined how root disease and arbuscular mycorrhizal fungi (two factors present in all fields, but absent in many glasshouse experiments) affected root morphology and the response of subterranean clover to P application. The effect of root disease was examined for three cultivars of subterranean clover and the effect of mycorrhizal fungi for two cultivars of subterranean clover and two species of serradella. Finally, the effect of plant growth in a mini-sward or without constraint to the spreading of the legume canopy on root trait expression was examined.

15.2 Experiment 1. The response to increasing P of six cultivars of subterranean clover

15.2.1 Background

The aim of this experiment was to determine whether cultivars of subterranean clover differed in their root morphology and their growth response to P.

15.2.2 Method

Six cultivars of subterranean clover (Woogenellup, Riverina, Daliak, Clare, Seaton Park and Geraldton) were grown in a low-P, disease-free, field soil. The soil was not pasteurised. Six P treatments were established (0, 3.47, 8.07, 16.15, 32.3, and 60 mg P kg soil^{-1}) by adding P to the top 5 cm of soil when it was dry. Pots were surrounded by reflective sleeves to mimic sward conditions. Appropriate rhizobia were applied, along with P-free basal nutrients. Harvest occurred after 6 weeks of growth. Data were collected on shoot and root dry mass and P concentration, root length, specific root length, average root diameter, root tissue density, percentage of root length colonised by mycorrhizal fungi and amount of rhizosphere carboxylates. Results were analysed with a 2-way ANOVA.

15.2.3 Results

The experiment demonstrated that a higher level of P addition than used in the experiment was necessary to maximise growth of most cultivars in this soil type and hence the critical external P requirement could not be calculated (Fig. 15.1A). However, this informed the levels of P addition used in the subsequent experiments and a number of interesting findings were made.

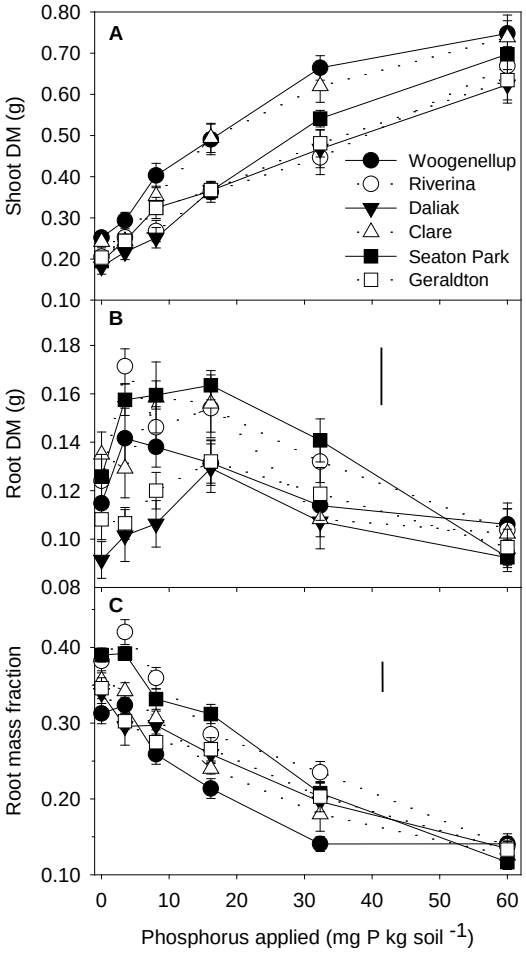


Figure 15.1 The effect of six levels of soil phosphorus (P) application on shoot DM (A), root DM (B) and root mass fraction (C) of six cultivars of *Trifolium subterraneum* grown for six weeks (mean±s.e., n=10, LSD at *P*=0.05). There was an effect of P level (*P* <0.001) and cultivar (*P*<0.001) on shoot DM with no significant interaction between P level and cultivar. There was a significant interaction between P level and cultivar for root DM (*P*=0.002, $LSD_{0.05}$=0.023) and root mass fraction (*P*<0.001, $LSD_{0.05}$=0.038). Six and two outliers were removed from (A) and (B), respectively.

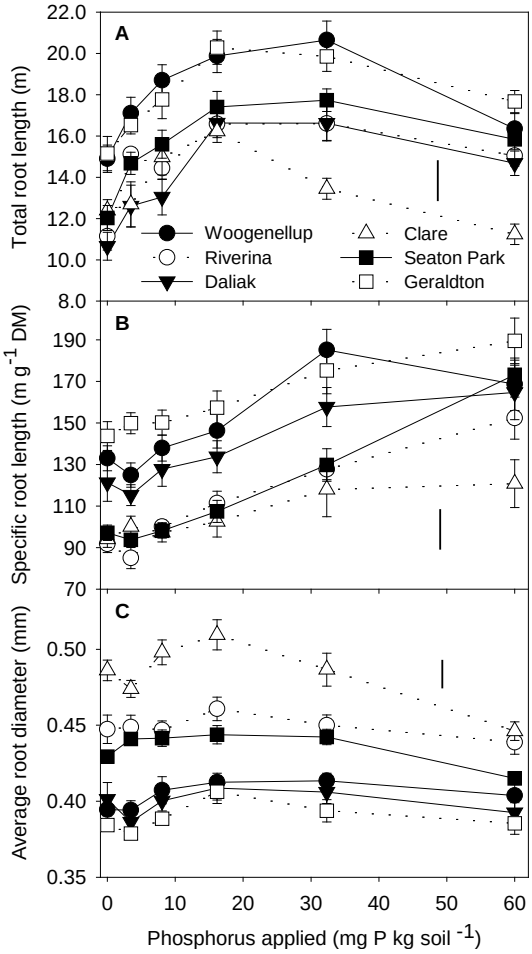


Figure 15.2 Total root length (**A**), specific root length (B) and average root diameter (C) of six cultivars of *Trifolium subterraneum* grown for six weeks with six levels of soil P application (mean±s.e., n=10, LSD at *P*=0.05). There was a significant interaction between P level and cultivar for total root length (*P*=0.008, $LSD_{0.05}$=1.94), specific root length (*P*<0.027, $LSD_{0.05}$=19.52) and average root diameter (*P*=0.012, $LSD_{0.05}$=0.017). Two, four and two outliers were removed from the data in (A), (B) and (C), respectively.

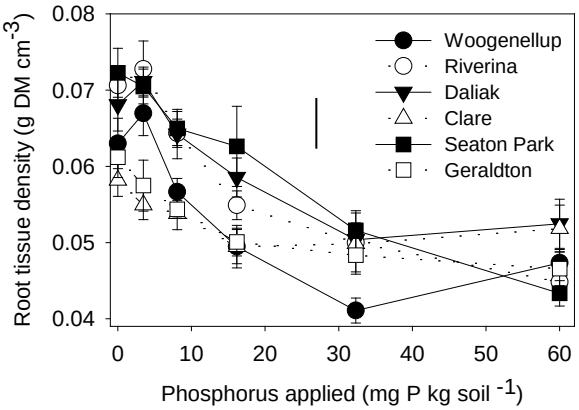


Figure 15.3 The effect of six levels of soil phosphorus (P) application on root tissue density of six cultivars of *Trifolium subterraneum* grown for six weeks (mean±s.e., n=10, LSD at *P*=0.05). There was a significant interaction between P level and cultivar (*P*<0.001, $LSD_{0.05}$=0.006). Seven outliers removed.

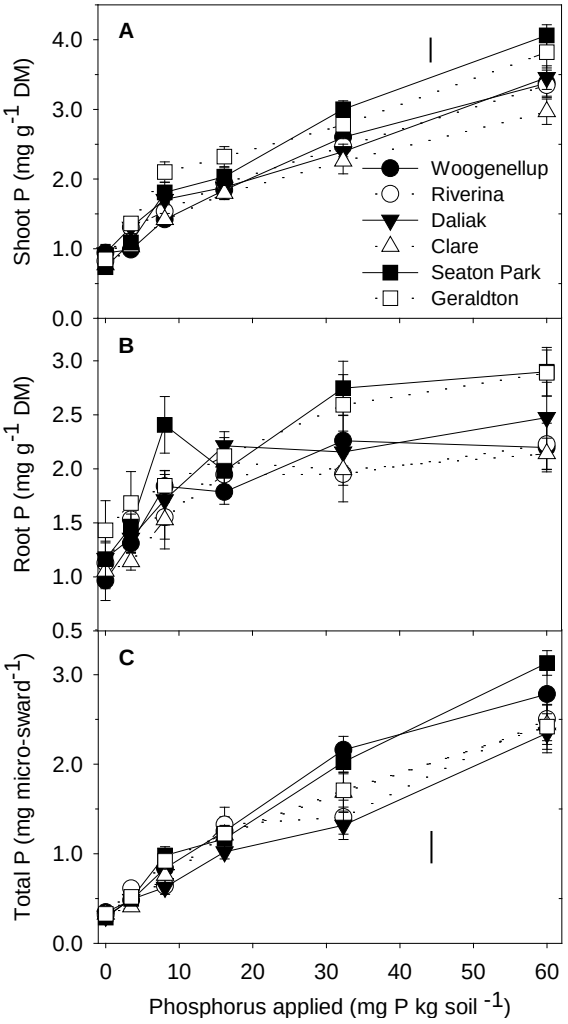


Figure 15.4 Phosphorus (P) concentration of shoot (A) and root (B) tissue, and total P content (C) of six cultivars of *Trifolium subterraneum* grown for six weeks with six levels of P application (mean±s.e., n=10, LSD at *P*=0.05). There was a significant interaction between P level and cultivar for shoot P (*P*=0.001, $LSD_{0.05}$=0.338) and total plant P content (*P*=0.009, $LSD_{0.05}$=0.344). There was a significant effect of P level (*P*<0.001) and cultivar (*P*<0.001) for root P with no significant interaction between P level and cultivar. Six, seven and two outliers were removed from (A), (B) and (C), respectively.

Shoot dry mass (DM) differed little among the six cultivars at the lowest two P levels, but was higher for Woogenellup and Clare at intermediate P levels (Fig. 15.1A). Root DM first increased, and then decreased with increasing P (Fig. 15.2B). Root DM varied greatly among cultivars, particularly at the lower three levels of P addition. The P level at which root DM was highest differed among cultivars (from P3.5 to P16). Root mass fraction decreased steadily with increasing P, and tended to be lowest at intermediate P levels for Clare and, in particular, Woogenellup (Fig. 15.1C). Total root length reached a maximum at P16–P32 with Clare only showing a marked decrease above P16. Woogenellup and Geraldton were notable for longer total root length (Fig. 15.2A). Specific root length increased with increasing P level, but cultivar rankings were consistent among P levels, with Woogenellup and Geraldton high and Clare low, especially at P60 (Fig. 15.2B). The increase in specific root length with P level reflected a decrease in root tissue density (Fig. 15.3). Average root diameter differed among cultivars, with Clare highest, but cultivar rankings were consistent among P levels and there was little effect of P level (Fig. 15.2C).

Shoot P concentration increased steadily with P level, with Seaton Park and Geraldton tending to be higher than other cultivars, and Clare lower (especially at the highest P level) (Fig. 15.4A). Root P concentration was more variable and stopped increasing by P30 (Fig. 15.4B). Differences among cultivars in root P concentration generally reflected differences in shoot P concentration. Shoot P-use efficiency decreased steadily with increasing P until P16 and then decreased only slightly thereafter (Fig. 13.5); differences among cultivars were small.

Three main carboxylates were present in the rhizosphere of the six clover cultivars: malate, malonate and citrate (Fig. 15.6). Malate was present only in Clare and decreased strongly with an increase in P level. Malonate was present in the rhizosphere of all cultivars, but did not respond to P. Citrate was present for most cultivars, but was much higher for Clare. Fumarate and maleate were also present, but only in trace amounts (Fig. 15.7A and 15.7B). Total carboxylates, the sum of these five carboxylates, was approximately three times higher for Clare than the other cultivars, which were all similar (Fig. 15.7C).

The percentage of root length colonised by indigenous mycorrhizal fungi was limited by low and high P supply (i.e. colonisation reached a maximum of ~ 30-40% of root length at an intermediate P supply level).

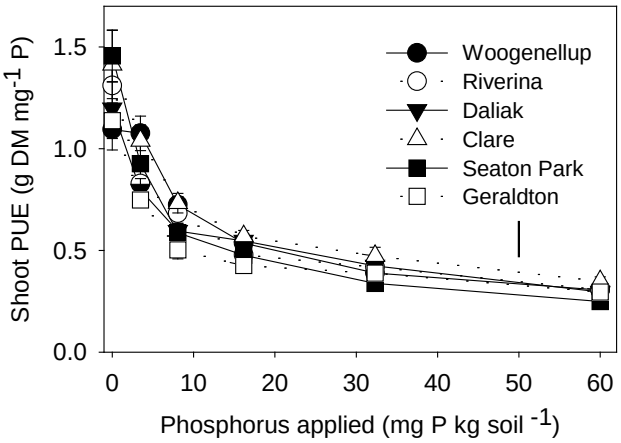


Figure 15.5 Internal phosphorus (P) utilisation efficiency (PUE) of shoots of six cultivars of *Trifolium subterraneum* grown for six weeks with six levels of soil P application (mean±s.e., n=10, LSD at P=0.05). There was a significant interaction between P level and cultivar (P=0.008, $LSD_{0.05}$=0.167). Three outliers were removed.

.

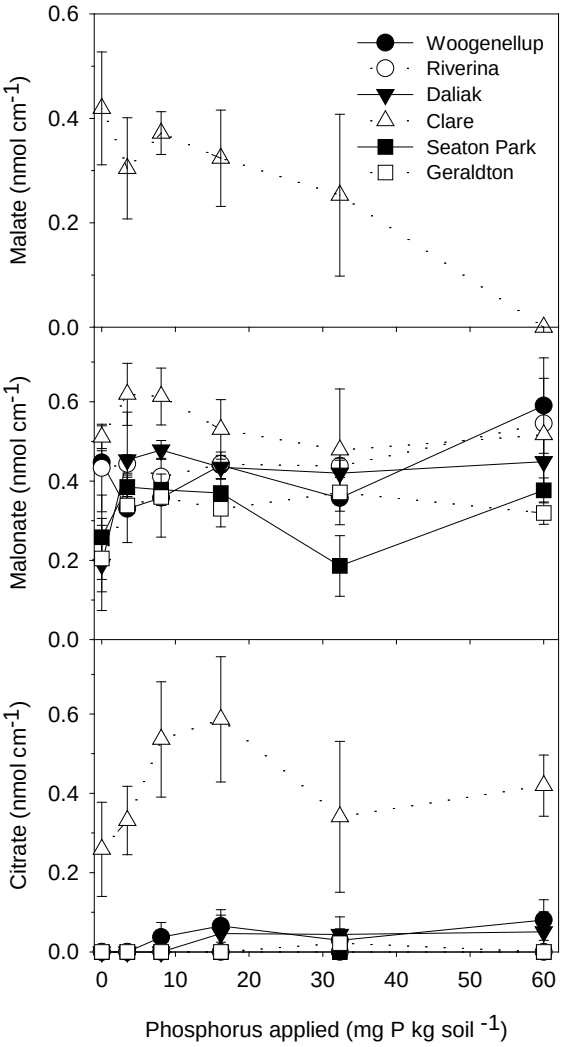


Figure 15.6 Amount of rhizosphere carboxylates (malate (A), malonate (B) and citrate (C) collected per cm root length from six cultivars of *Trifolium subterraneum* grown for six weeks with six levels of soil P application (mean±s.e., n=5, LSD at *P*=0.05). There was a significant effect of P level for malate (*P*=0.014, $LSD_{0.05}$=2.27) and an interaction between P level and cultivar for malonate (*P*=0.023, $LSD_{0.05}$=2.25). There was an effect of cultivar (*P*<0.001) but not P level for citrate, with no significant interaction between P level and cultivar. Note - some cultivars are not graphed because the specific organic acid was not detected for any samples of that cultivar. Three outliers were removed from (B).

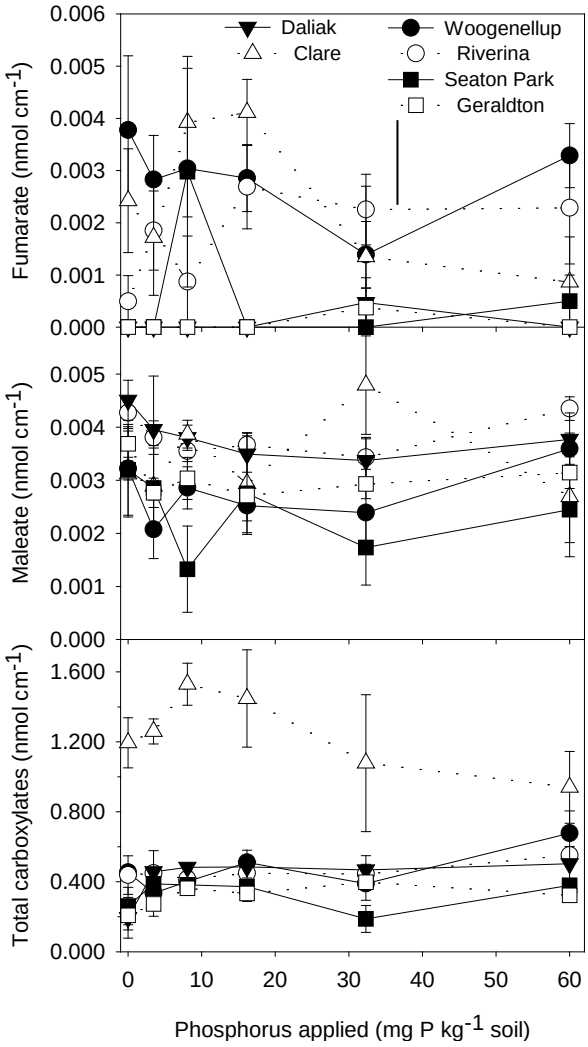


Figure 15.7 Amount of rhizosphere carboxylates (fumarate (A), maleate (B) and total carboxylates (excluding acetate and lactate) (C) collected per cm root length from of six cultivars of *Trifolium subterraneum* grown for six weeks with six levels of soil P application (mean±s.e., n=5, LSD at *P*=0.05). There was a significant interaction between P level and cultivar for fumarate (*P*=0.032, $LSD_{0.05}$=0.021) and total carboxylates (*P*=0.009, $LSD_{0.05}$=3.2). There was an effect of cultivar (*P*<0.001) and P level (*P*<0.001) for maleate with no significant interaction between P level and cultivar.

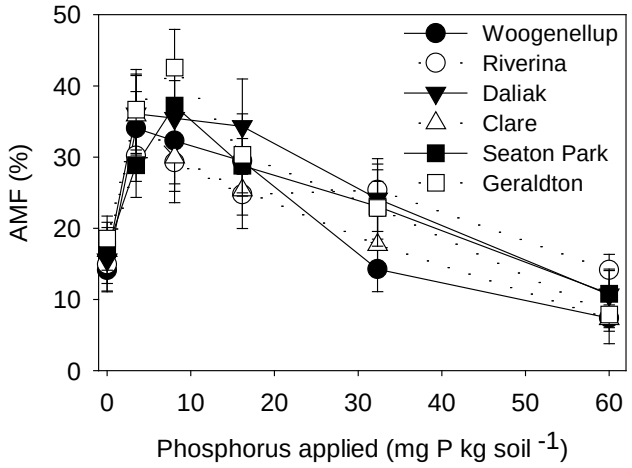


Figure 15.8 The effect of six levels of soil phosphorus (P) application on the percentage of root length colonised by arbuscular mycorrhizal fungi (AMF) of six cultivars of *Trifolium subterraneum* grown for six weeks with six levels of P application (mean±s.e., n=10, LSD at *P*=0.05). There was an effect of P level (*P*<0.001) and cultivar (*P*<0.001) with no significant interaction between P level and cultivar.

15.2.4 Discussion

Results from this experiment have a number of implications for future research. First, the maintenance of consistent differences among cultivars in specific root length and average root diameter with an increasing level of P supply suggests that screening of larger sets of germplasm can be accurately undertaken at a single level of P supply. However, the low and similar level of colonisation by AMF at the lowest P levels suggests that screening for this trait should be undertaken at a slightly higher level of P supply.

The increase in specific root length and decrease in root tissue density with increasing P was unexpected and contrary to much of the literature. We hypothesise that these trends reflect increased shading within the sward as the sward biomass increases with increasing P level: this is tested in Experiment 4.

The amount of carboxylates measured in this experiment are consistent with those reported for subterranean clover in Chapter 11. These amounts are low relative to known “high exuders”, such as *Lupinus* species and, as such, it seems unlikely that they play an important role in P uptake. However, it is possible that they play a role in other rhizosphere processes either directly, or indirectly through influencing the rhizosphere microbial community. Thus, the differences among cultivars of subterranean clover in amount and composition of rhizosphere carboxylates may be of interest to future research.

15.3 Experiment 2. The effect of a root pathogen, *Pythium irregulare*, on the response to P application by three cultivars of subterranean clover

15.3.1 Background

The aim of the experiment was to determine whether a root rot pathogen (*Pythium irregulare*) altered root morphology traits important for P uptake, their acclimation response to P supply and the growth response and critical external P requirements of three cultivars of subterranean clover.

15.3.2 Method

The same low P field soil used in Experiment 1 was used for this experiment, but it was first pasteurised (removing indigenous AMF along with root pathogens) to ensure the disease treatment was controlled. Disease inoculum was produced by growing the pathogen on sterilised millet seed. A preliminary experiment (three cultivars x three P levels x three inoculum levels) was used to allow choice of an appropriate level of disease inoculum for this main experiment (i.e. an inoculum level that resulted in damaged roots but did not kill the plants).

The main experiment consisted of three cultivars of subterranean clover (Woogenellup, Riverina and Seaton Park) grown in the low-P soil to which seven P treatments (0, 15, 30, 60, 90, 120 and 150 mg P kg soil^{-1}) and two root pathogen treatments (with and without *P. irregulare*) were applied. Pots were inoculated with a commercial inocula containing four species of arbuscular mycorrhizal fungi (AMF) (Microbesmart Pty Ltd., South Australia). Pots were surrounded by reflective sleeves to mimic sward conditions. Appropriate rhizobia were applied, along with P-free basal nutrients. Harvest occurred after 6 weeks. Data were collected as detailed for Experiment 1, except that rhizosphere carboxylates were not measured. Shoot and root P concentrations are currently being determined. For each species, the yield of shoot dry matter growth in response to P application was analysed by fitting a Mitscherlich non-linear curve (Equation 1, Chapter 6) and the critical external P requirement of each species was defined as the amount of P applied to achieve 90% of maximum yield. Critical P values were compared and significant differences were determined as described in Chapter 6

15.3.3 Results

The commercial inocula of AMF was ineffective and roots were largely uncolonised by AMF. Root disease symptoms (i.e. root browning) proved unexpectedly difficult to quantify in the glasshouse-grown plants and these data are not presented. However, there was a large negative effect on shoot DM of all cultivars (up to a 50% reduction) across most P levels, except at the two highest P levels where there was minimal effect of inoculation for Riverina and a lesser effect than at low P for Woogenellup (Fig. 15.9). The negative effect of inoculation on root DM was less marked than that for shoot DM and was more evident at the three lowest P levels (Fig. 15.10). In the absence of inoculation, Seaton Park had the lowest external critical P requirement and Riverina the highest (Table 15.1). As a consequence of the negative effects of inoculation on shoot DM, the critical P requirement of each cultivar was increased by inoculation with *Pythium irregulare*. The critical P level of Riverina showed the greatest increase.

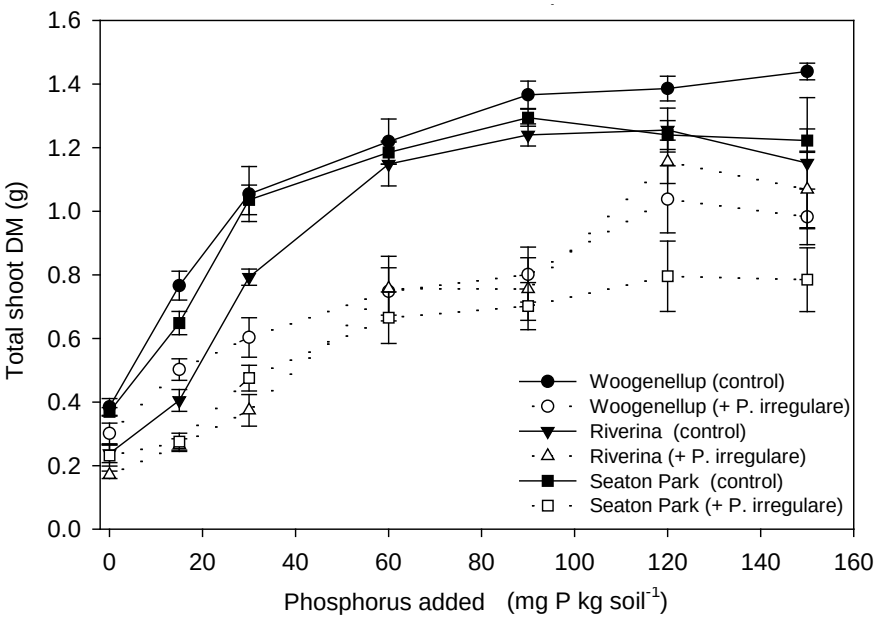


Figure 15.9 The effect of seven levels of phosphorus (P) application, on shoot DM of three cultivars of *Trifolium subterraneum* grown for six weeks in a pasteurised, low-P, soil with and without *Pythium irregulare* (mean±s.e., n=5).

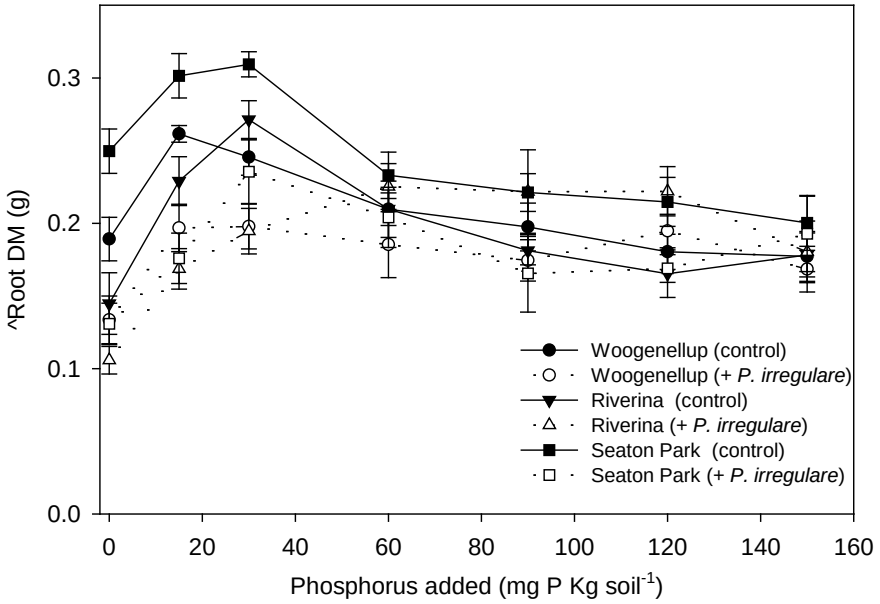


Figure 15.10 The effect of seven levels of phosphorus (P) application on root DM of three cultivars of *Trifolium subterraneum* grown for six weeks in a pasteurised, low-P, soil with and without *Pythium irregulare* (mean±s.e., n=5).

Table 15.1. Critical external P requirements, calculated from fitting a Mitscherlich curve.

Cultivar	*Pythium irregulare*	Critical P (mg added P kg soil^{-1})	s.e.	*
Riverina	Absent	79	11	b, c
	Present	278	25	e
Woogenellup	Absent	64	6	a, b
	Present	148	20	d, e
Seaton Park	Absent	53	4	a
	Present	124	22	c, d

Critical P values with the same letter do not differ from each other at $P<0.05$.

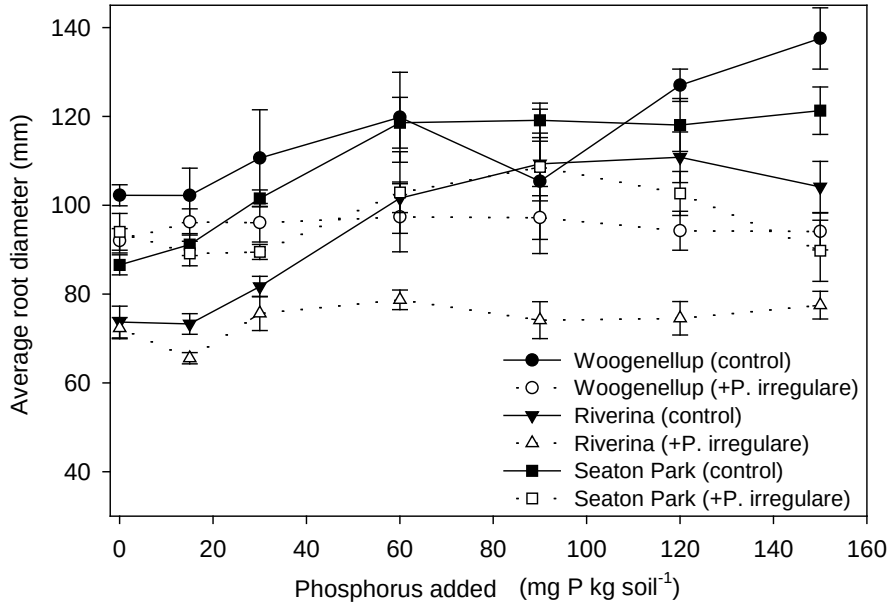


Figure 15.11 The effect of seven levels of phosphorus (P) applic-ation on the average root diameter of three cultivars of *Trifolium subterraneum* grown for six weeks in a pasteurised, low-P, sandy loam field soil with and without *Pythium irregulare* (mean±s.e., n=5).

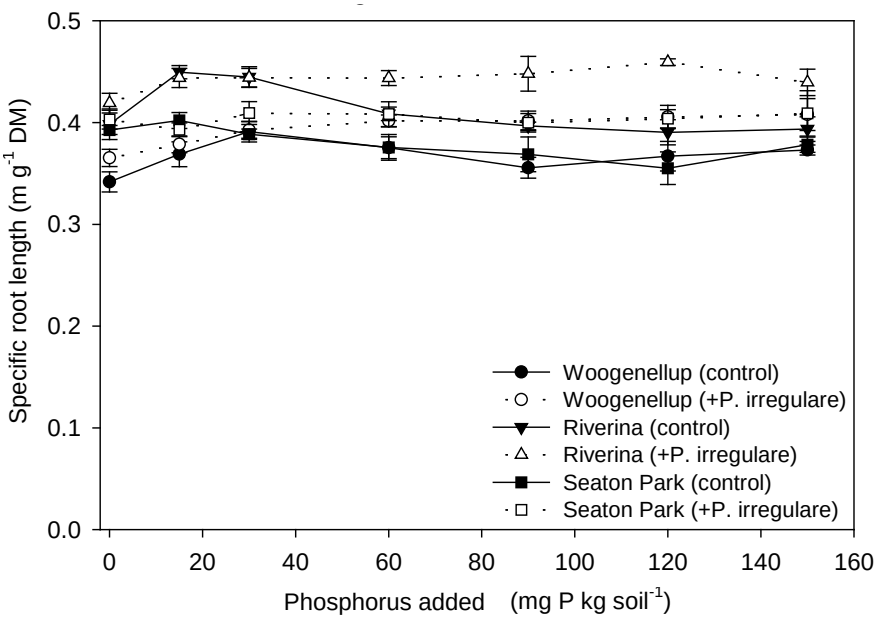


Figure 15.12 The effect of seven levels of phosphorus (P) application on the specific root length of three cultivars of *Trifolium subterran-eum* grown for six weeks in a pasteurised, low-P, sandy loam field soil both with and without *Pythium irregulare* (mean±s.e., n=5).

Average root diameter was little affected by inoculation at the lower P levels, but was higher (by around 15%) with inoculation at the higher P levels (Fig. 13.11). Similarly, specific root length was unaffected by inoculation at the lowest P levels. However, it then increased with P level, but only in the uninoculated treatments and by the highest two P levels the uninoculated plants had a specific root length up to 45% higher than the inoculated plants (Fig. 15.12). Root tissue density initially decreased with P level, but was little affected by cultivar or inoculation (results not shown).

15.3.4 Discussion

The negative effect of inoculation on root and shoot DM is an expected outcome of inoculation with a root pathogen. The exact reason for this negative effect is unknown, but its alleviation to some degree at high P in Woogenellup and Riverina suggests that impediment of plant P uptake contributed. The shoot P data (currently being processed) will aid the understanding of this effect. Any impediment to P uptake may have reflected morphological changes in the plant root system. For instance, the negative effect on shoot DM became more prominent at P30 and above, which was also the level of P at which average root diameter became a little thicker and specific root length lower; this was particularly the case for Riverina (which experienced the greatest shift in critical P with inoculation). The smaller root systems of the inoculated plants may also have reduced P uptake. Supply of nutrients has previously been shown to alleviate the effects of root pathogens under glasshouse conditions (O'Rourke *et al*., 2012). Whilst P alone did not have a favourable effect in the study of O'Rourke *et al*. (2012), this could have reflected multiple nutrient limitations in the field soil used.

The negative effect of inoculation on shoot DM resulted in a very large increase in critical P requirement for each of the three cultivars. Confirmation of these effects under field conditions. is now required. The ideal growth conditions in the glasshouse (e.g. regular watering, warm temperatures) may have caused the disease effects to become more prominent

15.4 Experiment 3. The effect of arbuscular mycorrhizal fungi on the P response of two cultivars of subterranean clover and two species of serradella

15.4.1 Background

Arbuscular mycorrhizal fungi (AMF) are a ubiquitous root symbiont of subterranean clover. The aim of this experiment was to determine if the response of root morphology to P and the critical external P requirements of two cultivars of subterranean clover and two species of serradella were altered when arbuscular mycorrhizal fungi (AMF) colonised their roots

15.4.2 Method

The experiments in this section used the low P field soil described for Experiments 1 and 2. In order to remove indigenous AMF, the soil was steam pasteurised twice at 60°C; the soil was treated on consecutive days. A cultivar of French serradella (Margurita), yellow serradella (Santorini) and two cultivars of subterranean clover (Woogenellup and Riverina) were used in the experiment for two reasons: (1) to enable inclusion of species with contrasting root systems to subterranean clover; and (2) in response to the findings of the main project

(Chapters 4, 6 and 8) that serradellas present the best option for immediate application for development of more P-efficient pastures.

After the failure of the commercial inoculant in Experiment 2, a preliminary experiment was conducted to establish the best AMF inoculum to use. Surprisingly, the commercial inoculum was the most effective (results not presented) and was, therefore, chosen for the experiment. The legumes were grown at seven P levels (0, 7.5, 15, 30, 60, 120 and 180 mg P kg soil^{-1}) with and without the commercial inoculum of AMF described in Experiment 2. "Sievings" of the inoculum were applied to the control pots to supply any soil microorganisms that accompanied the AMF. Pots were wrapped in reflective sleeves to mimic pasture sward conditions. Appropriate rhizobia were applied, along with P-free basal nutrients. Harvest occurred after 6 weeks.

Data were collected as described for Experiment 1. Root hairs have not yet been assessed, determination of P in shoots and roots is underway, rhizosphere carboxylate data has been collected but has not been analysed and statistical analyses have not yet been done. Values for critical external P requirements were determined as described for Experiment 2.

15.4.3 Results

Unexpectedly, it was found that the pasteurisation of the soil had not worked as expected and while most indigenous thick-hyphaed AMF ("coarse endophytes") had been removed, a root colonising fungus known as "fine endophyte", which is also thought to be a form of AMF, had survived and proliferated in the uninoculated control pots. The commercial inoculum that was applied to the AMF-treated pots contained four species of coarse endophyte AMF.

The proportion of colonised root length that contained coarse and fine endophyte was determined microscopically under high magnification for a single P level (P7.5). Statistical analysis showed no effect of cultivar or species, that is, differences were only due to inoculation (Table 15.2). The uninoculated controls had 22% of root length colonised by coarse endophyte which was presumed to result from some spores surviving pasteurisation.

Table 15.2 At P7.5 up to ~80% of total root length was colonised by one or both of the two AMF forms (i.e. coarse or fine endophyte) (see Fig.15.13). The proportion of colonised root length hosting each AMF form at P7.5 is reported here. NB: there is overlap in the numbers because the majority of the AMF-treated roots and some of the control roots contained both forms of fungi growing together. There was no effect of species/cultivar, consequently, means for all legume treatments are presented.

	Coarse endophyte (proportion of colonised root length)	Fine endophyte (proportion of colonised root length)
AMF inoculated	70	69
Control	22	92
LSD at $P<0.05$	13	12

Very high rates of root colonisation by, coarse endophyte (70% of root length colonised) was found in the AMF inoculated treatment. In contrast, 92% of root length in the uninoculated controls was colonised by fine endophyte. A lower proportion of root length (69%) was colonised by fine endophyte in the AMF inoculated treatment.

Instead of comparing plants with and without AMF, as was the original aim, the experiment became a comparison between plants with root colonisation dominated by fine endophyte (control) and plants with colonisation composed of similar amounts of fine and coarse endophyte (AMF inoculated treatment). Whilst unfortunate, the experiment still provides novel insights into whether root morphology is affected by presence of different communities of AMF.

In both subterranean clover and the two serradella species, the percentage of total root length colonised by mycorrhizal fungi (i.e. fine and coarse endophyte combined) reached a high level of almost 80% of root length (Fig. 15.13). The impact of P level and inoculation on colonisation level was very similar for the four legume genotypes. Colonisation initially increased with increasing P, reaching a maximum at 7.5 or 15 mg P kg^{-1}, and then decreased to be below 20% by 60 mg P kg^{-1}. In both of the subterranean clovers and the two serradella species, colonisation of the control plants, which was dominated by fine endophyte, was always lower than colonisation of the inoculated plants; it also declined more rapidly at higher P levels above P7.5.

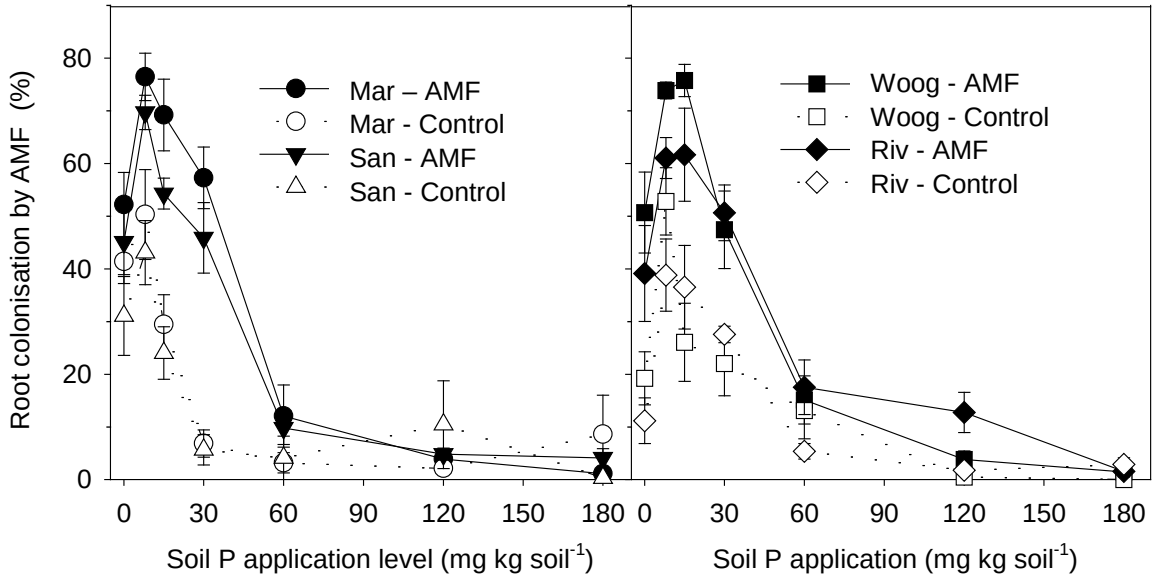


Figure 15.13 The effect of seven levels of soil phosphorus (P) application on the percentage of root length colonised by mycorrhizal fungi (fine and coarse endophyte combined) for French serradella cv. Margurita and yellow serradella cv. Santorini (left) and subterranean clover cvs. Woogenellup and Riverina (right) grown for six weeks in a pasteurised, low-P, sandy loam field soil (mean±s.e., n=5). Note: soil pasteurisation was only partially successful and hence the experiment is a comparison of plants with similar levels of colonisation by fine and coarse endophyte (AMF) and plants with colonisation dominated by fine endophyte (control).

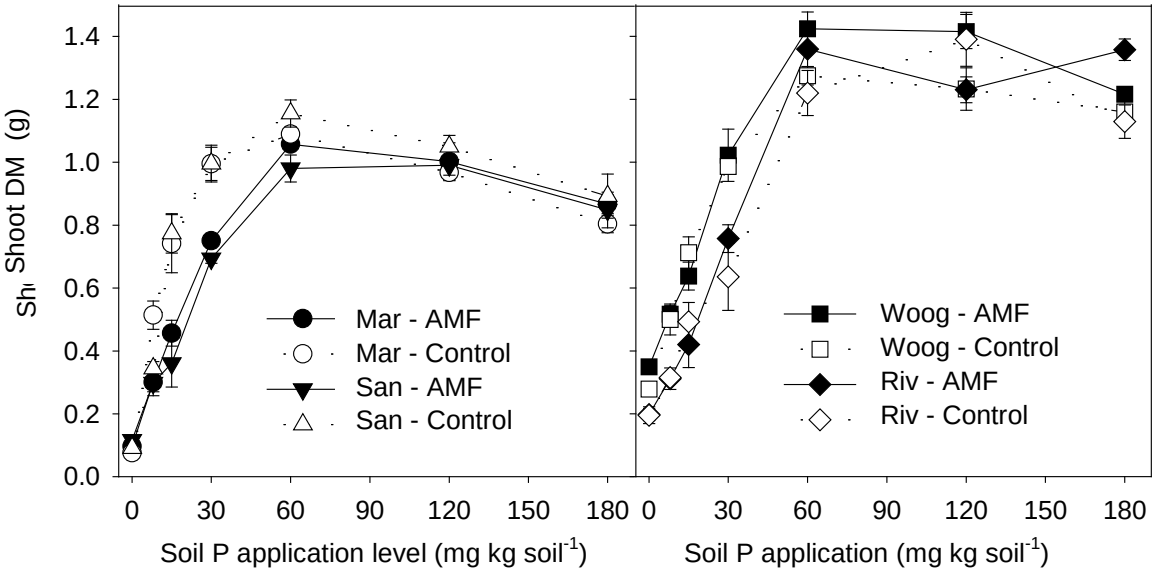


Figure 15.14 The effect of seven levels of soil phosphorus (P) application on the shoot DM of French serradella cv. Margurita and yellow serradella cv. Santorini (left) and subterranean clover cvs. Woogenellup and Riverina (right) grown for six weeks in a pasteurised, low-P, sandy loam field soil (mean±s.e., n=5). Note: soil pasteurisation did not successfully eliminate AMF and the experiment is a comparison of plants with similar levels of colonisation by fine and coarse endophyte (AMF) and plants with colonisation dominated by fine endophyte (control).

Table 15.3 Critical external P requirements, calculated from fitting a Mitscherlich curve.

Species	Cultivar	AMF	Critical P (mg added P kg soil^{-1})	s.e.	*
Subterranean clover	Riverina	Control	83	11	a
		AMF	81	8	a
	Woogenellup	Control	46	4	b
		AMF	56	6	b
French serradella	Margurita	Control	24	3	c
		AMF	50	5	b
Yellow Serradella	Santorini	Control	31	4	c
		AMF	58	7	a, b

*Critical P values with the same letter do not differ from each other at $P<0.05$.

Shoot DM increased with P level, before reaching a maximum at P60-P120 (Fig. 13.14). The effect of cultivar and AMF inoculation on shoot DM differed between the serradellas and subterranean clover (Fig. 15.14). For the serradellas, there was very little effect of cultivar, but a strong effect of inoculation with the control (fine endophyte dominated) treatment having a substantially higher shoot DM at P15-P60 than the AMF inoculated treatment. In contrast, for subterranean clover, there was little effect of inoculation, but Woogenellup had

a higher shoot DM than Riverina at P7.5-P60. As a consequence of these trends, the external critical P requirements for the serradellas were significantly lower in the control treatment than the AMF inoculated treatment, but unaffected by cultivar (Table 15.3). In contrast, the external critical P requirements for subterranean clover did not differ between the control and AMF inoculated treatments, but were significantly lower for Woogenellup than Riverina.

Root DM was much heavier for subterranean clover than serradella, but differed little between serradella species and subterranean clover cultivars (Fig. 15.15). Root DM first increased and then decreased with P addition, with the control (fine endophyte dominated) treatments peaking at a lower P than the AMF inoculated treatment for the serradellas (Fig. 15.15). Total root length showed trends similar to those of root DM (Fig. 15.16), other than Woogenellup being markedly higher than Riverina in the control (fine endophyte dominated) treatment, especially at P15 and P30 (reflecting a higher specific root length and lower average root diameter; Figs 15.18 and 15.19). Root mass fraction (Fig. 15.17) decreased with increasing P level and was higher for subterranean clover than the two serradella species, especially at the lower P levels. At P30 in the fine-endophyte dominated controls in the serradellas, root mass fraction was lower than in the AMF inoculation treatment. Specific root length was higher for the serradellas than subterranean clover (Fig. 15.18). In the serradellas, specific root length was little affected by P level or the AMF inoculated treatment, however, for subterranean clover, it increased with P level and was higher in the fine endophyte dominated controls than the AMF inoculated treatment. Average root diameter was lower for the two serradella species than for subterranean clover and was little affected by P level (Fig. 15.19). In all species, average root diameter was lower in the fine endophyte dominated controls than the AMF inoculated treatments at the higher P levels (where total colonisation was lower).

15.4.4 Discussion

Fine endophyte is presumed to be a form of AMF. It is widely found and has been reported in Australian agricultural systems previously (Ryan and Kirkegaard, 2012). However, as pure cultures are difficult to produce its role in plant growth has been rarely studied and its classification as a mycorrhizal fungus is uncertain. Whilst the outcome of this experiment was not as planned, the results of the study are novel and interesting. For instance, the experiment indicated that root colonisation in the fine endophyte-dominated (control) treatments was negatively impacted by lower levels of P availability than the mixed coarse and fine endophyte (AMF-treated) treatment.

The improved growth of the serradellas in the fine-endophyte dominated control at P15-30, and the resulting large reduction in critical P requirements, is particularly interesting. First, it may reflect a greater ability of fine endophyte to access soil P than coarse endophyte. This is assuming both treatments grew better than an uncolonised control – probably a reasonable

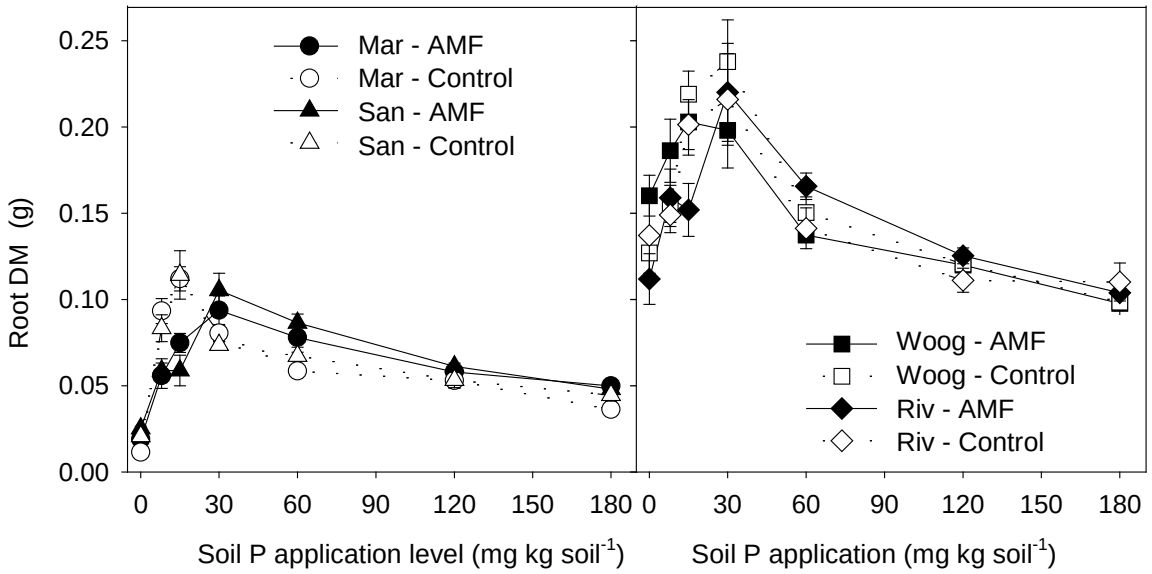


Figure 15.15 The effect of seven levels of soil phosphorus (P) application on the root DM of French serradella cv. Margurita and yellow serradella cv. Santorini (left) and subterranean clover cvs. Woogenellup and Riverina (right) grown for six weeks in a pasteurised, low-P, sandy loam field soil (mean±s.e., n=5). Note: soil pasteurisation partially failed and hence the experiment is a comparison of plants with similar levels of colonisation by fine and coarse endophyte (AMF) and plants with colonisation dominated by fine endophyte (control).

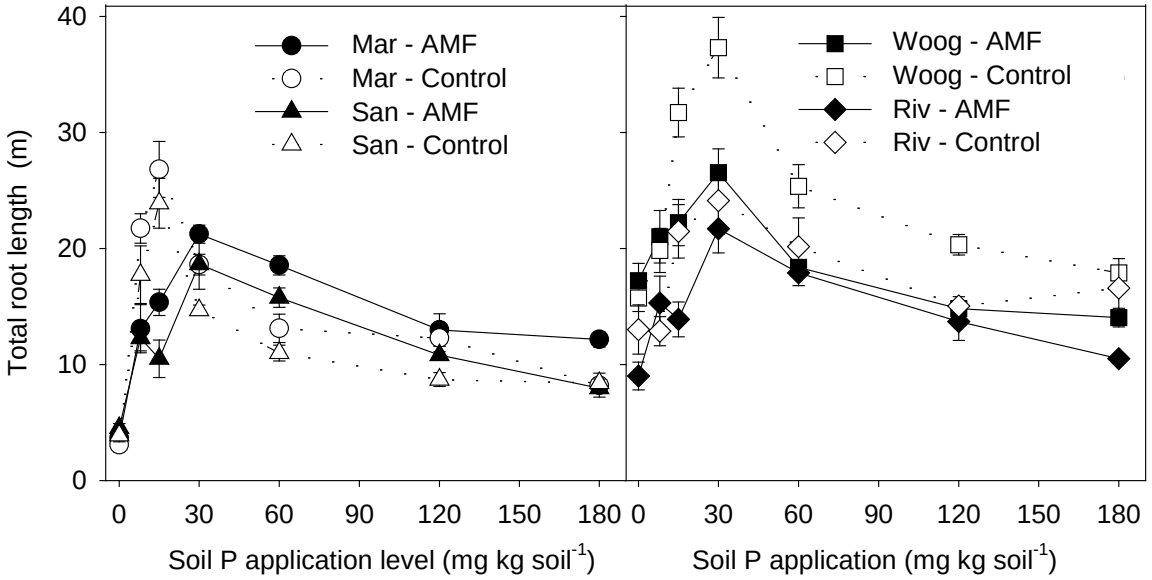


Figure 15.16 The effect of seven levels of soil phosphorus (P) application on total root length of French serradella cv. Margurita and yellow serradella cv. Santorini (left) and subterranean clover cvs. Woogenellup and Riverina (right) grown for six weeks in a pasteurised, low-P, sandy loam field soil (mean±s.e., n=5). Note: soil pasteurisation partially failed and hence the experiment is a comparison of plants with similar levels of colonisation by fine and coarse endophyte (AMF) and plants with colonisation dominated by fine endophyte (control).

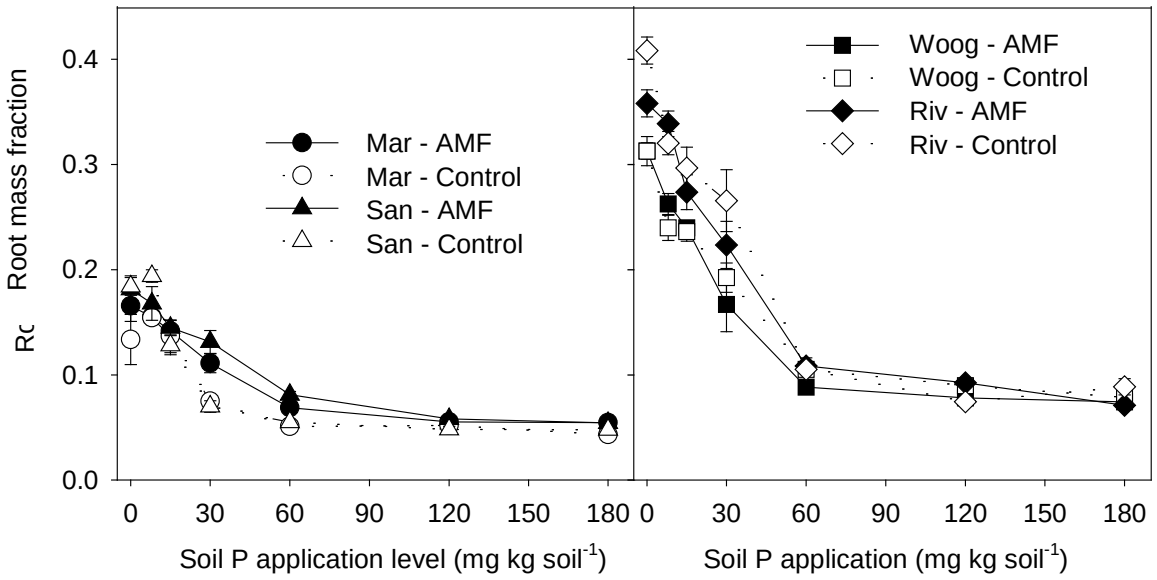


Figure 15.17 The effect of seven levels of soil phosphorus (P) application on root mass fraction of French serradella cv. Margurita and yellow serradella cv. Santorini (left) and subterranean clover cvs. Woogenellup and Riverina (right) grown for six weeks in a pasteurised, low-P, sandy loam field soil (mean±s.e., n=5). Note: soil pasteurisation partially failed and hence the experiment is a comparison of plants with similar levels of colonisation by fine and coarse endophyte (AMF) and plants with colonisation dominated by fine endophyte (control).

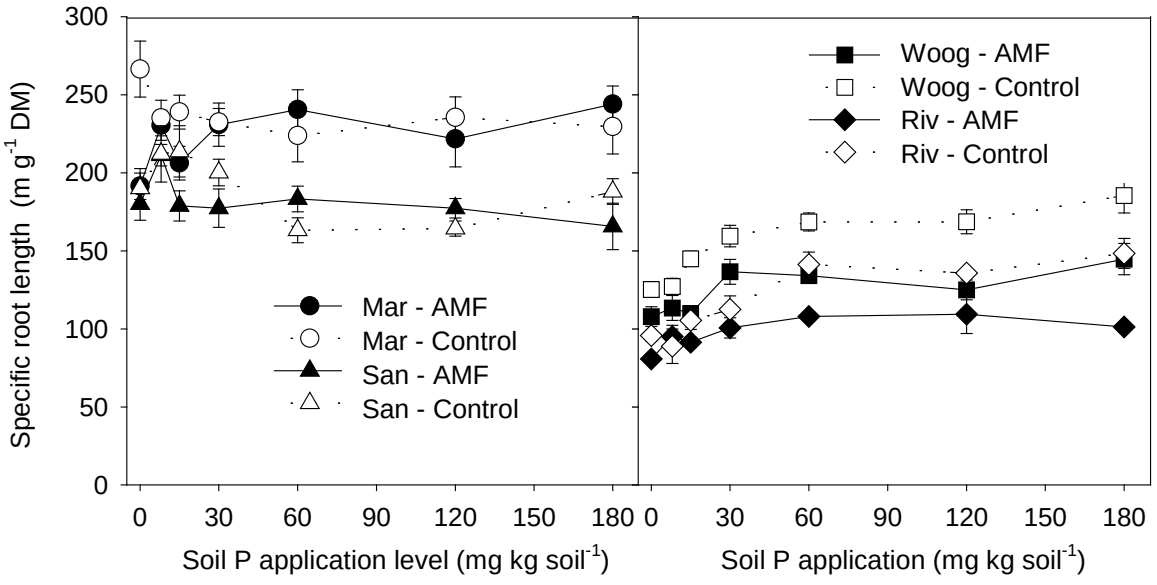


Figure 15.18 The effect of seven levels of soil phosphorus (P) application on the specific root length of French serradella cv. Margurita and yellow serradella cv. Santorini (left) and subterranean clover cvs. Woogenellup and Riverina (right) grown for six weeks in a pasteurised, low-P, sandy loam field soil (mean±s.e., n=5). Note: soil pasteurisation partially failed and hence the experiment is a comparison of plants with similar levels of colonisation by fine and coarse endophyte (AMF) and plants with colonisation dominated by fine endophyte (control).

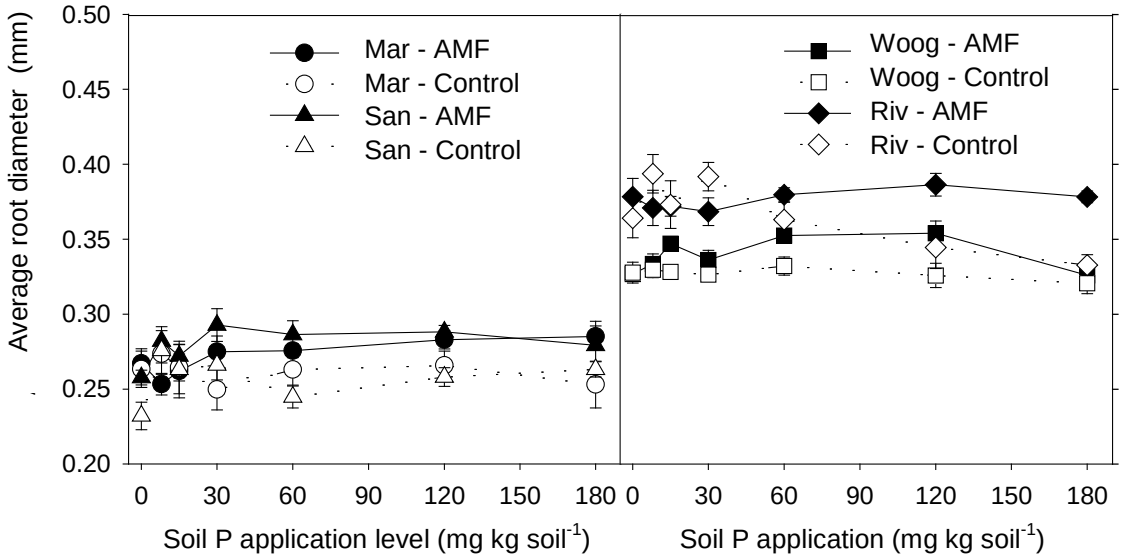


Figure 15.19 The effect of seven levels of soil phosphorus (P) application on the average root diameter of French serradella cv. Margurita and yellow serradella cv. Santorini (left) and subterranean clover cvs. Woogenellup and Riverina (right) grown for six weeks in a pasteurised, low-P, sandy loam field soil (mean±s.e., n=5). Note: soil pasteurisation partially failed and hence the experiment is a comparison of plants with similar levels of colonisation by fine and coarse endophyte (AMF) and plants with colonisation dominated by fine endophyte (control).

assumption (see Schweiger *et al*. (1995)). Such a difference could potentially be the result of differences between soil exploration ability or P transport ability of the external hyphae of fine and coarse fungi (Jakobsen *et al*., 1992a, b). However, higher specific root length (subterranean clover only) and lower average root diameter (all species) were also associated with the fine-endophyte dominated controls. These results have not been reported before and cannot be readily explained at this stage. They may, for example, reflect different carbon demands by fine and coarse endophytes which, presumably, obtain all their carbon (energy) requirements from their host plant.

It is known that the effects if coarse endophyte species on growth of a host plant can differ among AMF strains and species (e.g. Graham and Abbott 2000) and that fine endophyte colonisation may involve more than one species (Thippayarugs *et al*. 1999). Consequently, it cannot be assumed, at this stage, that the differences associated with fine endophyte in this experiment are characteristic of fine endophyte colonisation in general. However, the results do show that dramatic changes in critical P requirement can occur with shifts in non-pathogenic components of the soil biological community. It is very possible that the impacts of such shifts are magnified in pasteurised soil and under the ideal growth conditions of a glasshouse and it remains to be tested whether the differences would prove significant under field conditions. Unfortunately, any benefits that may be associated with particular forms of AMF cannot be easily managed in fields where AMF are ubiquitous and are likely to always colonise subterranean clover and serradellas (e.g. see Chapter 4).

A number of other interesting aspects of this experiment are consistent with previous experiments:
(1) The generally lower critical P requirement of the serradellas than the subterranean clover (Chapters 4, 6, 8).
(2) The lower critical P requirement of Woogenellup compared with Riverina (Experiment 2).
(3) The increase in specific root length of subterranean clover with increasing P (in the absence of root disease) (Experiments 1 and 2; Chapters 7 and 8). The absence of this trend in serradella may reflect better light penetration into the sleeves surrounding the pots due to its more upright shoot morphology or species-specific differences in root morphology responses.
(4) The minimal effect of P level on average root diameter and hence the maintenance of differences among cultivars with increasing P (Experiments 1, 2 and 3; Chapters 7, 8 and 9).

[Note: MLA-funded UWA-based PhD student Suzanne Orchard is studying the fine AMF endophyte and will assist in the interpretation and preparation of results of this experiment for publication.]

15.5 Experiment 4. Effects on root morphology of subterranean clover and serradella of simulating pasture sward growth conditions in glasshouse experiments.

15.5.1 Background

In this project we attempted to closely mimic field conditions by growing plants in mini-swards with each pot wrapped in a reflective sleeve that was raised in line with plant height as the sward grew. It was observed that specific root length of subterranean clover was higher under high soil-P condition in earlier experiments. This is the opposite response to soil P fertility that is often expected and we previously hypothesised that it was a consequence of self-shading in the mini-swards. In this experiment, root traits of plants grown in mini-swards were compared with those of plants grown without the sleeves, where shoots were able to grow out past the edge of the pots. The hypothesis being examined was that the shading of the lower leaves of the plants in mini-swards changes the carbon dynamics of the plants and affect the availability of carbon to the roots and AMF colonising the roots.

15.5.2 Method

The experiment used a similar low P field soil to that described for all of the previous experiments (see Experiment 1). In order to remove indigenous AMF, it was pasteurised at 60°C on two consecutive days. A commercial inoculant was also added. The pasteurisation did not remove all indigenous AMF and thus the experiment contained a similar community of AMF as the 'AMF inoculation treatment' in Experiment 3.

The experiment consisted of subterranean clover cv. Riverina and yellow serradella cv. Santorini, grown at two P levels (15 and 60 mg P kg soil^{-1}), with and without reflective sleeves. Appropriate rhizobia were applied, along with P-free basal nutrients. Harvest occurred after 6 weeks. Data are currently being analysed.

15.5.3 Results

The collection and analysis of data from this experiment is not yet completed. A number of observations can be reported. However, they remain to be confirmed by a full analysis of the data.

(1) For both species, shoot DM was decreased a little when sleeves were used to mimic growth in pasture sward conditions, especially at under high soil P conditions (P60).

(2) For both species, root DM and total root length was decreased 30-40% in mini-swards and, therefore, root mass fraction was also lower.

(3) For yellow serradella cv. Santorini, at both P levels, and subterranean clover cv. Riverina at P15, specific root length was increased by ~15-20% in mini-swards.

(4) For both species, root tissue density was decreased in mini-swards.

15.5.4 Discussion

The use of reflective sleeves to mimic pasture sward conditions resulted in thinner, less dense roots which was associated with an increase in specific root length. This is consistent with lower rates of photosynthesis due to the restriction to canopy spreading and the consequent self-shading of lower leaves. This inevitably would decrease the absolute and proportional allocation of carbon to the roots. The experiment confirmed previous observations (Experiment 1; Chapter 7) of concurrent increases in specific root length and decreases in root tissue density when plants are grown in mini-swards with higher rates of applied P.

Overall, the results of this experiment provide support for the use of mini-swards in many of the experiments conducted as part of this project. If shoots are allowed to expand to outside the confines of the pot more light is captured than would be expected in a pasture sward and this changes root characteristics. The change can be presumed to result in root systems that are not a realistic imitation of what would be found in the field in a pasture sward.

Acknowledgement

RJ held and Australian Postrgraduate Award and a top-up scholarship funded by MLA as part of PUE0104.

16 Discussion and Conclusions: feasibility and roadblocks to development of pasture systems based on more P-efficient plants.

16.1 Feasibility of developing pasture systems that can be operated with substantially less phosphorus (P)-fertiliser

(Objective 1: Prove that highly productive pasture systems can be operated with substantially less phosphorus (P)-fertiliser by using plants with low 'critical' P requirements.)

16.1.1 Further evidence to back the underpinning hypothesis

Phosphorus is an essential input for the majority of pastures in southern Australia because most soils in their native condition are deficient in P for plant growth. In temperate, legume-based pasture systems, P fertiliser plays a “keystone” role in production because the main legumes that are used (subterranean clover, white clover and annual medics) have the highest P requirement of all species grown in the pasture mix (e.g. Ozanne *et al*. 1969; Ozanne *et al*. 1976; Hill *et al*. 2006). Consequently, P inputs determine pasture legume composition and persistence, and the annual rate of nitrogen input to pastures via nitrogen fixation. This in turn drives overall productivity and the efficiency of water and land-use by each grazing enterprise and the industry as a whole.

The amount of P that must be applied annually to maintain soil P fertility is the sum of: (i) the P removed from the pasture when animal or plant products are removed, (ii) P lost in leaching, runoff or by soil erosion, and (iii) P accumulated as sparingly-available phosphate and organic P in the soil. If soil fertility is being improved, an additional input is also required to build the “plant-available” soil P pool.

$$P_{fertiliser} = P_{exported} + P_{lost} + P_{accumulated\ in\ soil}\ (+ P_{\text{to build soil P if required}})$$

The P-balance efficiency (P exported expressed as a proportion of P imported) of Australian temperate pasture systems is low. Median values for sheep, beef and dairy enterprises are 11%, 19% and 29%, respectively (Weaver and Wong 2011). In contrast, cropping systems on equivalent soil achieve about 50% efficiency. In a pasture system with stable soil P fertility, it is P “losses” and “accumulations” that degrade the efficiency of P use. P losses can be very significant in coastal and other sandy soil areas of Australia (Ozanne *et al.* 1961; Lewis *et al.* 1987), but the major inefficiency term for most soils is the accumulation of P that occurs when they are fertilised.

P accumulates in grazed fields in: (i) forms of phosphate that are only sparingly soluble in the soil solution (e.g. Ca-phosphates in alkaline and calcareous soils) or are bound to aluminium and iron within the clay lattice of soil particles (e.g. acid soils) (McLaughlin *et al*. 2011; Barrow 2015);
(ii) in organic compounds that resist mineralisation (e.g. humic P, phytate and other (mainly monoester P) forms of soil organic matter) (McLaren *et al*. 2015b; Jarosch *et al*. 2015); and
(iii) as a result of P-enriched excreta accumulations in animal camps.

The rate at which phosphate slowly reacts with soil rendering it sparingly-available to plants (P sorption reactions) is proportional to the concentration of phosphate in soil solution and the time it is in contact with the soil:

$$Ps = a \cdot C^{b1} \cdot t^{b2}$$

where: Ps is the amount of P sorbing, C is the phosphate concentration of the solution in which the soil bathed, *a* approximates the amount of sorbing capacity within a soil, and *b1* and *b2* are coefficients that describe the shape of the sorption relationship. These coefficients vary widely between soils; however, *b1* and *b2* are reasonably well correlated when compared across a wide range of soils (Barrow 1980a; Barrow 1980b). In calcareous soils this equation also applies initially, but precipitation of calcium phosphates decreases the phosphate concentration of the soil solution to levels that are determined by the solubility product (Barrow 1980a).

This is analogous to the driving force behind accumulation of P in grazed fields because it has been demonstrated recently that the sum of phosphate and organic P accumulation in soil and P accumulations in camps is also positively related to the concentration of extractable-P (soil test P [STP] concentrations) at which a field soil is being maintained (Fig. 1.3; Simpson *et al*. 2015). The major component of this was accumulation of P in the soil.

Because the STP concentration for optimal soil P management (*Gourley et al. 2007*; Moody 2007; Simpson *et al*. 2014) is determined by the pasture legume (Ozanne *et al*. 1969; Ozanne *et al*.1976; Hill *et al*. 2006) it was hypothesised that legumes with lower critical P requirements would result in slower accumulation of P in grazed fields and this, in turn, would reduce the P fertiliser input required to maintain soil P fertility.

Absolute proof of this hypothesis would require P balance studies over several years, of grazed pasture systems based on legumes that differ in their critical P requirements. However, at the beginning of this project there was only limited evidence that there may be pasture legumes with critical P requirements that were substantially lower than that of subterranean clover. Consequently, we sought initially to test the claimed relationship between STP concentration and the rate of P accumulation in fertilised fields, by evaluating evidence from long-term grazing experiments where soil P fertility had been altered (see summary of this survey in Table 1 of Simpson *et al*. 2014).

There were numerous examples of partial and complete P balances of Australian pasture systems that demonstrate P accumulations in soil under fertilised pasture. However, many of these studies do not specify extractable STP concentrations and it cannot be determined whether soil fertility was stable or changing. Under these circumstances the component of P accumulation associated with increasing soil fertility (an efficient use of P) cannot be distinguished from inefficient P accumulations resulting from P sorption or organic P accumulation. Consequently, data capable of supporting the hypothesis that grazing systems managed at lower STP concentrations will accumulate less P are scarce.

Nevertheless, a few experiments have been conducted over several years with relatively stable soil P fertility.

For example, pasture in long-term grazed phosphorus experiment at Hamilton, Victoria managed with a stable, low and deficient STP concentration (4-6 mg Olsen P/kg soil) over seven years accumulated 3 kg P/ha/year (McCaskill and Cayley 2000). In contrast, pasture managed with rising soil fertility (10 increasing to18 mg Olsen P/kg) over the same period

accumulated 16 kg P/ha/year. According to Burkitt *et al*. (2001), a rise of 1.1-1.2 Olsen P units/year (on a soil with PBI = 250) is likely to require ~11.5 kg of additional P to be applied annually per hectare in addition to the maintenance P rate. Thus, it is estimated that about 50% more P/ha was required annually to maintain the higher STP concentration in the long-term grazing experiment.

Where comparison of pasture systems managed with contrasting STP concentrations can be made, the data support the notion that P accumulations are lower in fields managed with low extractable soil P concentrations and relatively high in fields managed at higher soil P fertility (Simpson *et al*. 2014).

16.1.2 Pasture legumes with low critical P requirements

At the commencement of the present experiments, the only consistent evidence that it may be possible to find pasture legumes with a critical P requirement that was lower than that of subterranean clover came from studies of yellow serradella when grown on relatively sandy soils in Western Australia (Bolland and Paynter 1992; Schweiger *et al*. 1995). Despite these reports, it has never been recommended that yellow serradella should be fertilised to reflect its lower P requirement. Yellow serradella was introduced to Australia mainly for use on deep acid sands where subterranean clover grew poorly or failed to persist (Nichols *et al*. 2012), so it was also possible that the reported differences in critical P requirement were more a reflection of differences in the edaphic adaptation of the species, than an intrinsic difference in their P requirements.

The present experiments have now demonstrated that there are significant differences in critical P requirements among a number of the alternative pasture legumes (Chapters 4, 6, and 8). In controlled-environment and glasshouse experiments, relatively fine differentiation between the critical P requirements was possible among the species (Chapters 6 and 8), with very large differences in the requirements of the least and most P-efficient species. In Chapter 7, a low critical P requirement was shown to be associated with roots that enabled a species to explore soil efficiently for P when the extractable-P concentration was relatively low.

The fine distinctions in critical P requirements among some of the legumes were not detectable when they were grown in the field (Chapter 4). Indeed, a range in critical Colwell P concentration was recorded for every species in the field experiments (Table 4.11) and the ranking of some of the species varied between sites or growing seasons (Tables 4.7 – 4.10). There are at least four reasons why it was expected that it would be more difficult to find differences in critical P requirements under field conditions:

(i) Experimental errors are typically larger in field experiments because less variables can be controlled. Small differences among species will be harder to prove.
(ii) Critical Colwell P varies among soil types because Colwell P test results differ with PBI (Moody *et al*. 2007). Consequently, apparent differences in the critical Colwell P value of a species when grown at different sites could potentially be due to different soil P-sorption characteristics at the sites.
(iii) Seasonal conditions that alter plant growth rates can also modify the apparent critical P requirement of a plant. For example, conditions that slow growth during spring (e.g. low temperatures) would result in maximum yield being achieved at a lower STP. Dry spring conditions (e.g. potentially Belfrayden and Beckom in 2014; Chapter 4) can lengthen the

diffusion path for P in surface soil well before drought directly slows plant growth (i.e. the water-filled spaces around soil particles dry and become more tortuous or broken). Under these conditions a higher soil solution phosphate concentration is required to achieve an equivalent rate of P diffusion to the root and this increases the apparent critical P requirement of a plant.

(iv) Lack of adaptation of a species to a site's edaphic and/or climatic conditions may potentially confound the determination of a species' critical-P requirement. If the growth rate of a species is compromised by poor adaptation (soil chemistry, waterlogging, root diseases, etc.) it is highly likely that the apparent critical P requirement will be site-specific and will not reflect the intrinsic potential of the genotype. In the present experiments, there were a number of alternative legumes that were found to be unsuitable for growth at particular field sites (Table 4.3). This proved to be a much larger problem than had been anticipated because reliable information about the adaptation ranges of the alternative legume species is scarce

For these reasons, it is important to compare among species or growing seasons relative to the critical P value of a control species, such as subterranean clover. When this was done, the alternative legumes fell into three or four groupings with respect to their critical P requirements (Table 4.11). There was considerable overlap in the critical Colwell P ranges of the low- and moderate-P requiring groups of species and the moderate- and high-P requiring species, so it is debatable whether these groups can be claimed to differ by a meaningful amount. However, it was clear that some members of the low-P requiring group (particularly French and yellow serradellas) always had a substantially lower critical P requirement than subterranean clover. Lucerne had a substantially higher P requirement than subterranean clover.

16.1.3 Further comments on adaptation range of the alternative legumes

The clearest example of poor adaptation by a species to the field sites was the high susceptibility of tedera to frost at Yass. This eliminated our ability to reliably determine its response to P. Other examples included the excessive thinning of stands of *Lotus corniculatus* line LC07AUYF over the summer periods; *B. pelecinus*, *O compressus, T. spumosum* and other species did not yield as well as subterranean clover at some sites (e.g. Yass, Burrinjuck); *T. hirtum* was often outcompeted and did not persist after its first growing season if subterranean clover had emerged amongst it from the soil seed bank.

The issues of poor persistence and low yield reduce the practical value of these species at certain sites but also they potentially compromise assessments of critical P requirements. This was a major factor in the decision to develop two new field sites near Wagga in 2014. This environment was considered likely to be more favourable for some of the alternative legumes including *B. pelecinus*, *T. spumosum, T. vesiculosum.*

Biserrula pelecinus grew considerably better at Belfrayden and Beckom but good growth was not accompanied by the low critical P requirement expected from its root morphology and the glasshouse experiment results (Table 4.9 and 4.10; Chapter 8). The reason for this was not known, but the sorts of field constraint that may modify a plant's response to P in this way include sensitivity to soil acidity (e.g. Delhaize *et al*. 2009) and susceptibility to root diseases (e.g. Chapter 15).

16.2 Critical-P benchmarks for the alternative pasture legumes

(Objective 2: Quantify (benchmark) the critical P requirements of key pasture legume species relative to subterranean clover. P-fertiliser management guidelines, strategies for targeted fertiliser use and objective information concerning the P-fertility requirements of emerging, novel and alternative pasture legumes.)

16.2.1 Soil test P benchmarks for pasture management

16.2.1.1 Method

The mathematical determination of the critical-P requirement of a species is most accurate when the inflexion point in the asymptotic relationship between soil P fertility and pasture yield occurs at about the centre of the fitted relationship (e.g. Fig. 16. 1a). This creates an experimental design problem when comparing species that may have very different critical P requirements. The range in soil P fertility required for fitting a mathematical function to one species will not be ideal for fitting it to another. By necessity, the number of P application rates that could be deployed in the field experiments was also limited by the resources available to manage and harvest the experiment. The P application rates required for all of the species being compared were also initially unknown. The rates of P application to establish the range of STP levels over which the species were to be examined was initially an educated “guess” and adjustments to soil P fertility were made cautiously when maintenance P dressing were applied in subsequent years.

This project had two competing requirements: (i) the primary aim was to discover legumes with lower critical P requirements than subterranean clover, with (ii) a secondary objective of using the experimental results to benchmark the critical STP concentrations of the alternative legumes. The field experiments were designed primarily to facilitate the discovery of novel low-P legumes. Six rates of P (with 3 replicates) were applied with the intention of achieving the inflection in their P response function between about the fourth and fifth highest P rates for subterranean clover. It was expected this would highlight species with lower critical P requirements than subterranean clover and would allow the critical P levels of any “P-efficient” legumes to be defined over about three or four P levels (as a minimum) (Fig. 16. 1b). However, this design incurred the potential risk that there would be inadequate P levels at the maximum yield of subterranean clover to adequately define the asymptote of its P response function. In addition, species with P requirements that were higher than that of subterranean clover would be identified, but their critical STP concentrations would not be accurately determined.

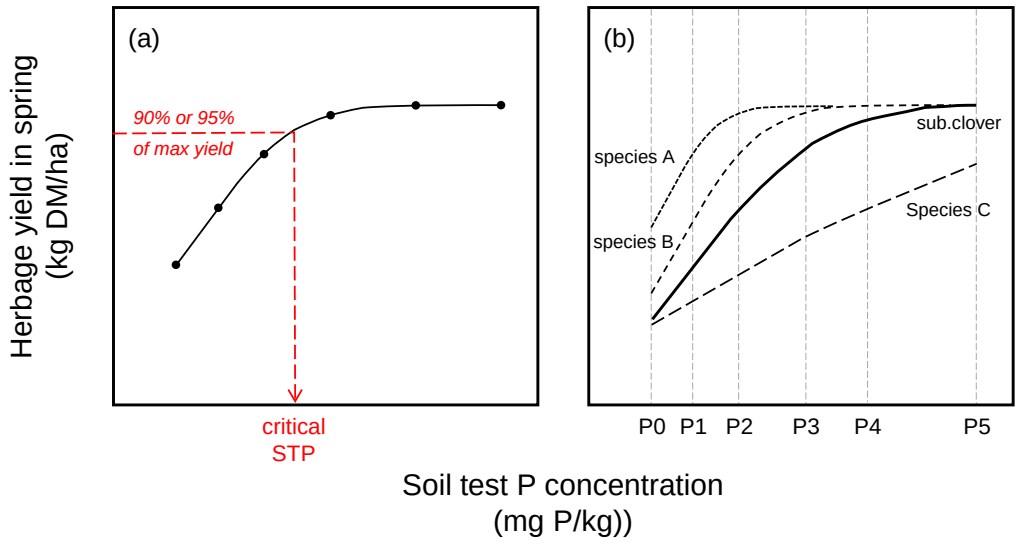


Figure 16.1 Theoretical yield responses of legumes to soil P fertility showing: (a) the ideal spread of P treatments in relation to yield for the mathematical determination of critical STP from an asymptotic yield function; (b) the compromise in deployment of soil P treatments necessary for discovery of species with lower critical P requirements than subterranean clover. Species A and B represent hypothetically P-efficient species and species C a less P-efficient species than subterranean clover.

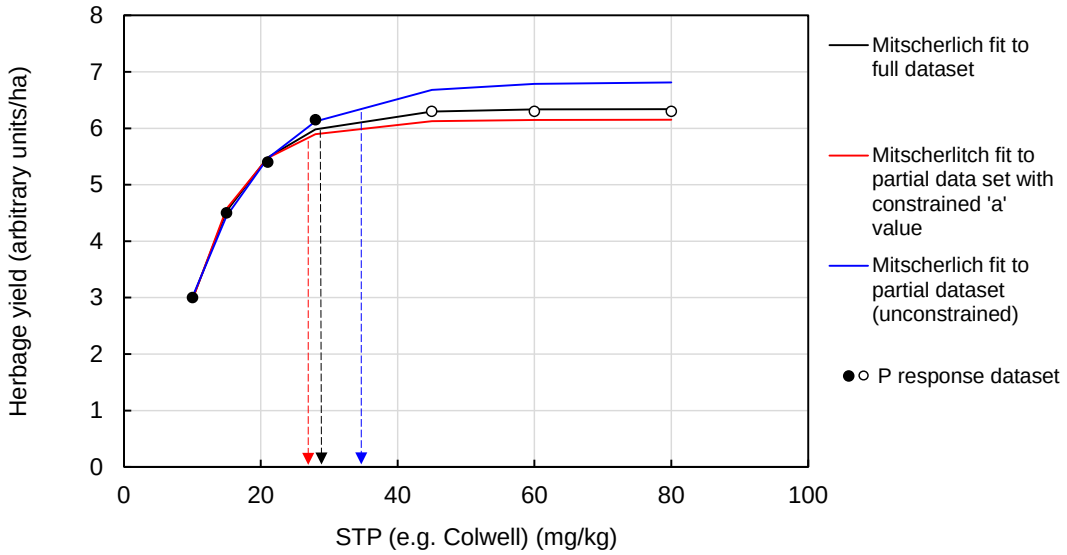


Figure 16.2 An hypothetical yield response of a pasture legume to soil P fertility showing how the Mitscherlich function ($y = a - b^{*}e^{-cx}$) would fit a dataset where the inflexion in the relationship occurred near the middle (black lines and all data points). The blue lines show how the Mitscherlich function would fit a partial dataset (closed circles) for the same species when the maximum yield was only achieved at the highest P application rate. This is similar to the datasets available for subterranean clover in a number of the present experiments. The Mitscherlich function inevitably over-predicts the asymptote (*a*) and consequently predicts a higher critical STP concentration than is correct. One way to correct for the over-prediction is to constrain *a* to equal the yield at the highest rate of P application (red lines). The validity of this approach relies on the inherent assumption that sufficient P was applied at the highest P application rate to achieve maximum herbage yield.

Estimates of critical STP values were made for all species sown in the field experiments. However, estimates of STP benchmarks were only considered reasonable when a species had been grown and tested over the full P fertility range at two or more of the four field sites (Tables 4.7–4.10) and only when the species achieved relatively high spring yields. If the critical P requirement of a species was not ranked consistently across the sites or seasons, it was assumed that this may indicate a limited adaptation range and the species was assigned conservatively to highest of the P-requirement categories in which it ranked (Table 14.1).

In recent years in Australia, critical STP benchmarks for pasture production have been defined as the STP that corresponds to 95% of maximum herbage yield (*Gourley et al. 2007*). However, 90% of maximum yield is also used (e.g. Moody 2007) and is the criterion used for crop STP benchmarks (Dyson and Conyers 2013). In Chapter 4, most analyses used the 90% criterion for critical Colwell P concentrations of the topsoil (0-10 cm depth) and equivalent critical P levels at 95% of max yield were also determined. Estimates of the corresponding critical Olsen P concentration were made using relationships between Olsen P and Colwell P determined for each site and growing season (Table 4.6)

16.2.1.2 Results and discussion

Four possible categories of P requirement were identified among the legumes assessed in the field experiments (Table 4.11). However, for the purposes of practical management on farms only three P-requirement categories were realistic (Table 16.1). The reasons for this were: (i) the species identified as having a moderate P requirement were either not sufficiently differentiated from other species or were inconsistent in their critical P rankings among sites or seasons, and (ii) soil test P results are intrinsically variable[5] reflecting season conditions (biological error) and variability associated with sampling and laboratory measurements (experimental error) so it is misleading and impractical to set STP benchmarks that give the impression of high “precision”

The three STP benchmark categories were:

Very high P requirement – the maximum yield of *Medicago sativa* (lucerne) was not achieved by *M. sativa* in any of the P response experiments. Its critical STP requirement was estimated to exceed 47 mg P/kg (Colwell) and to be >17 mg P/kg (Olsen). Given the importance of this species, experiments designed to specifically determine the very high STP benchmark for managing lucerne (and possibly other annual *Medicago* species) should be considered.

High P requirement - most *Trifolium* species fell into this category along with subterranean clover. A few species were also assigned to this category, despite occasionally exhibiting lower P requirements, because they did not consistently achieve a low critical P. The species that was most surprising was *Biserrula pelecinus* because it had root morphology traits typical of a very P-efficient species, but did not consistently achieve a low critical P requirement in either field or glasshouse experiments where it also often exhibited a relatively low yield. Biserrula may have quite specific edaphic preferences.

[5] Based on observations of farm and experimental soil testing results, Simpson *et al*. (2009) have suggested that it is sensible to assume the a Colwell STP concentration may at best be within ±2-3 mg P/kg soil, and an Olsen STP concentration within ±1-1.5 mg P/kg of the true soil fertility level.

Table 16.1. Estimated mean (±standard error) critical Colwell P and Olsen P benchmarks for managing pastures based on alternative legumes when grown in soil with PBI = 40-82. The values of means of data from all sites except from Belfrayden (Table 4.5). The values from Belfrayden were excluded from the benchmark estimates because the site returned consistently high critical P levels for all species. A possible reason for this is discussed in Chapter 4. Values in **bold** text are considered the most appropriate to use as benchmarks for soil P management (see text for discussion). (Unit: mg P/kg soil; 0-10 cm soil layer; critical STP corresponding with 95% of maximum yield).

Pasture legume	Cultivar	Critical Colwell $P_{(0.95)}$ (*unadjusted*) (mg/kg)	Critical Colwell $P_{(0.95)}$ (*constrained asymptote*) (mg/kg)	Number of experiments available for estimate	Inferred# critical Olsen $P_{(0.95)}$ (mg/kg)	Comment
			Very high P requirement			
Medicago sativa	SARDI 10	**>47**	N/A	5	**>17**	Maximum yield in response to P was not achieved by *M. sativa* in any of the present experiments.
			High P requirement			
Trifolium glanduliferum	Prima	40 (±3)	**36 (±1)**	7	**14 (±1)**	The critical requirements of these species were similar to that of *T. subterraneum* or were not reliably lower than it. The critical P requirement of the category is set using the data available for *T. subterraneum* because it is the most widely grown species.
Medicago truncatula	Sultan-SU					
Trifolium subterraneum	Leura					
T. subterraneum	Narrikup					
T. subterraneum	Izmir					
Trifolium spumosum	Bartolo					
*Biserrula pelecinus**	Casbah					
*Trifolium incarnatum**	Dixie					
*Trifolium hirtum**	Hykon					
			Low P requirement			
Trifolium purpureum	Electra	**25 (±4)**		3	**10 (±1)**	
Lotus corniculatus	LC07AUYF	**27 (±3)**		2	**10 (±0.4)**	
Ornithopus sativus	Margurita	**23 (±4)**	21 (±3)	5	**8 (±1)**	
Ornithopus compressus	Santorini	**22 (±5)**	21 (±4)	4	**8 (±1)**	

critical Olsen P derived from estimate of critical Colwell P using relationships between Olsen and Colwell P tests of soil samples from each site and year (Table 4.6)

* these species relegated to this higher critical P category as a consequence of inconsistent lower critical P rankings among sites or seasons.

Trifolium subterraneum (subterranean clover) was the control species used in all of the field and glasshouse experiments because a major objective of the work was to find legumes with lower critical P requirements than that of subterranean clover. It is appropriate that the benchmark STP should be based on the P requirement of subterranean clover because the critical requirements of most species in this category were similar to that of *T. subterraneum* or were not reliably lower than it, and because it is the most widely-used pasture legume in temperate southern Australia.

The expected critical P requirements of clover-based pastures in Australia have been published (*Gourley et al. 2007*; Moody *et al*. 2007) and are used to guide fertiliser practice. For the PBI range encountered at the field sites (PBI = 40-82), the critical Colwell P concentration for 95% of maximum yield was expected to be 28-32 mg P/kg soil (*Gourley et al. 2007*). The equivalent critical Olsen $P_{(0.95)}$ is 15 mg P/kg.

The average critical Colwell $P_{(0.95)}$ predicted from the field experiments using an unamended Mitscherlich asymptotic function was 40 mg P/kg. However, it was anticipated that the experimental design would over-predict the critical requirement for subterranean clover as described above (Section 16.2.1.1). An interim solution to the mathematical problem created by the experimental design was to constrain the asymptote of each fitted Mitscherlich relationship by making its asymptote equal to the yield achieved at the highest P-application rate in each experiment. An example which demonstrates how this should counteract the problem of over-prediction is shown in Figure 16.2. The validity of this solution relied on the assumption that sufficient P had been applied at the highest P application rate to achieve maximum herbage yield. When constrained asymptotes were applied to the subterranean clover data the average critical Colwell $P_{(0.95)}$ was predicted to be 36 mg P/kg (Olsen P equivalent = 14 mg P/kg; Table 16.1). The validity of this interim solution was also tested by constraining the Mitscherlich asymptote for data from the serradella treatments. This did not significantly alter the predicted critical P for the serradellas as should be expected for a species where the critical P requirement falls approximately in the middle of the fitted function (Table 16.1)

Low P requirement – four species were found to consistently achieve critical P requirements that were substantially lower than that of subterranean clover. Of these species, *Lotus corniculatus* (birdsfoot trefoil) was relatively low yielding, did not persist over the dry summer period. This perennial legume is better suited to areas with more summer rainfall. It could only be tested in two growing seasons due to shortages of breeder's seed. *Trifolium purpureum* (purple clover) is a forage species and, as such, is not a suitable alternative for permanent pastures based on subterranean clover.

The outstanding pasture types were *Ornithopus sativus* (French serradella) and *O. compressus* (yellow serradella) which consistently achieved a low critical P requirement. Yellow serradella did not yield as well as subterranean clover during spring in most experiments, especially in the year it was sown. At Yass, a substantial improvement in yield was seen in subsequent years. Yield during winter was not measured, but yellow serradella clearly grew slowly in the cool winter conditions of the southern tableland sites. This species is a preferred pasture legume in some areas of southern Australia (e.g. Western Australia, Coonabarabran, NSW) where it grows as well or better than subterranean clover. In contrast, French serradella grew vigorously during cool winters and yielded as well as subterranean clover during spring and did so in low P soil that constrained the growth of

subterranean clover. The critical Colwell $P_{(0.95)}$ concentration of the serradellas was 22-23 mg P/kg (8 mg P/kg, Olsen; Table 16.1).

The *Ornithopus* species had the largest potential of all of the legumes examined for development of more P-efficient pasture systems.

16.3 How do pasture legumes achieve a low critical P requirement?

(Objective 3: Identify the root morphology traits that have the largest influence on the critical P requirements of subterranean clover and alternative legume species.)

16.3.1 High capacity for nutrient foraging by roots; the key to a low critical P requirement among pasture legume species

P is supplied to roots by diffusion from the bulk soil to the root surface in the soil solution. The process is described by Fick's Law (see Chapter 7). The rate of diffusion is directly proportional to the phosphate concentration gradient established between the soil and the root surface. There are consequently two things that can alter the amount of P that a plant is able to take up:
(i) The concentration of plant-available phosphate in the soil can be increased. This increases the rate of P diffusion towards the root surface. The usual way to do this is by applying P fertiliser. However, the objective of the present experiments is to find plants that can achieve equivalent P uptake when the concentration of phosphate is lower than is presently needed for rapid plant growth.

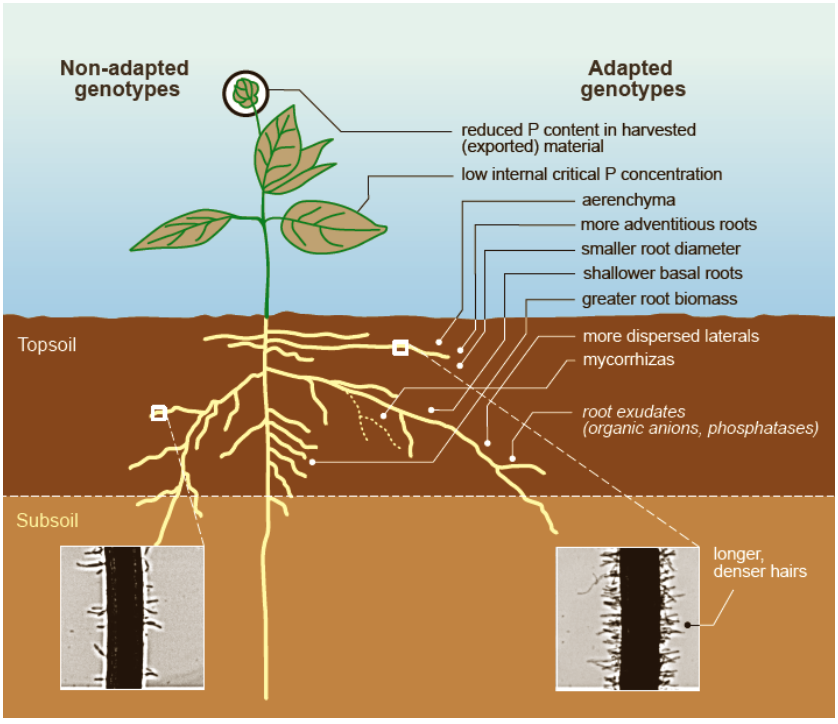


Figure 16.2 Root morphology, P-stress acclimation and P-utilisation traits that can assist plants to achieve greater P uptake in P-deficient soil and/or a low critical P requirement for maximum growth (from Richardson *et al*. 2011).

(ii) Plants may counter the impact of low P concentrations by increasing the area of their root-soil interface ("nutrient foraging") and/or by modify their rhizosphere ("nutrient mining") so that more P is intercepted and absorbed. (see Fig. 16.2 for the diversity of ways that plant may potentially do this, Richardson *et al*. 2011).

When P acquisition by a diverse group of the alternative legumes was examined in detail (Chapters 6 and 7) it was concluded that P acquisition could be explained by diffusion to the surface of the root hair cylinder of each species (nutrient foraging). The similarity with which P acquisition could be explained this way among the five legume species studied, indicated that nutrient mining benefits were either small or were essentially equivalent for all of the legumes. The experiments did not test whether colonisation of roots by AMF would have modified these conclusions. However, any potential for differences due to AMF colonisation appeared to be less likely when it was observed that roots of serradella and subterranean clover were highly colonised by AMF at four field sites in 2014 (Chapter 4). The relative abilities of subterranean clover and serradellas for P acquisition were maintained in field experiments with high rates of AMF colonisation, and were similar to the observations made in the more detailed controlled-environment experiments with lower rates of AMF colonisation (Chapters 6 and 7).

Rates of P uptake per unit surface area of the root hair cylinder were similar for all species (Chapter 7) because phosphate diffusion in low P soil was a rate limiting process. High P uptake in low P soil and lower critical P requirements for maximum growth were, consequently, achieved by developing a large root hair cylinder surface area for P uptake in P-enriched patches of the soil profile. This was especially effective when a species was able to combine high root length density with long root hairs.

Different species achieved a high root length density by different means. The *Ornithopus* spp. deployed long, fine roots with high specific root lengths (i.e. high length per unit dry matter). By contrast *T. subterraneum* had roots with low specific root length but allocated more dry matter to nutrient foraging in the nutrient-enriched topsoil. This was extremely effective for achieving a high root length density but is surmised to come at a relatively high carbon cost for the plant.

Although *T. subterraneum* had an exceptional ability to develop a high root length density, it was unable to fully utilise this capability and had low rates of P uptake and the highest critical P requirement because its root hairs were very short. Unlike some other species (e.g. *Arabidopsis thaliana*, Bates and Lynch 1996), *T. subterraneum* also did not adapt to low P conditions by growing longer root hairs. In direct contrast to *T. subterraneum*, the *Ornithopus* spp. had long root hairs (2- to 3-fold longer than any *T. subterraneum*) that approached the length measured on many temperate grasses and also marginally increased their length (+20%) when growing in low P soil. The *Ornithopus* spp. achieved high P uptake and the lowest critical P requirements of the legumes by combining high root length density with long root hairs.

16.3.2 Differences in "nutrient mining" attributes

The potential for organic acid (carboxylate) exudation from roots was extensively examined (Chapter 11) because of their potential to enhance phosphate desorption from sparingly-available sources and accumulated P in fertilised soils. Most of the pasture legumes and *T.*

subterraneum in particular had relatively low rhizosphere carboxylate exudation and did not approach the levels observed for crop species that are known to gain P uptake benefits as a consequence of carboxylate exudation. The *Ornithopus* spp. were an interesting exception. They had very high total rhizosphere carboxylates when expressed on a per gram of root basis; levels equivalent to, or approaching those of *Lupinus* spp. However, the effectiveness of carboxylates in desorbing P in soil is concentration dependant (e.g. Ryan *et al*. 2014). When total carboxylates were expressed on a root length basis, the *Ornithopus* spp. still had moderately high levels for a pasture legume, but now equivalent to that of other species such as lucerne (*M. sativa*).

As such, the question of whether carboxylate exudation is a significant factor in the P nutrition of some pasture legumes remains open, but for most of the species examined (e.g. subterranean clover) it is unlikely that they are important for P nutrition in low P soils because most species exhibited low rates of rhizosphere carboxylate exudation.

16.3.3 Highly P-efficient species have root systems with a combination of favourable root traits

The results from a series of experiments emphasise the importance of having a package of favourable nutrient foraging traits for achieving high P acquisition efficiency. Biserrula (*B. pelecinus*) also has relatively long root hairs but when grown in soil from Canberra (Chapter 7) it did not combine this with high root length density and did not achieve a marked improvement in its critical P requirement. When grown in soil from Wagga (Chapter 8), biserrula developed a high root length density combined with long root hairs and achieved a low critical P requirement, close to that of the serradellas. Rose clover (*T. hirtum*) had a high specific root length density in all experiment (Chapters 5, 7, 8 and 13) and could achieve relatively high root length densities in the nutrient layer of a soil profile without the very large commitment of root dry matter that subterranean clover needed to allocate to nutrient foraging. However, it had very short root hairs similar to those of subterranean clover and achieved only a marginally lower critical P requirement as a consequence.

It is noteworthy that the serradellas appear to combine many P-efficient root system traits: high specific root length, high root length density in a nutrient patch, very long root hairs, roots that are highly colonised by AMF and a potentially higher capacity for organic anion exudation.

The importance of achieving a package of beneficial root traits has implications for plant improvement. Some species have clear weaknesses in the P-efficiency attributes of their root morphology. For examples, many of the cultivated *Trifolium* spp. have very short root hairs. However, a focus on only one root trait in a plant improvement program may only improve P efficiency whilst that trait remains the rate-limiting attribute of the root system.

16.3.4 P acquisition efficiency vs P utilisation efficiency

Plants can also achieve improved P efficiency by using P internally in a more efficient manner (i.e. more dry matter yield per unit of P taken up; or grain yield with a lower P content). For example, in crops which have a P balance efficiency of ~50% (i.e. this means half of the P applied as fertiliser is harvested in the grain), it is argued that reducing the P content of grain would potentially lift the P efficiency of the system by reducing the amount of P removed from fields at harvest (e.g. Veneklaas *et al*. 2012).

It is debatable whether this is a useful strategy to pursue for P efficiency in pastures, at least in the first instance. The major component of P inefficiency in Australian grazing enterprises is the 70-90% of applied P that accumulates in the soil. Rates of P accumulation are related to the STP concentration of the soil (Simpson *et al*. 2014) which is not altered by improving the P utilisation efficiency of pasture plants. In contrast, reducing the critical external P requirement of a "rate limiting" species in a mixed pasture (i.e. the pasture legume) directly reduces the largest component of P inefficiency.

Plants that have improved internal P (utilisation) efficiency should, nevertheless, be able to yield more per unit of P uptake. For example, if a 20% gain in P utilisation efficiency of all species in a pasture were possible, it is inferred that ~20% more pasture growth and animal product could be obtained from the same amount of P input. However, in grazing enterprises, where the range in median P balance efficiencies is presently 11-29% (Weaver and Wong 2011), fertiliser costs per unit of production would only be reduced by about 2-6%.

Pasture is not the product being removed from a field except during forage conservation, so changes to P utilisation efficiency will also only indirectly effect P removal in products. The P content of animals (the main product removed from fields) is only determined by the pasture when the herbage has insufficient P content to support animal growth or lactation. Reducing P concentrations of plant tissues to this extent would be counterproductive because it would limit production. Many pasture species are already close to these P concentration limits for pregnant and lactating females and young, growing ruminants; any further reductions may carry a risk of impaired feeding value (Ozanne 1980; Betteridge 1986). (NB. Lower P concentrations are much less of a problem for mature animals.)

In Chapter 6, the contribution of P utilisation efficiency to legume P use was examined briefly because the ability of plants to more efficiently distribute and utilise P internally could potentially contribute to a lower external P requirement. However, with the exception of *O. compressus* (which had marginally higher internal P efficiency), the legumes had very similar critical internal P concentrations. For all of the species examined, P utilisation was not an overriding influence on their critical external P requirements. *Trifolium hirtum* has been reported to have a low critical shoot P concentration (Pinkerton *et al*. 1997) but, it was not different to *T. subterraneum* in the present study. The grass used in this study (*Dactylis glomerata*) had a lower critical internal shoot P concentration than most of the legumes. This is typical of many temperate grasses (Pinkerton *et al*. 1997).

16.4 Variation in P-efficiency root traits of subterranean clover

(Objective 4: Assess the variation in P-efficient root traits of subterranean clover and quantify the potential for breeding P-efficient clovers.)

16.4.1 Species-wide assessment of P-efficiency root traits in subterranean clover.

The potential to improve the P-acquisition efficiency of *Trifolium subterraneum* is of particular interest because it is the most widely used pasture legume in Australia (Nichols *et al*. 2012) but has a relatively high external critical P requirement. The "core collection" of *T. subterraneum* lines (representing ~80% of the variation in the subterranean clover genome; Ghamkar *et al*. 2008) provided a unique opportunity to evaluate the variation in root

morphology traits in this species and consequently the potential for improving the P-efficiency of *T. subterraneum* through breeding.

The root morphology traits of subterranean clover related to P acquisition that have been examined were:

(i) root mass fraction (the proportion of total plant mass that is allocated to roots),
(ii) specific root length (the length of roots per unit root mass),
(iii) root proliferation in a nutrient-enriched soil patch (ability of the plant to sense and forage for P in soil),
(iv) average root diameter (a measure of root fineness),
(v) root hair length (single cell projections on roots that extend the root-soil interface),
(vi) lateral root angle (the propensity for a genotype to have a shallow or deep rooting pattern),
(vii) AMF colonisation (the proportion of root length colonised by AMF),
(viii) amount of carboxylates in the rhizosphere (carboxylates [organic acids] can assist mobilisation of sparingly-available P in the rhizosphere),

Variation in the magnitude of most root traits among *T. subterraneum* lines was substantial. For many key traits (specific root length, root hair length, root proliferation) a 2-fold range was observed (Chapters 9, 10 and 11). However, the range in root colonisation by AMF was 3-fold. For a few traits (e.g. root mass fraction and lateral root angle) the range, while substantial, was relatively small (~1.5 fold range). In contrast, the amounts of carboxylate exuded into the rhizosphere was small compared with other legumes and the exuded amounts did not vary among the *T. subterraneum* lines examined.

The capacity of *T. subterraneum* to proliferate root length in a P-enriched patch of soil was exceptional when compared with almost all other legume species (Figs. 7.1, 8.4, 11.4a and 14.3a). Root proliferation was primarily the product of preferential allocation of root dry mass to roots in the P-enriched zone of a soil profile. *T. subterraneum* appeared incapable of adapting its root morphology favourably to enhance the root proliferation response. Consequently, it was the cultivars with the most favourable specific root length and root hair length that captured the most benefit from root proliferation (Chapter 11).

It is interesting and valuable to note that for most root traits the ranges in their magnitude among the core collection lines was similar or only marginally greater than the range observed among the 30 or so cultivars in the reference collection. A possible exception to this was root colonisation by AMF, which appeared to be less wide-ranging among the 30 cultivars (Chapter 12).

There are two important consequences of this observation:

- It may be feasible and highly effective to select or breed for P efficiency among the existing *T. subterraneum* cultivars because the phenotypic range in the core collection is only marginally wider. There are potential advantages in doing this because the cultivars are already selected for superior yield, low-toxin and disease-resistance attributes (e.g. Nichols *et al*. 2012).
- It is also useful to be able to use the cultivars for studies of P nutrition and root system physiology/ morphology because of their superior yield, disease resistance and other agronomic and adaptation attributes. This avoids pitfalls and inconclusive results when

examining lines that lack agronomic “fitness” (as observed for some of the core collection lines).

In retrospect, it was clear that it would have been a mistake to have only examined P-efficiency traits within *T. subterraneum*. The wide range in critical external P requirements of the alternative pasture legumes (Chapters 4, 6, 7, 8 and 13) provided critical insights into the root morphology traits that can ultimately deliver reduced P costs in Australian pasture systems because the variation among the alternative legume species in these traits (especially specific root length and root hair length) was much wider than found within the *T. subterraneum* genome. Work with the wide diversity of legume root traits demonstrated that estimates of specific root hair cylinder volume (a measure that integrates the influence of the key root morphology traits) was found to be useful as a “P-foraging index” and broadly indicative of the critical P requirements of the legume species (Chapters 5 and 8).

The P-foraging index of *Ornithopus* roots (Table 5.2) illustrates the peak in potential P-acquisition efficiency among the pasture legumes. *Ornithopus* roots combined most of the P-efficient traits that are presently recognised as important, and this is why they achieved the lowest critical P requirements among the pasture legumes examined to date. Their consistent low critical P requirement provides a useful benchmark against which initial comparisons of the P-efficient root morphology of pasture legumes can be made.

The P-foraging index was, consequently, proposed as a potential primary screening tool for discovering putative P-efficient legume species (Chapter 8). Calculation of the P-foraging index also provides us with a possible way to estimate the progress that may be made in breeding for improved P-acquisition efficiency using the phenotype of pasture legume roots.

16.4.2 Which root traits are most important for P acquisition efficiency in subterranean clover

The experiments examining root acclimation to P stress and the features of root systems that are associated with low critical P requirements have highlighted the importance of three root traits: root length proliferation, specific root length and root hair length, that either improve of constrain the clover’s P acquisition capacity.

Root length proliferation in P-enriched soil patches: this is a complex trait that is the product of root mass fraction x specific root length. The very high capacity of *T. subterraneum* roots to forage for P in nutrient-rich patches of soil indicates that root proliferation is a critical trait for P acquisition by subterranean clover. The root proliferation response under low soil P conditions is so strong that we now suspect that lateral root angle (a proven P-efficiency trait in some other species; e.g. common bean, soybean, etc.) may not be important for P acquisition in subterranean clover. The interplay between these root traits is yet to be resolved, but it may prove more valuable to select subterranean clover for ‘low root angles’ (i.e. deep roots for drought tolerance) because ‘root proliferation’ will be sufficient to ensure high root length density in regions of soil where P can be found.

Specific root length: the attributes of *T. subterraneum* roots that modify the ability of the legume to capitalise on root proliferation are its specific root length (i.e. high dry matter cost per unit length) and root hairs. Compared with the *Ornithopus* species, *T. subterraneum* has relatively low specific root length (i.e. a high dry matter cost per unit length) and short root hairs (i.e. inability to develop a high specific root cylinder volume) (Fig. 16.3). Cultivars of *T.*

subterraneum with intrinsically higher specific root length were advantaged in their foraging for P in low-P soil, did not experience P-stress until grown in soils with substantially lower soil P levels than those with low specific root length, and had a lower critical P requirement (Chapter 11).

Root hair length: very short root hairs which limit the development of a high specific root cylinder volume with a large surface for P uptake stands out as a major constraint to achieving a low critical P requirement equivalent to that of the *Ornithopus* spp.. There was a 2-fold range observed among the core and cultivar lines for root hair lengths which may prove useful for plant improvement, but it is a difficult trait to assess and we have sometimes noted relatively large variation in root hair length for single cultivars when growth in different experiments. Substantive improvement in the size of the root hair cylinder volume due to small increases in root hair length were observed among subterranean clover cultivars in an experiment where AMF colonisation of roots was low (Chapter 11). However, in the field, AMF colonisation of roots is ubiquitous and it is known that there is an interaction between root hair length and AMF colonisation for plant P uptake (Schweiger *et al*. 1995; Caradus 1981). To some extent, AMF can compensate for short root hairs and this may negate apparent P-acquisition advantages that longer root hairs could provide. This must be considered if attempting to improve P-acquisition efficiency by selecting for longer root hairs (e.g. Caradus 1981).

On the basis of the present studies, these three root morphology traits appear to be critically important for P-acquisition by subterranean clover and should be a primary focus for genetic improvement. However, contributions to P acquisition by the other P-efficiency traits that were examined are also likely to be important in some instances. For example, the differences P-acquisition, persistence of root foraging and yield in low P soil among five cultivars of *T. subterraneum* (that differed mainly in their specific root lengths) was essentially explained by differences in their root morphology (Chapter 11). However, when the 97 subterranean clover core collection lines (representing a much wider array of *T. subterraneum* genotypes) were examined, root morphology alone only explained ~54% of the variation in P uptake from a low P soil (Fig. 10.6c). Time did not permit further examination of the factors involved in the ‘unaccounted component’ of variation in P-efficiency among the core collection lines. However, over such a wide diversity of genotypes, variation in internal P efficiency is feasible. For reasons outlined previously, it is debateable whether pursing internal P efficiency would be wise or a productive use of time in a pasture context.

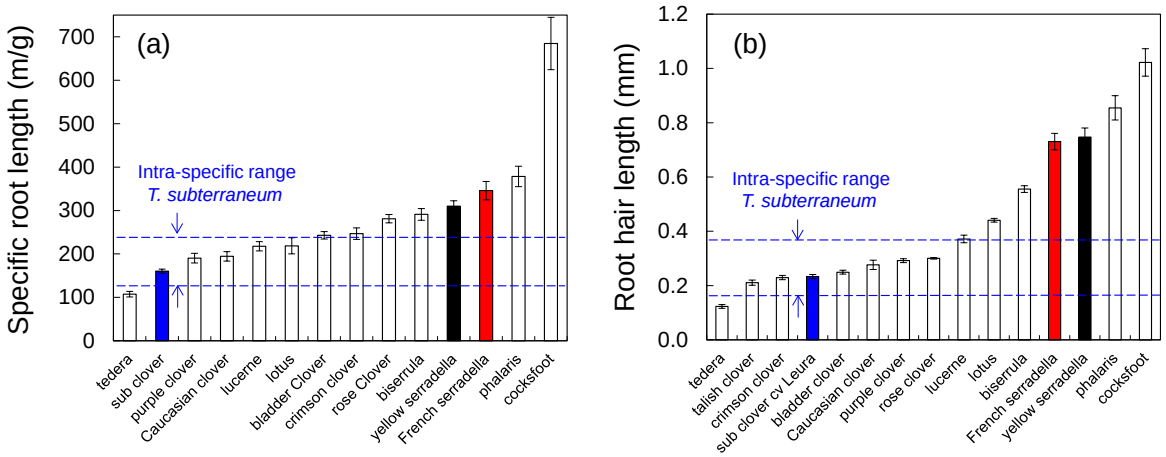


Figure 16.3 The ranges in magnitude of (a) specific root length and (b) root hair length in subterranean clover (Chapter 9) compared with the inter-specific range observed for a number of alternative legumes and two grasses (Chapter 5).

16.4.3 What is the potential for identifying and/or breeding more P-efficient cultivars of subterranean clover?

16.4.3.1 P-efficient subterranean clover cultivars

On face value, there was sufficient variation in key P-efficiency root traits to suggest that significant differences may exist among the lines and cultivars of subterranean clover for improved growth in low-P soils and for differences in critical external P requirements. Jones *et al*. (1970) reported variation in response to P among subterranean clover cultivars, but most of the genotypes in that study are now outclassed and are not recommended for use. Differences in shoot yield of up to 2-fold were also recorded for growth in moderately P-deficient soil among subterranean clover cultivars in this project (Figs 10.1, 10.2, 11.1, 13.1 and 13.7). However, no significant difference in the critical P requirement of three subterranean clover cultivars (cvs Leura, Narrikup and Izmir) was detected in the field experiments (Chapter 4).

The differences in yield in moderately P-deficient soils among the *T. subterranean* cultivars could be potentially useful on many farms where, for a variety of reasons, farmers are unable or unwilling to lift soil P fertility to levels sufficient for maximum growth by subterranean clover-based pastures. Choosing the right (P-efficient) cultivar could potentially double the production of low P paddocks in some instances. However, in the present project cultivar comparisons cold only be made in controlled-environment, pot experiments. We know that these experiments can detect subtle differences in P-efficiency among legume genotypes (e.g. Chapter 8), that cannot be detected in the more variable edaphic and climatic environment of farm paddocks (Chapter 4). Testing the apparent P-efficiency differences among subterranean clover cultivars in the field must occur before any recommendations regarding cultivar choice can be made to farmers.

16.4.3.2 Can new cultivars of subterranean clover be developed to achieve the high P-efficiency of serradellas?

Initial comparison of key P-efficiency root traits of *T. subterraneum* with those of serradellas indicates that it should be feasible to develop clovers with lower critical P requirements than is available in many of the current subterranean clover cultivars. However, it is also essential to understand whether subterranean clover lines can be developed to emulate the very low critical P requirements of serradella. While clover cultivars that are more productive in moderately P-deficient soils will be valuable, a low critical P requirement equivalent to that of serradella is required for the substantial savings in P fertiliser costs (as outlined in Section 1.3) to be realised on farms.

The three root traits that have interim support as key to improvements in the P efficiency of subterranean clover are identified above (Section 16.4.2). The potential we see for developing these traits in subterranean clover to match the phenotype observed in serradella is as follows:

Root proliferation and specific root length: subterranean clover was exceptionally good at proliferating root length for nutrient foraging and developed a marginally higher root length density in a P-enriched soil patch than the serradella species (Chapters 6, 7 and 8). On face value, it is possible that root length density by subterranean clover should not be considered a constraint to achieving a very low critical P requirement. However, serradellas and subterranean clovers developed their high root length densities by different means: the serradellas had an intrinsically long, fine root system (high specific root length) and could develop high root length density without having to allocate large amounts of dry mass to their root systems (Figs. 6.2 and 8.2); subterranean clover, with its relatively low specific root length, had to allocate significantly more root dry mass to nutrient foraging roots in response to P stress to achieve the same outcome (Figs. 6.2 and 6.4b). All of the comparisons of P-efficiency among the legumes species (Chapters 6, 7 and 8) indicated that the intrinsic root morphology characteristics of their root systems defined the critical P requirement of the species. Root acclimation to P stress was also clearly important in defining how well a species could tolerate low soil P conditions and did modify a species' critical P requirement. However in Chapter 11, where the root acclimation to low soil P among a number of subterranean cultivars was examined, it was apparent that root adaptation was predominantly a response to P stress (sensed within the plant) and came at a cost to shoot yield in low P soil. The cultivars that had intrinsically higher specific root lengths were able to achieve higher yields in moderately P-deficient soil because they avoided extreme P-stress and could more readily maintain root foraging in the low P soil.

We conclude that reactive deployment of roots to nutrient patches (e.g. the subterranean clover strategy) will not be as effective as intrinsic deployment of root length (e.g. the serradella strategy) in a novel low-P cultivar. Consequently, the relatively coarse root system of many subterranean clover lines is a potential disadvantage for P acquisition because it requires the plant to allocate more dry matter to root growth to achieve equivalent root length. However, the range in the specific root lengths among subterranean clover lines was large (2-fold variation) and lines with the highest specific root length achieved about 70-80% of the specific root length found in French and yellow serradella (Fig. 16.3a). This indicates that lines with relatively high specific root lengths (approaching the levels found in serradella)

can and should be selected to improve the effectiveness of root foraging in subterranean clover cultivars.

Root proliferation was an identifiable root trait in its own right, albeit the result of two separable root attributes (root mass fraction and specific root length). There was also some indication in the comparison of P-efficiency among clover cultivars (Chapter 11) that the persistence of root proliferation in soil P patches (and thus opportunity for P acquisition) may differ among genotypes. Irrespective of these observations, cultivars with an intrinsically higher specific root length had a clear advantage when nutrient foraging and selecting for higher specific root length (as opposed to root proliferation) should be the initial focus for subterranean clover improvement.

Unfortunately, the specific root length trait varied with the plant's growth environment (Chapters 7, 8, 11 and 15). This may complicate selection of plants for high specific root length as selections may be subject to high levels of environmental variance. Further work is needed to clarify the conditions under which screening lines for specific root length should be conducted to ensure minimal interference from environmental influences. It is encouraging that intrinsic differences in specific root length were expressed at all levels of soil P supply, so it is feasible that selection may be made successfully by comparing plants in high soil P conditions where variation due to other environmental factors, appeared to be less (e.g. Fig. 11.5a). Specific root length is itself the product of two root properties: root diameter and root tissue density (Chapter 10).

$$\underset{(m/g)}{\text{Specific root length}} = \pi * (\text{root diam} / 2)^2 \ / \ \underset{(g/m^3)}{\text{root tissue density}}$$

Plant growth conditions that clearly affected the specific root length phenotype of subterranean clover did not alter root diameter (e.g. Figs. 8.5 and 8.6). This indicates that the impacts of environment on specific root length occur via an effect on root tissue density. The basis of differences in root tissue density was not examined due to lack of time, but should be considered in future research.

In a number of experiments representing different plant growth conditions, the specific root lengths of diverse subterranean clover lines were often highly correlated with their average root diameters. The amount of variation in specific root length explained by variation in root diameter varied among the experiments from 33% to 91% of (Chapters 9 and 10). However, in two-thirds of the experiments, the correlation co-efficients were sufficiently high (>0.7) that it appeared likely that selecting for finer roots (small root diameters) could prove to be a simple and rapid way to select lines with relatively high specific root length. However, the data presented in Figure 10.5 demonstrate that root tissue density and root diameter vary independently and more effective selection for high specific root length would be made by concurrent selection for small root diameters and low root tissue densities.

Root hair length: the root hairs of subterranean clovers are among the smallest observed among the alternative legumes. It was clear from the responses of a number of the alternative legumes to P supply that short root hairs are a major disadvantage for P acquisition and can even negate the advantages of high root length density. The range in root hair lengths in subterranean clover was also large (2-fold) but the size of even the longest root hairs fell well short of that observed on the serradellas (at best about half of the

root hair length achieved by serradella, Fig. 14.3b). Caradus (1981) has attempted to select longer root hairs in *Trifolium repens* (white clover) which has root hairs of similar length to subterranean clover. His objective was to improve P efficiency but he was defeated by the low diversity in root hair lengths in *T. repens* which resulted in only a small improvement in length and no improvement in the critical P requirement of the clover unless it was grown without AMF. There is some evidence to suggest that root hairs must be longer than about 0.5 mm before the influence of AMF on P uptake is overtaken (Schweiger *et al*. 1995; also see discussion in Simpson *et al*. 2011).

There are two potential strategies identified to break this nexus: (i) widening the gene pool for root hair length through interspecific hybridisation, and (ii) a molecular genetic approach that uses novel gene editing technologies to produce cultivars that may not be classed as a GMO[6].

Widening the gene pool for root hair length: interspecific hybridisation enables introduction of traits from outside the existing genetic variation of a species using traditional plant breeding techniques. Some major improvements in root system attributes have been achieved in pasture species by this method. The acid soil (aluminium) tolerance of *Phalaris aquatica* has been increased substantially by introgression of genes from *P. arundinaceae*, a weedy relative (Culvenor *et al*. 2004). Drought tolerance in *Lolium multiflorum* has also been improved after hybridisation with *Festuca arundinacea* (Thomas *et al.* 2003) and waterlogging tolerance improved by hybridising *Brachiaria* spp. (Cardoso *et al*. 2013). Interspecific crosses are now also being utilised in the genus *Trifolium*, particularly between *T. repens* and its close relatives, to develop novel hybrid clovers (Williams *et al.* 2010; Jahufer *et al.* 2012). In this way some of the limitations of white clover may be overcome. Notably, *T. repens* x *T. uniflorum* hybrids show potential for substantial changes in root morphology and development of P efficient forage legumes (Nichols *et al*. 2014a, Nichols *et al*. 2014b; Williams *et al.* 2013).

An initial examination of root hair lengths and other root morphology traits was made in genetically-allied *Trifolium* species (Chapter 14). For some of these species there is published evidence of potential for successful interspecific crosses (Katznelson 1967). Some of the *Trifolium* species exhibited markedly different responses in their root morphology in response to low P supply. *Trifolium pauciflorum*, *T. meduseum* and *T. pilulare* were the only *Trifolium* species to increase their root hair lengths in response to low P supply. Most notably, *T. pauciflorum* and *T. meduseum* increased their root hair length 1.2 to 1.3-fold and maintained longer root hair lengths (~0.45 mm) than subterranean clover (0.25 mm) or any of the other *Trifolium* species in low P soil (Fig. 12.3c).

A molecular genetic approach to development of long root hairs: in recent years a series of papers have been published that demonstrate large increases in root hair length can be achieved in dicots and monocots by manipulation of ExpansinRSL4 genes for longer root hairs in *Arabidopsis thaliana* (Yi *et al*. 2010) and *Brachypodium distachyon (*L. Dolan *pers. commun.)*. The work has also demonstrated that most plants have comparable regulatory genes (Pires and Dolan 2010) and may potentially be activated to produce longer

[6] Presently in the USA, gene editing is classified as a mutagenesis procedure and plants produced using the technology not classified or marketed as a GMO (Ainsworth (2015)). However, the status of plants produced by these new technologies is yet to be determined in Europe and Australia (Nature, 17 December 2015).

root hairs (Datta *et al*. 2015). Novel technologies for gene editing (e.g. CRISPR) can also now be used to produce plants that may not be regarded as genetically-modified organisms (Ainsworth 2015), so there is a distinct possibility that a breakthrough in root hair length in subterranean clover may be feasible by this means.

17 Recommendations.

17.1 Soil and pasture management for optimum and improving P efficiency

17.1.1 Soil P fertility management

On Australian grazing farms, it is recommended practice to build STP slowly towards a target STP that suits the goals of the farm business, and then to maintain this level (Reuter *et al*., 1995; Simpson *et al*. 2009). There are many pragmatic reasons why this is the preferred practice. For example, it allows input costs to be managed conservatively and stocking rate to be increased in line with the increase in soil fertility and pasture yield. Once optimum soil P fertility is achieved, the efficiency of land-use (fertilised pasture has a higher carrying capacity) and water and nitrogen use are also maximised (P fertiliser promotes maximum pasture growth rates per mm of rainfall and high pasture legume content). The target STP for temperate pastures is determined by the critical P requirement of the pasture legume. The published soil test guidelines for southern Australia (*Gourley et al. 2007*) are considered suitable for pastures based on *T. repens* (white clover) and *T. subterraneum* (subterranean clover). However, there are relatively few independent published sources of data that support these recommendations (e.g. one such paper is Moody 2007) and there no information about the critical P requirements of other pasture legumes.

The results from this project strongly support the current soil P management guidelines for subterranean clover-based pastures. Our critical Olsen P concentration (14 mg P/kg) was well-matched to the recommended level for pasture management (15 mg P/kg), whilst the critical Colwell P concentration determined in the field experiments (36 mg P/kg) was slightly higher than the level that would be recommended (28-32 mg P/kg) for soils with equivalent PBI.

The data collected in the experiments allow an expansion of the pasture soil P management guidelines. **Three categories of soil P management are recommended depending on the legume that is being used in the pasture:**

Very high P requirement **(Olsen P >17; Colwell P[7] >50 mg/kg):** *Medicago sativa* (lucerne)

High P requirement **(Olsen P = 15, Colwell P = 35 mg/kg):** *Biserrula pelecinus* (biserrula)*, Medicago truncatula* (barrel medic)*, Trifolium glanduliferum* (gland clover)*, Trifolium hirtum* (rose clover)*, Trifolium incarnatum* (crimson clover)*, Trifolium spumosum* (bladder clover)*, Trifolium subterraneum* (subterranean clover)

Low P requirement **(Olsen P = 10; Colwell P = 25 mg/kg):** *Ornithopus compressus* (yellow serradella)*, Ornithopus sativus* (French serradella)*, Lotus corniculatus* (birdsfoot trefoil)*, Trifolium purpureum* (purple clover)

[7] Colwell P benchmarks are rounded to the nearest 5- or 10-unit equivalent and are applicable to soils with PBI = 40-80.

Medicago sativa (lucerne) pasture had a much higher critical P requirement than any other legume (Colwell P > 50 mg P/kg). A definitive estimate could not be determined for lucerne because its critical value was "off-the-scale" of the P fertility levels used in the field experiments. **Given the high importance of lucerne pastures for meat production and its various uses in all temperate grazing systems and in phase farming, it is recommended that high priority be given to determining the critical P requirement of lucerne.**

17.1.2 "Low-P" pastures based on P-efficient, alternative legumes

The consistent low critical P requirements of the *Ornithopus* species opens up an opportunity for development of low-P pasture systems that can yield as well as subterranean clover pastures but with substantially less P fertiliser. P input savings of the order estimated in Section 1.3 (i.e. ~30% less P fertiliser annually) should be possible in optimally-fertilised pasture systems because the difference in the critical P requirement of subterranean clover and the serradellas is similar in magnitude to that used in the original calculations.

In areas where serradella pastures are already used (e.g. permanent pastures in WA and Coonabarabran NSW; in phase farming systems WA and the Riverina of NSW) immediate adoption of "low-P" technology should be feasible. However, to ensure adoption on farms, it will almost certainly be necessary to build confidence (e.g. district level demonstrations) that the lower critical P benchmarks do not reduce pasture yield. Currently, serradellas are grown in some areas with STP concentrations that are up to twice that indicated as necessary by the present research.

It is also clear that the P-efficient legumes yield considerably more herbage in soils that have not been fertilised to optimum levels. Their use will enable substantial increases in production in paddocks that are not regularly fertilised. The practical reality for many farms is that there are paddocks that do not always get fertilised. The reasons for this are diverse: cash flow may limit the amount of fertiliser that can be obtained each year, paddocks regarded as under-productive are often given a low priority for fertiliser spreading, some paddocks are difficult to access or are too steep to safely spread from the ground. Both the field and the glasshouse experiments indicated that it is possible to double legume herbage yields in moderately P-fertile soils by growing a serradella in place of subterranean clover. A production gain such as this could substantially change the value of paddocks that are not fertilised regularly.

The research project has shown that *Biserrula pelecinus* has root characteristics suited to high P-efficiency but its critical P requirements in field trials have been variable and have not reflected its root morphology. The species did not grow particularly well at some sites. **Biserrula is an emerging species with growing interest from farmers in particular farming districts where it grows exceptionally well (e.g. Riverina, NSW). The reason(s) for its variable field P requirements should be investigated further with the objective of being able to capitalise on the anticipated high P efficiency of the species.**

The lower critical external P requirements of *Trifolium purpureum* and *Lotus corniculatus* may also prove useful in the niche farming environments to which they are suited. However, these species are not expected to have wide industry impacts on P efficiency.

17.2 Towards wider adoption of highly P-efficient pasture systems

(Objective 5: Clear decision point for breeding improved subterranean clovers, and/or evaluation of alternative legume species for P-efficient farming systems.)

This project has shown that there are opportunities to increase the P-efficiency of pastures by increasing the use of serradellas because they have very low critical P requirement when compared with subterranean clovers. However, the industry-wide success of this option depends on the effective adaptation range of the serradellas.

The project is also indicating that there are opportunities within the existing cultivars of subterranean clover to select lines that will deliver better production in moderately P-deficient soils. This has been a surprising outcome and it is still under investigation. It is unlikely that P-efficient lines of subterranean clover can generate the P-fertiliser savings that a serradella is surmised to deliver, because their root system morphology is not as suitable for nutrient foraging as that of serradellas. However, the known and widespread adaptation range and easy cultural management of subterranean clovers ensures that there is a place for cultivars that can be more productive when soils are not optimally fertilised The recommendations associated with these options are outlined below.

17.2.1 Serradella-based pastures and constraints to their wider adoption

In many permanent pasture areas, the *Ornithopus* species are still an almost “unknown” quantity. There has been limited trialling of the available cultivars that have shown promise for growth of serradellas in some permanent pasture areas (e.g. Freebairn 1990; Hackney *et al*. 2013; Loi *et al*. 2007). However, there is a lack of longer-term and mixed pasture data with many unknowns that have the potential to stall adoption of serradella-based pasture systems: e.g.

- At all sites (Yass and Burrinjuck, particularly) the yields of yellow serradella in spring were well behind that of French serradella. Winter yield of yellow serradella was also low. In contrast, winter yield of French serradella and its suppression of weed competition during winter appeared to be as good as that of subterranean clover. The yield issues of the current yellow serradella cultivar suggest it may be unsuitable for cold, wet tableland environments. However, it was only feasible to examine limited genetic material for any legume species in the present experiments. In the case of yellow serradella most work was conducted with cv. Santorini, but on some occasions cv. Avila was also included. The edaphic constraints of serradellas and their potential for wider use in permanent pasture systems essentially remains untested for many farming districts.
- Longer term persistence of the serradellas and disease incidence were out of scope for the current experiments, but casual observations made during the work indicate that they should also be examined if wider use of serradellas is to occur. During the research project, a number of paddocks on the southern Tableland that had been sown to serradellas by farmers were visited. In most cases, no serradella plants could be found. The reasons for this were unclear: sowing rates and technique were unknown,

establishment success was never recorded, and grazing management was unspecified. In most cases the farmer did not realise the species was no longer present.

- There are few late-season cultivars of the serradellas for use in higher rainfall areas where permanent pasture are the dominant land use, and there has not been any specific selection work undertaken in Australia for serradella persistence and performance in such environments (e.g. tablelands, NSW, etc.).

The suitability, production and longer-term persistence of serradella species for use in permanent pasture areas (such as the tablelands of NSW) needs to be established.

There is a pressing need to define the seed production, hardseed breakdown patterns and seedling survival characteristics that are required in a serradella cultivar for adaptation to permanent pastures outside its existing areas of use. The value of undertaking this research can be gauged from the recent dramatic expansion in use of French serradella in ley-farming and in other soil types (e.g. WA, NSW Riverina) that occurred when the cv. Margurita was developed by selecting for higher levels of hardseededness. Ninety years of subterranean clover research and development have defined how edaphic and climatic boundaries limit the use of legume species and how to expand these boundaries by appropriate cultivar development. Use of serradella is in its infancy with cultivar development akin to the situation for subterranean clover in the 1920s-30s when inadequate cultivar options limited its use across southern Australia.

The different species of serradella are known to differ in their tolerances of cold temperature, waterlogging and acid soils. The attributes of the species need to be quantified to inform their wider use and to assist cultivar development, especially for permanent, high-rainfall pasture environments.

17.2.2 P-efficient subterranean clovers

Controlled-environment experiments (Section 10.4.3.1) indicate that there are potentially significant differences among the existing cultivars of subterranean clover for improved production in moderately P-deficient soils. The potential advantages of legumes that can grow more effectively under these circumstances are discussed above (Section 16.1.2). **To capitalise on the early indications that particular subterranean clover cultivars can yield substantially better in moderately P-deficient soils, it will be necessary to test the yielding ability of the "elite" cultivars under low-P field conditions before they are promoted to farmers.**

Breeding for more favourable root morphology is feasible in subterranean clover and will further improve the P-efficiency of clover clutivars. The main root morphology traits to be emphasised are: high specific root lengths (small root diameter with low root tissue density**), root proliferation, and longer root hairs.**

There is reasonable evidence to suggest that the intra-specific boundary for root hair length in subterranean clover may be overcome by inter-specific hybridisation and/or directed mutagenesis. This is a feasible but longer-term objective that builds on the huge Australian investment in subterranean clover improvement over 90 years. The potential pay-off for the grazing industries will be large because subterranean clover is so widely adapted and persistent, and has many easy-care advantages for pasture management.

17.2.3 Can "*companion planting*" deliver the next jump in P-efficiency of pastures?

This project has shown that serradellas have critical STP requirements that are close to that of grasses with which they are commonly grown and it is estimated that fertilising pastures to this target STP concentration could reduce P fertiliser costs by ~30% without reducing pasture yield. The STP concentration strategy for improving P-efficiency is based on knowledge that P-accumulation in soils is positively correlated with the STP concentration at which they are managed. Once the critical STP concentration of a legume has been matched to that of the grasses with which it is grown, further gains by this strategy are impractical because it is fanciful to think that it would be feasible to attempt to lower the critical requirements of grasses as well as the key legume in a pasture mix. The sparingly-available P that accumulates in soil under pastures can potentially be accessed in other ways. At the Yass site in 2014, subterranean clover was interplanted with *Lupinus albus* (white lupin cv. Luxor), a species known to "mine" sparingly-available P by exuding citrate from its roots (Dinkelaker *et al*. 1989). When other crop species have been interplanted with or closely follow white lupin, the crop species can also access P mobilised from the sparingly-available pools in soil by the lupin (Gardner and Boundy 1983; Hocking *et al*. 1997; Horst *et al*. 2001; Cu *et al*. 2005). Despite these reports, we found the impact of interplanting subterranean clover with white lupin to be surprising. The interplant was not beneficial in unfertilised soil and had its greatest benefit for yield in the moderately P-fertile treatments (Chapter 4). The critical STP concentration of the interplanted lupin-clover sward was significantly lower than that of the subterranean clover monoculture and equivalent to the critical STP requirement of serradella. This result needs to be verified with further experimentation but, if repeatable, it demonstrates a potential further step towards even greater P-efficiency in pastures. The modes of action of the interplant (P-mining) and serradella (P-foraging) are independent and may prove to be additive in their effect on P efficiency**. It is recommended that there be further research to confirm and advance the strategy of companion planting to "mine" accumulated P in pasture soils, and to assess the potential additive benefits for lowering P fertiliser costs by companion planting in a serradella-based pasture system.**

17.3 Why strive for P-efficiency in pasture production systems?

(Objective 6: Improved environmental credentials for grazing industries with respect to efficiency of fertiliser use, reduced over-applications, and less loss of P to the wider environment.)

The results of this project demonstrate that substantially more P-efficient pasture systems are within grasp for temperate southern Australia. It is estimated that pastures based on either yellow or French serradella could deliver up to 30% less P fertiliser cost, if they are fertilised to the critical STP requirement of serradella rather than to the STP concentrations recommended for optimum production from subterranean clover or white clover pastures. In areas where serradella is already used, adoption of the technology only requires a minor change in fertiliser practice. The serradellas also deliver better yields in P-deficient soil. It will be beneficial to adopt serradellas for use in pastures that are not fertilised regularly. Serradella-based pastures operated at their lower critical STP requirement should reduce

the risks of P loss from farms to waterways because this risk is linearly related to the concentration of STP of at which a soil is maintained.

Wider use of serradellas in pastures, however, depends on whether the serradella species prove adaptable to farming areas where they are not already used. It is clear that the different serradella species can be quite specific in their soil and climatic requirements. For example, yellow serradella is the preferred species in the Coonabarabran area because of its yield and persistence compared with French serradella and subterranean clover. However, in the present experiments (southern tablelands and Riverina) yellow serradella did not yield as well as French serradella or subterranean clover. There is very little information about the persistence of either species in areas and pasture systems other than where they are already grown. The maturity types available in the current cultivars are limited and the maturity type of the most widely-available cultivars are not a good match for higher rainfall areas.

17.3.1 Why should Australia pursue low-P pasture systems?

Global P reserves: The known P deposits of the world are usually described in two categories. “P-reserves” (deposits that are economic to mine at current prices) and “P-resources” (deposits that are not economic to mine because they are difficult to access or of lower quality for fertiliser manufacturing). P-fertiliser is manufactured from the P-reserves. Despite relatively recent claims (Cordell *et al*. 2009), the world does not face an imminent shortage of P because “peak P” and reserve depletion are not close at hand. However, it is likely that global reserves will only last ~300-400 years are the current rate of use (van Kauwenburg 2010). Forward projections of global demand for P suggest that stable demand is probably a ‘best-case’ scenario; many projections indicate rising global demand for P (Sutton *et al*. 2013). The largest P reserves are controlled by Morocco (85%) followed by China (6%), USA (3%), etc. (van Kauwenburg 2010). Within 30 years it is likely that Morocco (and China) will control the world market. This is a scenario that is not conducive to stable pricing for P (Reijnders 2014). Indeed, global prices have doubled since 2000 with a major price spike (a transient 6-fold increase) in 2007-08. The magnitude of increase in the underlying price of P should not be a surprise. Frantel *et al*. (1985; 1988) predicted a price increase would occur as new mines are developed to accommodate the exhaustion of mines based on existing (easier-to-access) reserves.

Socio-economic issues: P is required for agriculture in many regions of the world but there is a large disparity in the affordability and use of P by farmers. In developed economies, P-fertiliser is an expensive but affordable input for agriculture. However, in developing economies the current cost of P is beyond reach for many farmers (Cordell and White 2011; Reijnders 2014) The fertiliser price spike experienced in 2007-08 was followed by food price increases and violent food riots in 40 countries, including major unrest in Bangladesh, Haiti, India, Mexico and other nations (Childers *et al*. 2011).

The disparity in access to P, the emerging potential for a monopoly in supply, the finite nature of the high-quality global P reserves and the potential for disruption to global food security are among the reasons why the European Union placed phosphate rock on the European Union Critical Raw Materials list in 2014. Materials are placed on this list as a consequence of their perceived high risk for supply security which, in the case of phosphate

rock, was considered to be compounded by non-substitutability and high economic importance. (http://phosphorusplatform.eu/images/download/ScopeNewsletter104.pdf)

Animal production systems are commonly nominated as one of the more inefficient uses of P and it is often said that scarce P resources can be conserved by reducing meat and dairy consumption (e.g. Cordell and White 2013). These analyses are usually conducted at a global level that does not distinguish highly P-efficient animal production systems from less efficient systems, and does not distinguish disparities due to housed animal industries, with their mass P transfer and disposal issues, from rangeland and pasture-based production systems. Animal production is, nonetheless, continually promoted as part of the P-scarcity problem and an industry that can potentially contribute to greater global P efficiency (Childers *et al*. 2011; van den Berg *et al*. 2016).

The P-dependence of Australian agriculture: With very few exceptions, Australian agriculture is based mainly on P-deficient soils and P fertiliser inputs are required to maximise production and profitability, land-use efficiency and water and nitrogen use in the pasture-based grazing industries of southern Australia.

P fertiliser inputs are a significant cost for sheep and beef grazing enterprises in particular, with P estimated to accounts for ~20-25% of annual variable costs; often the largest cost after labour and debt-servicing (e.g. McEachern and Brown 2010). Increases in the cost of P at the rate seen over the last 15 years (i.e. at twice the rate of inflation in Australia) are a significant threat to the terms of trade for Australian farmers. How P prices will move in the future cannot be predicted with any accuracy. Models of global demand for P indicate that P use may stabilise at its current rate of use, but it may also continue to rise as it has done since 1945. Scenarios differ depending on the assumptions that are made about the P status of world soils and the need for additional P inputs to meet rising demand for food (Sutton *et al*. 2013). Either way, it seem highly probable that global P supplies will tighten and/or prices will to continue to rise. This is a particular problem for Australian agriculture because most Australian soils are P deficient and have a moderate to high P-sorption ("P-fixation") capacity.

A major reason why Australia should be developing a more P-efficient agriculture is to future-proof its primary industries against the inevitability of P cost increases and the adverse impact this will have on the terms of trade for Australian farm industries.

P use and the environment: Everywhere that P fertilisers have been used in the world there has been an environmental consequence. Even small losses of P to waterways degrades stream health and, in some cases, leads to eutrophication. There are very widespread and major pollution issues in Europe, China, USA and many other countries. In many cases, this has led to strict regulation of P fertiliser use. Inevitably, farmlands in Australia are a source of diffuse P loss to waterways either as a result of soil erosion, P in runoff and in leaching losses. However, there are also very significant examples of P pollution of waterways especially associated with P application to sandy soils with very low capacity to retain P. Lowering the STP concentrations at which soils are managed on farms will reduce the risks of P loss to the wider environment. It is widely documented that all forms of P loss are positively and linearly related to the STP concentrations of soil (e.g. Melland *et al*. 2008)

Development of low P pasture systems can improve profitability, will mitigate against future price rises and will assist grazing industries to lift their socio-economic and environmental credentials with respect to the efficient use of scarce global resources (P, land, water and N) whilst also protecting against risks of P loss to the wider environment.

18 Key messages

- The most efficient *current* use of P fertiliser for pasture production in southern Australia is achieved by using critical soil test P (STP) benchmark concentrations to guide soil P management and fertiliser practice. The benchmarks are set by the relatively high P requirements of pasture legumes.

- Three STP benchmark groups for a range of alternative pasture legumes are proposed for practical and efficient soil P management:

  Very high P requirement (Olsen P >17; Colwell P[8] >50 mg/kg): *Medicago sativa* (lucerne)

  High P requirement (Olsen P = 15, Colwell P = 35 mg/kg): *Biserrula pelecinus* (biserrula), *Medicago truncatula* (barrel medic), *Trifolium glanduliferum* (gland clover), *Trifolium hirtum* (rose clover), *Trifolium incarnatum* (crimson clover), *Trifolium spumosum* (bladder clover), *Trifolium subterraneum* (subterranean clover)

  Low P requirement (Olsen P = 10; Colwell P = 25 mg/kg): *Ornithopus compressus* (yellow serradella), *Ornithopus sativus* (French serradella), *Lotus corniculatus* (birdsfoot trefoil), *Trifolium purpureum* (purple clover)

- The actual critical STP requirement of lucerne (*M. sativa*) is yet to be determined. As this is an important species for all of the grazing industries and also important as a pasture phase for high nitrogen inputs in crop systems, this should be a high research priority.

- Substantially more P-efficient pasture systems *than currently in use* are within reach for temperate southern Australia.

- Pastures based on either yellow or French serradella can potentially deliver up to 30% less P fertiliser cost for farmers if these pastures are fertilised to the critical STP requirement of serradella, rather than to the higher STP concentrations recommended for subterranean clover or white clover pastures. In P-deficient soils, the serradellas also substantially out-yield subterranean clover provided they are growing in a soil to which they are well adapted.

- The most rapid adoption of low-P pasture systems can be achieved in areas that already use the serradella species, but it may be necessary to initiate district-level demonstrations to show that P fertiliser inputs to serradella pastures can be reduced without loss of production. Otherwise, this notion may be considered a significant “leap of faith” by farmers in these areas.

- Further work is required to assess how widely serradellas can be used as the key legume in mixed, permanent pastures. There is a major gap in knowledge about the adaptation range for these species. It may prove necessary to develop new serradella cultivars better suited to higher rainfall, permanent pasture areas because cultivar development within the serradellas is still in its infancy.

[8] Colwell P benchmarks are rounded to the nearest 5- or 10-unit equivalent and are applicable to soils with PBI = 40-80.

- There is scope to identify among the existing subterranean clover lines, cultivars that can deliver substantially improved yields in moderately P-deficient soil. Informed cultivar choice will, thus, help farmers to get more production from areas of farms that that are unable to, or choose not to fertiliser regularly.

- Significant variation exists within subterranean clover for root traits known to confer P acquisition efficiency (root proliferation, high specific root length, longer root hairs). Targeted breeding for these root traits will: (i) improve the yield of subterranean clover grown on moderately P-deficient soils, and (ii) will move the P efficiency of subterranean clover closer to that of the highly P-efficient serradellas.

  However. there are also longer-term opportunities (e.g. intra-specific gene introgression; directed mutagenesis) to radically alter the root morphology of subterranean clover to deliver cultivars with very long root hairs and, in this way, to achieve the high P-efficiency presently observed in the serradellas.

19 Bibliography

Abbott L, Robson A (1982) Infectivity of vesicular arbuscular mycorrhizal fungi in agricultural soils. Australian Journal of Agricultural Research 33: 1049-1059.
Abbott L, Robson A, Hall I (1983) Introduction of vesicular arbuscular mycorrhizal fungi into agricultural soils. Australian Journal of Agricultural Research 34: 741-749.
Abbott LK, Robson AD (1977) Growth stimulation of subterranean clover with vesicular arbuscular mycorrhizas. Australian Journal of Agricultural Research 28: 639-649.
Abbott LK, Robson AD (1978) Growth of subterranean clover in relation to the formation of endomycorrhizas by introduced and indigenous fungi in a field trial. New Phytologist 81: 575-585.
Agresti A (2002) Categorical data analysis. Wiley, New York.
Ainsworth C (2015) A new breed of edits. Nature 528: S15-S16.
Allen DC, Jeffery RC (1990) Methods for the analysis of phosphorus in Western Australian soils. In Report on Investigation No 37. Chemistry Centre of Western Australia, Perth.
An GH, Kobayashi S, Enoki H, Sonobe K, Muraki M, Karasawa T, Ezawa T (2010) How does arbuscular mycorrhizal colonization vary with host plant genotype? An example based on maize (*Zea mays*) germplasms. Plant and Soil 327: 441-453.
Anderson A, Moye D (1952) Lime and molybdenum in clover development on acid soils. Australian Journal of Agricultural Research 3: 95-110.
Ayres JF, Blumenthal MJ, Lane LA, O'Connor JW (2006) Birdsfoot trefoil (*Lotus corniculatus*) and greater lotus (*Lotus uliginosus*) in perennial pastures in eastern Australia II. Adaptation and applications of lotus-based pasture. Australian Journal of Experimental Agriculture 46: 521-534.
Baligar VC, Fageria NK, He ZL (2001) Nutrient use efficiency in plants. Communications in Soil Science and Plant Analysis 32: 921-950.
Barber SA (1984) Soil nutrient bioavailability. John Wiley & Sons.
Barrow NJ (1969) The accumulation of soil organic matter under pasture and its effect on soil properties. Australian Journal of Experimental Agriculture and Animal Husbandry 9: 437-444.
Barrow NJ (1975) Response to phosphate of two annual pasture species I. Effect of soils ability to adsorb phosphate on comparative phosphate requirement. Australian Journal of Agricultural Research 26: 137-143.
Barrow NJ (1980a) Differences among Some North-American Soils in the Rate of Reaction with Phosphate. Journal of Environmental Quality 9: 644-648.
Barrow NJ (1980b) Differences Amongst a Wide-Ranging Collection of Soils in the Rate of Reaction with Phosphate. Australian Journal of Soil Research 18: 215-224.
Barrow NJ (1983) A mechanistic model for describing the sorption and desorption of phosphate by soil. Journal of Soil Science 34: 733-750.
Barrow NJ (1999) The four laws of soil chemistry: the Leeper lecture 1998. Australian Journal of Soil Research 37: 787-829.
Barrow NJ (2015) Soil phosphate chemistry and the P-sparing effect of previous phosphate applications. Plant and Soil 397: 401-409.
Bates TR, Lynch JP (1996) Stimulation of root hair elongation in Arabidopsis thaliana by low phosphorus availability. Plant Cell Environ 19: 529-538.
Betteridge K (1986) A survey of the phosphorus-content of pastures and the serum inorganic phosphorus-content of dairy cows. New Zealand Veterinary Journal 34: 22-26.
Blair GJ, Chinoim N, Lefroy RDB, Anderson GC, Crocker GJ (1991) A soil sulfur test for pastures and crops. Australian Journal of Soil Research 29: 619-626.
Blair GJ, Cordero S (1978) Phosphorus efficiency of three annual legumes. Plant and Soil 50: 387-398.
Bolland MDA (1985) Responses of serradella and subterranean clover to phosphorus from superphosphate and duchess rock phosphate. Australian Journal of Experimental Agriculture 25: 902-912.
Bolland MDA, Paynter BH (1992) Comparative responses of annual pasture legume species to superphosphate applications in medium and high rainfall areas of Western Australia. Fertilizer Research 31: 21-33.
Bonser AM, Lynch J, Snapp S (1996) Effect of phosphorus deficiency on growth angle of basal roots in Phaseolus vulgaris. New Phytologist 132: 281-288.
Brouwer R (1962) Distribution of dry matter in the plant. Netherlands Journal of Agricultural Science 10: 361-376.

Brown LK, George TS, Dupuy LX, White PJ (2013) A conceptual model of root hair ideotypes for future agricultural environments: what combination of traits should be targeted to cope with limited P availability? Annals of Botany 112: 317-330.
Burkitt LL, Gourley, C.J.P., Sale, P.W.G., Uren, N.C., Hannah, M.C. (2001) Factors affecting the change in extractable phosphorus following the application of phosphatic fertiliser on pasture soils in southern Victoria. Australian Journal of Soil Research 39 759-771.
Caradus JR (1981) Effect of root hair length on white clover growth over a range of soil phosphorus levels. New Zealand Journal of Agricultural Research 24: 353-358.
Caradus JR (1994) Selection for improved adaptation of white clover to low phosphorus and acid soils. Euphytica 77: 243-250.
Caradus JR, Dunn A (2000) Adaptation to low fertility hill country in New Zealand of white clover lines selected for differences in response to phosphorus. New Zealand Journal of Agricultural Research 43: 63-69.
Cardoso JA JJ, Rincón J, Guevara E, van der Hoek R, Jarvis A, Peters M, Miles J, Ayarza M, Cajas S, Rincón A, Mateus H, Quiceno J, Barragán W, Lascano C, Argel P, Mena M, Hertentains L, Rao I (2013) Advances in improving the tolerance to waterlogging in Brachiaria grasses. In 'Revitalising grasslands to sustain our communities'. Proceedings of the 22nd International Grassland Congress pp. 118-121. http://www.igc2013.com
Cawthray GR (2003) An improved reversed-phase liquid chromatographic method for the analysis of low-molecular mass organic acids in plant root exudates. Jounal of Chromatography A 1011: 233-240.
Childers D.L. CJ, Edwards M., Elser J.J. (2011) Phosphorus and food: Solutions from closing the human phosphorus cycle. . BioScience 61: 117-124.
Colwell JD (1963) The estimation of the phosphorus fertiliser requirements of wheat in southern New South Wales by soil analysis. Australian Journal of Experimental Agriculture 3: 190-197.
Colwell JD (1965) An automatic procedure for the determination of phosphorus in sodium hydrogen carbonate extracts of soils. Chemistry and Industry 10: 893-895.
Coombes N (2009) DiGGer design generator under correlation and blocking. R package version 0.2-1.
Corbin E, Brockwell J, Gault R (1977) Nodulation studies on chickpea (*Cicer arietinum*). Australian Journal of Experimental Agriculture 17: 126-134.
Cordell D, Drangert JO, White S (2009) The story of phosphorus: Global food security and food for thought. Global Environmental Change-Human and Policy Dimensions 19: 292-305.
Cordell D, White S (2011) Peak Phosphorus: Clarifying the Key Issues of a Vigorous Debate about Long-Term Phosphorus Security. Sustainability-Basel 3: 2027-2049.
Cordell D, White S (2015) Tracking phosphorus security: indicators of phosphorus vulnerability in the global food system. Food Security 7: 337-350.
Cordell D Whitw S (2013) Sustainable Phosphorus Measures: Strategies and Technologies for Achieving Phosphorus Security. . Agronomy Journal 3: 86-116.
Crush J, Ouyang L, Nichols S (2010) Loss of weight in ryegrass and clover roots preserved in ethanol prior to image analysis for root traits. Acta Physiologiae Plantarum 32: 605-606.
Crush JR, Boulesteix-Coutelier ARL, Ouyang L (2008) Phosphate uptake by white clover (*Trifolium repens* L.) genotypes with contrasting root morphology. New Zealand Journal of Agricultural Research 51: 279-285.
Cu STT, Hutson J, Schuller KA (2005) Mixed culture of wheat (*Triticum aestivum* L.) with white lupin (*Lupinus albus* L.) improves the growth and phosphorus nutrition of the wheat. Plant and Soil 272: 143-151.
Culvenor RA, Davidson IA, Simpson RJ (1989) Regrowth by swards of subterranean clover after defoliation I. Growth, non-structural carbohydrate and nitrogen content. Annals of Botany 64: 545-556.
Culvenor RA, Wood JT, Avery AL, Dempsey W, McDonald SE, Ronnfeldt G, Veness PE (2004) Multi-site evaluation on acid soils of a Phalaris aquatica x P-arundinacea x P-aquatica backcross population bred for acid soil tolerance. Australian Journal of Agricultural Research 55: 681-692.
Daniels Hetrick BA, Thompson Wilson G, Gerschefske Kitt D, Schwab AP (1988) Effects of soil microorganisms on mycorrhizal contribution to growth of big bluestem grass in non-sterile soil. Soil Biol Biochem 20: 501-507.
Datta S, Prescott H, Dolan L (2015) Intensity of a pulse of RSL4 transcription factor synthesis determines Arabidopsis root hair cell size. Nature Plants 1: Article Number: 15138, DOI: 10.1038/NPLANTS.2015.138.

de Ruiter JM (1981) The phosphate response of eight Mediterranean annual and perennial legumes. New Zealand Journal of Agricultural Research 24: 33-36.
Dear BS, Zorin M (1985) Persistence and productivity of *Trifolium ambiguum* M Bieb (Caucasian Clover) in a high-altitude region of southeastern Australia. Australian Journal of Experimental Agriculture 25: 124-132.
Dear BS, Virgona JM (1996) Legumes in low-input perennial pastures of southern Australia: Historical role and future development. New Zealand Journal of Agricultural Research 39: 579-589.
Delhaize E, James RA, Ryan PR (2012) Aluminium tolerance of root hairs underlies genotypic differences in rhizosheath size of wheat (*Triticum aestivum*) grown on acid soil. New Phytologist 195: 609-619.
Delhaize E, Taylor P, Hocking PJ, Simpson RJ, Ryan PR, Richardson AE (2009) Transgenic barley (Hordeum vulgare L.) expressing the wheat aluminium resistance gene (TaALMT1) shows enhanced phosphorus nutrition and grain production when grown on an acid soil. Plant Biotechnology Journal 7: 391-400.
Dinkelaker B, Romheld V, Marschner H (1989) Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin (*Lupinus albus* L). Plant Cell Environ 12: 285-292.
Drew MC (1975) Comparison of the effects of a localised supply of phosphate, nitrate, ammonium and potassium on the growth of the seminal root system, and the shoot, in barley. New Phytologist 75: 479-490.
Dyson CB, Conyers MK (2013) Methodology for online biometric analysis of soil test-crop response datasets. Crop & Pasture Science 64: 435-441.
Eichler B, Caus M, Schnug E, Koppen D (2004) Soil acid and alkaline phosphatase activities in regulation to crop species and fungal treatment. Landbauforschung Volkenrode 54: 1-5.
Ellison NW, Liston A, Steiner JJ, Williams WM, Taylor NL (2006) Molecular phylogenetics of the clover genus (Trifolium - Leguminosae). Molectular Phylogenetic Evolution 39: 688-705.
Evans PS (1977) Comparative root morphology of some pasture grasses and clovers. New Zealand Journal of Agricultural Research 20: 331-335.
Fan L, Dalpe Y, Fang CQ, Dube C, Khanizadeh S (2011) Influence of arbuscular mycorrhizae on biomass and root morphology of selected strawberry cultivars under salt stress. Botany 89: 397-403.
Fan MS, Zhu JM, Richards C, Brown KM, Lynch JP (2003) Physiological roles for aerenchyma in phosphorus-stressed roots. Functional Plant Biology 30: 493-506.
Fita A, Nuez F, Pico B (2011) Diversity in root architecture and response to P deficiency in seedlings of *Cucumis melo* L. Euphytica 181: 323-339.
Fohse D, Claassen N, Jungk A (1991) Phosphorus efficiency of plants II. Significance of root radius, root hairs and cation-anion balance for phosphorus influx in seven plant species. Plant and Soil 132: 261-272.
Frantel RJ, Hurdelbrink RJ, Shields DJ, Johnson RL (1988) Phosphate availability and supply, a minerals availability appraisal. Information Circular 1987, United States Department of the Interior, Bureau of Mines. 70 pp.
Frantel RJ, Peterson GR, Stowasser WF (1985) The worldwide availability of phosphate rock. Natural Resources Forum 9: 5-24.
Freebairn RD (1990) The history of serradella (*Ornithopus* spp) in NSW – a mirace pasture for light soils. NSW Agriculture and Fisheries (Agdex 137). 33 pp.
Gahoonia TS, Care D, Nielsen NE (1997a) Root hairs and phosphorus acquisition of wheat and barley cultivars. Plant and Soil 191: 181-188.
Gahoonia TS, Nielsen NE (1997b) Variation in root hairs of barley cultivars doubled soil phosphorus uptake. Euphytica 98: 177-182.
Gahoonia TS, Nielsen NE (2003) Phosphorus (P) uptake and growth of a root hairless barley mutant (bald root barley, brb) and wild type in low- and high-P soils. Plant Cell and Environment 26: 1759-1766.
Gahoonia TS, Nielsen NE (2004) Barley genotypes with long root hairs sustain high grain yields in low-P field. Plant and Soil 262: 55-62.
Gardner WK, Barber DA, Parbery DG (1983) The acquisition of phosphorus by *Lupinus albus* L. III. The probable mechanism by which phosphorus movement in the soil/root interface is enhanced. Plant and Soil 70: 107-124.
Gardner WK, Boundy KA (1983) The acquisition of phosphorus by *Lupinus albus* L. IV. The effect of interplanting wheat and white lupin on the growth and mineral composition of the two species. Plant and Soil 70: 391-402.

Gavito ME, Curtis PS, Mikkelsen TN, Jakobsen I (2000) Atmospheric CO_2 and mycorrhiza effects on biomass allocation and nutrient uptake of nodulated pea (*Pisum sativum* L.) plants. Journal of Experimental Botany 51: 1931-1938.

Gerke J (2015) The acquisition of phosphate by higher plants: Effect of carboxylate release by the roots. A critical review. Journal of Plant Nutrition and Soil Science 178: 351-364.

Ghamkhar K, Isobe S, Appels R, Nichols P, Snowball R, Ryan M (2009) Initial linkage map of a subterranean clover (*Trifolium subterraneum* L.) F2 population for multiple traits. SABRAO Journal of Breeding and Genetics 41 Special Supplement: 213-220.

Ghamkhar K, Nichols P, Erskine W, Snowball R, Murillo M, Appels R, Ryan M (2015) Hotspots and gaps in the world collection of subterranean clover (*Trifolium subterraneum* L.). The Journal of Agricultural Science 153: 1069-1083.

Ghamkhar K, Revell C, Erskine W (2012) *Biserrula pelecinus* L. – genetic diversity in a promising pasture legume for the future. Crop and Pasture Science 63: 833-839.

Ghamkhar K, Snowball R, Wintle B, Brown A (2008) Strategies for developing a core collection of bladder clover (*Trifolium spumosum* L.) using ecological and agro-morphological data. Crop and Pasture Science 59: 1103-1112.

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytologist 84: 489-500.

Gourley CJP Melland AR, Waller RA, Awty IM, Smith AP, Peverill KI, Hannah MC (2007) Making better fertiliser decisions for grazed pastures in Australia. Victorian Government Department of Primary Industries, Melbourne. http://www.asris.csiro.au/downloads/BFD/Making%20Better%20Fertiliser%20Decisions%20for%20Grazed%20Pastures%20in%20Australia.pdf.

Graham JH, Abbott LK (2000) Wheat responses to aggressive and non-aggressive arbuscular mycorrhizal fungi. Plant and Soil 220: 207-218.

Greenwood EAN, Hallsworth EG (1960) Studies on the nutrition of forage legumes II. Some interactions of calcium, phosphorus, copper and molybdenum on the growth and chemical composition of *Trifolium subterraneum*. Plant and Soil 12: 97-127.

Guo T, Zhang JL, Christie P, Li XL (2006) Influence of nitrogen and sulfur fertilizers and inoculation with arbuscular mycorrhizal fungi on yield and pungency of spring onion. J Plant Nutr 29: 1767-1778.

Hackney B, Rodham C, Piltz J (2013) Using French serradella to increase crop and livestock. Meat and Livestock Australia. http://www.mla.com.au/CustomControls/PaymentGateway/ViewFile.aspx?HXa6IXXUQPvfd2veYIjZC0aMMrgcI4yNPCUoX9RpS3KwVCIPa9RLWs7tTUNdvlyM3EYMKKAfsht7d1Tnt3BqiA.

Haling RE, Richardson AE, Culvenor RA, Lambers H, Simpson RJ (2010) Root morphology, root-hair development and rhizosheath formation on perennial grass seedlings is influenced by soil acidity. Plant and Soil 335: 457-468.

Haling R, Yang Z, Shadwell N, Culvenor R, Stefanski A, Ryan M, Sandral G, Kidd D, Lambers H, Simpson R (2016) Growth and root dry matter allocation by plants with contrasting external critical phosphorus requirements. Plant and Soil 407: 670-679.

Hall EJ, Hughes SJ, Humphries AW, Corkrey R (2013) Habitat and plant diversity of *Trifolium tumens* (Steven ex M. Bieb.) collected in Azerbaijan and its characterisation and field evaluation in Tasmania, Australia. Crop and Pasture Science 64: 374-387.

Helyar KR, Anderson AJ (1970) Responses of five pasture species to phosphorus, lime and nitrogen on an infertile acid soil with a high phosphate sorption capacity. Australian Journal of Agricultural Research 21: 677-692.

Hill JO, Simpson RJ, Moore AD, Chapman DF (2006) Morphology and response of roots of pasture species to phosphorus and nitrogen nutrition. Plant and Soil 286: 7-19.

Hill JO, Simpson RJ, Ryan MH, Chapman DF (2010) Root hair morphology and mycorrhizal colonisation of pasture species in response to phosphorus and nitrogen nutrition. Crop & Pasture Science 61: 122-131.

Hill JO, Simpson RJ, Wood JT, Moore AD, Chapman DF (2005) The phosphorus and nitrogen requirements of temperate pasture species and their influence on grassland botanical composition. Australian Journal of Agricultural Research 56: 1027-1039.

Ho MD, Rosas JC, Brown KM, Lynch JP (2005) Root architectural tradeoffs for water and phosphorus acquisition. Functional Plant Biology 32: 737-748. doi: 10.1071/fp05043.

Hocking PJ, Keerthisinghe G, Smith FW, Randall PJ (1997) Comparison of the ability of different crop species to access poorly-available soil phosphorus. In: T Ando, K Fujita, T Mae, H

Matsumoto, S Mori, J Sekiya (eds) Plant Nutrition for Sustainable Food Production and Environment. Springer, Netherlands.
Hodge A (2006) Plastic plants and patchy soils. Journal of Experimental Botany 57: 401-411.
Horst WJ, Abdou M, Wiesler F (1993) Genotypic differences in phosphorus efficiency of wheat. Plant and Soil 155: 293-296.
Horst WJ, Kamh M, Jibrin JM, Chude VO (2001) Agronomic measures for increasing P availability to crops. Plant and Soil 237: 211-223.
IFDC (2014) International Fertilizer Development Centre. http://ifdc.org/
Indorante SJ, Follmer LR, Hammer RD, P.G. K (1990) Particle-size analysis by a modified pipette procedure. Soil Science Society of America Journal 54: 560-563.
Irving GCJ, Mclaughlin MJ (1990) A rapid and simple field-test for phosphorus in Olsen and Bray No 1 extracts of soil. Communications in Soil Science and Plant Analysis 21: 2245-2255.
Isbell RF (1996) The Australian soil classification. CSIRO Publishing, Melbourne.
Jahufer MZZ, Ford JL, Widdup KH, Harris C, Cousins G, Ayres JF, Lane LA, Hofmann RW, Ballizany WL, Mercer CF, Crush JR, Williams WM, Woodfield DR, Barrett BA (2012) Improving white clover for Australasia. Crop and Pasture Science 63: 739-745.
Jahufer MZZ, Nichols SN, Crush JR, Ouyang L, Dunn A, Ford JL, Care DA, Griffiths AG, Jones CS, Jones CG, Woodfield DR (2008) Genotypic variation for root trait morphology in a white clover mapping population grown in sand. Crop Science 48: 487-494. doi: DOI 10.2135/cropsci2007.03.0161.
Jakobsen I, Abbott LK, Robson AD (1992a) External Hyphae of Vesicular-Arbuscular Mycorrhizal Fungi Associated with Trifolium-Subterraneum L .1. Spread of Hyphae and Phosphorus Inflow into Roots. New Phytologist 120: 371-380.
Jakobsen I, Abbott LK, Robson AD (1992b) External hyphae of vesicular arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. II. hyphal transport of ^{32}P over defined distances. New Phytologist 120: 509-516.
Jakobsen I, Chen BD, Munkvold L, Lundsgaard T, Zhu YG (2005) Contrasting phosphate acquisition of mycorrhizal fungi with that of root hairs using the root hairless barley mutant. Plant Cell and Environment 28: 928-938.
Jarosch KA, Doolette AL, Smernik RJ, Tamburini F, Frossard E, Bunemann EK (2015) Characterisation of soil organic phosphorus in NaOH-EDTA extracts: A comparison of P-31 NMR spectroscopy and enzyme addition assays. Soil Biology and Biochemistry 91: 298-309.
Johnson JF, Allan DL, Vance CP, Weiblen G (1996) Root carbon dioxide fixation by phosphorus-deficient *Lupinus albus* (Contribution to organic acid exudation by proteoid roots). Plant Physiol 112: 19-30.
Jones MB, Lawler PW, Ruckman JE (1970) Differences in annual clover responses to phosphorus and sulfur. Agronomy Journal 62: 439-442.
Katznelson J (1967) Interspecific Hybridization in Trifolium. Crop Science 7: 307-310.
Khorassani R, Hettwer U, Ratzinger A, Steingrobe B, Karlovsky P, Claassen N (2011) Citramalic acid and salicylic acid in sugar beet root exudates solubilize soil phosphorus. BMC Plant Biology 11: 212-129.
Kim MK, Edwards DG, Asher CJ (1985) Tolerance of *Trifolium subterraneum* cultivars to low pH. Australian Journal of Agricultural Research 36: 569-578.
Kirk AP, Entz MH, Fox SL, Tenuta M (2011) Mycorrhizal colonization, P uptake and yield of older and modern wheats under organic management. Canadian Journal of Plant Sciences 91: 663-667.
Kirkegaard JA, Ryan MH (2014) Magnitude and mechanisms of persistent crop sequence effects on wheat. Field Crops Research 164: 154-165.
Kitson RE, Mellon MG (1944) Colorimetric determination of phosphorus as molybdivanadophosphoric acid. Industrial & Engineering Chemistry Analytical Edition 16: 379-383.
Lambers H, Clements JC, Nelson MN (2013) How a phosphorus-acquisition strategy based on carboxylate exudation powers the success and agronomic potential of lupines (Lupinus, Fabaceae). Am J Bot 100: 263-288.
Lambers H, Plaxton WC (2015) Phosphorus: back to the roots. In Annual Plant Reviews Volume 48: Phosphorus Metabolism in Plants. Wiley-Blackwell Publishing, Chicester.
Lean GR, Vizard AL, Ware JKW (1997) Changes in productivity and profitability of wool-growing farms that follow recommendations from agricultural and veterinary studies. Aust Vet J 75: 726-731.

Lehmann A, Barto EK, Powell JR, Rillig MC (2012) Mycorrhizal responsiveness trends in annual crop plants and their wild relatives-a meta-analysis on studies from 1981 to 2010. Plant and Soil 355: 231-250.
Leiser WL, Olatoye MO, Rattunde HFW, Neumann G, Weltzien E, Haussmann BG (2015) No need to breed for enhanced colonization by arbuscular mycorrhizal fungi to improve low-P adaptation of West African sorghums. Plant and Soil: 1-14.
Lewis DC, Clarke AL, Hall WB (1987) Accumulation of Plant Nutrients and Changes in Soil Properties of Sandy Soils under Fertilized Pasture in Southeastern South-Australia .1. Phosphorus. Australian Journal of Soil Research 25: 193-202.
Li HG, Zhang FS, Rengel Z, Shen JB (2013) Rhizosphere properties in monocropping and intercropping systems between faba bean (*Vicia faba* L.) and maize (*Zea mays* L.) grown in a calcareous soil. Crop & Pasture Science 64: 976-984.
Li J, Copeland L (2000) Role of malonate in chickpeas. Phytochemistry 54: 585-589.
Li L, Tilman D, Lambers H, Zhang FS (2014) Plant diversity and overyielding: insights from belowground facilitation of intercropping in agriculture. New Phytologist 203: 63-69.
Liao H, Ge ZY, Yan XL (2001) Ideal root architecture for phosphorus acquisition of plants under water and phosphorus coupled stresses: From simulation to application. Chinese Science Bulletin 46: 1346-1351.
Linderman RG, Davis EA (2004) Varied response of marigold (*Tagetes* spp.) genotypes to inoculation with different arbuscular mycorrhizal fungi. Scientia Horticulturae 99: 67-78.
Lipton DS, Blanchar RW, Blevins DG (1987) Citrate, malate, and succinate concentration in exudates from P-sufficient and P-stressed *Medicago sativa* L. seedlings. Plant Physiology 85: 315-317.
Loi A, Howieson JG, Nutt BJ, Carr SJ (2005) A second generation of annual pasture legumes and their potential for inclusion in Mediterranean-type farming systems. Australian Journal of Experimental Agriculture 45: 289-299.
Loi A, Nutt BJ, Howieson JG, Yates RJ, Norman HC (2012) Preliminary assessment of bladder clover (*Trifolium spumosum* L.) as an annual legume for ley farming systems in southern Australia. Crop and Pasture Science 63: 582-591.
Loi A RC, Nutt B, Howieson J, Dear B. (2007) Yellow serradella. Pastures Australia Factsheet. http://keys.lucidcentral.org/keys/v3/pastures/Html/Factsheet%20%20Yellow%20serradella.pdf
Lolicato S, Rumball W (1994) Past and present improvement of cocksfoot (*Dactylis glomerat*a L.) in Australia and New Zealand. New Zealand Journal of Agricultural Research 37: 379-390.
Lynch JP (2007) Roots of the second green revolution. Australian Journal of Botany 55: 493-512.
Lynch JP (2011) Root phenes for enhanced soil exploration and phosphorus acquisition: tools for future crops. Plant Physiol 156: 1041-1049.
Lynch JP, Brown KM (2001) Topsoil foraging - an architectural adaptation of plants to low phosphorus availability. Plant and Soil 237: 225-237.
Lynch JP, Ho MD (2004) Rhizoeconomics: Carbon costs of phosphorus acquisition. Plant and Soil 269: 45-56.
Ma Z, Walk TC, Marcus A, Lynch JP (2001) Morphological synergism in root hair length, density, initiation and geometry for phosphorus acquisition in *Arabidopsis thaliana*: A modeling approach. Plant and Soil 236: 221-235.
Mackay AD, Caradus JR, Wewala S (1991) Aluminium tolerance of forage species. In: W R.J, B V.C, M R.P (eds) Plant-soil interactions at low pH. Springer, Dordrecht.
Manske GGB, Ortiz-Monasterio JI, Van Ginkel M, Gonzalez RM, Rajaram S, Molina E, Vlek PLG (2000) Traits associated with improved P-uptake efficiency in CIMMYT's semidwarf spring bread wheat grown on an acid Andisol in Mexico. Plant and Soil 221: 189-204.
Mao L, Liu YJ, Shi GX, Jiang SJ, Cheng G, Li XM, An LZ, Feng HY (2014) Wheat cultivars form distinctive communities of root-associated arbuscular mycorrhiza in a conventional agroecosystem. Plant and Soil 374: 949-961.
McCaskill MR, Cayley JWD (2000) Soil audit of a long-term phosphate experiment in South-Western Victoria: total phosphorus, sulfur, nitrogen and major cations. Australian Journal of Agricultural Research 51: 737-748.
McEachern S FJ, Brown D (2010) AgInsights 2009 (Holmes Sackett Pty Ltd, Wagga Wagga) 118 pp.
McKell CM, Williams WA, Wilson AW (1962) Effect of temperature on phosphorus utilization by native and introduced legumes. Agronomy Journal 54: 109-113.
McLaren TI, Simpson RJ, McLaughlin MJ, Smernik RJ, McBeath TM, Guppy CN, Richardson AE (2015a) An assessment of various measures of soil phosphorus and the net accumulation of phosphorus in fertilized soils under pasture. Journal of Plant Nutrition and Soil Science 178: 543-554.

McLaren TI, Smernik RJ, McLaughlin MJ, McBeath TM, Kirby JK, Simpson RJ, Guppy CN, Doolette AL, Richardson AE (2015b) Complex Forms of Soil Organic Phosphorus-A Major Component of Soil Phosphorus. Environmental Science and Technology 49: 13238-13245.

McLaughlin M, McBeath T, Smernik R, Stacey S, Ajiboye B, Guppy C (2011) The chemical nature of P accumulation in agricultural soils - implications for fertiliser management and design: an Australian perspective. Plant and Soil 349: 69-87.

McLaughlin MJ, Fillery IR, Till AR (1992) Operation of the phosphorus, sulphur and nitrogen cycles. In: Australia's renewable resources: sustainability and global change. Bureau of Rural Resources Proceedings 14: 67 – 116

Melland AR, Mc Caskill MR, White RE, Chapman DF (2008) Loss of phosphorus and nitrogen in runoff and subsurface drainage from high and low input pastures grazed by sheep in southern Australia. Australian Journal of Soil Research 46: 161-172.

Mendoza R (2001) Phosphorus nutrition and mycorrhizal growth response of broadleaf and narrowleaf birdsfoot trefoils. Journal of Plant Nutrition 24: 203-214.

Mengel K, Kirby EA, Kosegarten H, Appel T (2001) Principles of plant nutrition. Springer, Netherlands.

Moody PW (2007) Interpretation of a single-point P buffering index for adjusting critical levels of the Colwell soil P test. Australian Journal of Soil Research 45: 55-62.

Mortvedt JJ (1981) Nitrogen and molybdenum uptake and dry matter relationships of soybeans and forage legumes in response to applied molybdenum on acid soil. Journal of Plant Nutrition 3: 245-256.

Motomizu S, Wakimoto T, Toei K (1983) Spectrophotometric determination of phosphate in river waters with molybdate and malachite green. Analyst 108: 361-367.

Nature ((17 December 2015)) Crop conundrum. pp. 307-308.

Navascues J, Perez-Rontome C, Sanchez DH, Staudinger C, Wienkoop S, Rellan-Alvarez R, Becana M (2012) Oxidative stress is a consequence, not a cause, of aluminum toxicity in the forage legume Lotus corniculatus. New Phytologist 193: 625-636.

Nazeri N, Lambers H, Tibbett M, Ryan M (2013) Do arbuscular mycorrhizas or heterotrophic soil microbes contribute toward plant acquisition of a pulse of mineral phosphate? Plant and Soil 373: 699-710.

Nazeri NK, Lambers H, Tibbett M, Ryan MH (2014) Moderating mycorrhizas: arbuscular mycorrhizas modify rhizosphere chemistry and maintain plant phosphorus status within narrow boundaries. Plant, Cell & Environment 37: 911-921.

Neumann G, Massonneau A, Langlade N, Dinkelaker B, Hengeler C, Romheld V, Martinoia E (2000) Physiological aspects of cluster root function and development in phosphorus-deficient white lupin (*Lupinus albus* L.). Annals of Botany 85: 909-919.

Nichols PGH, Collins WJ, Barbetti MJ (1996) Registered cultivars of subterranean clover: their characteristics, origin and identification, Agriculture Western Australia Bulletin No. 4327. Perth, Western Australia.

Nichols PGH, Foster KJ, Piano E, Pecetti L, Kaur P, Ghamkhar K, Collins WJ (2013) Genetic improvement of subterranean clover (*Trifolium subterraneum* L.) I. Germplasm, traits and future prospects. Crop and Pasture Science 64: 312-346.

Nichols PGH, Loi A, Nutt BJ, Evans PM, Craig AD, Pengelly BC, Dear BS, Lloyd DL, Revell CK, Nair RM, Ewing MA, Howieson JG, Auricht GA, Howie JH, Sandral GA, Carr SJ, de Koning CT, Hackney BF, Crocker GJ, Snowball R, Hughes EJ, Hall EJ, Foster KJ, Skinner PW, Barbetti MJ, You MP (2007) New annual and short-lived perennial pasture legumes for Australian agriculture - 15 years of revolution. Field Crops Research 104: 10-23.

Nichols PGH, Revell CK, Humphries AW, Howie JH, Hall EJ, Sandral GA, Ghamkhar K, Harris CA (2012) Temperate pasture legumes in Australia-their history, current use, and future prospects. Crop and Pasture Science 63: 691-725.

Nichols SN (2012) Introgression of root and shoot characteristics in *Trifolium repens* x *Trifolium uniflorum* interspecific hybrids. PhD thesis, 323 pp. Lincoln University, New Zealand.

Nichols SN, Crush JR, Ouyang L (2014b) Phosphate responses of some *Trifolium repens* x *T.uniflorum* interspecific hybrids grown in soil. Crop and Pasture Science 65: 382-387.

Nichols SN, Hofmann RW, Williams W.M., Crush J.R (2014a) Nutrient responses and macronutrient composition of some *Trifolium repens* x *Trifolium uniflorum* interspecific hybrids. Crop and Pasture Science 65: 370-381.

Nielsen KL, Eshel A, Lynch JP (2001) The effect of phosphorus availability on the carbon economy of contrasting common bean (*Phaseolus vulgaris* L.) genotypes. Journal of Experimental Botany 52: 329-339.

O'Rourke TA, Ryan MH, Scanlon TT, Sivasithamparam K, Barbetti MJ (2012) Amelioration of root disease of subterranean clover (Trifolium subterraneum) by mineral nutrients. Crop and Pasture Science 63: 672-682.

O'Rourke TA, Scanlon TT, Ryan MH, Wade LJ, McKay AC, Riley IT, Li H, Sivasithamparam K, Barbetti MJ (2009) Severity of root rot in mature subterranean clover and associated fungal pathogens in the wheatbelt of Western Australia. Crop and Pasture Science 60: 43-50.

Oburger E, Dell'mour M, Hann S, Wieshammer G, Puschenreiter M, Wenzel WW (2013) Evaluation of a novel tool for sampling root exudates from soil-grown plants compared to conventional techniques. Environmental and Experimental Botany 87: 235-247.

Oburger E, Kirk GJD, Wenzel WW, Puschenreiter M, Jones DL (2009) Interactive effects of organic acids in the rhizosphere. Soil Biology and Biochemistry 41: 449-457.

Olsen SR, Cole C, Watanabe CV, Dean LA (1954) Estimation of available phosphorus in soils by extraction with sodium bicarbonate. USDA Circular No. 939.

Otani T, Ae N, Tanaka H (1996) Phosphorus (P) uptake mechanisms of crops grown in soils with low P status II. Significance of organic acids in root exudates of pigeonpea. Soil Science and Plant Nutrition 42: 553-560.

Ozanne PG, (1980) Phosphate nutrition of plants - a general treatise. In 'The Role of Phosphorus in Agriculture'. (Eds FE Khasawneh, EC Sample, EJ Kamprath) pp. 559-616. (American Society of Agronomy, Crop Science Society of America, Soil Science Society of America: Madison, WI, USA).

Ozanne PG, Howes KMW, Petch A (1976) Comparative phosphate requirements of four annual pastures and two crops. Australian Journal of Agricultural Research 27: 479-488.

Ozanne PG, Keay J, Biddiscombe EF (1969) Comparative applied phosphate requirements of eight annual pasture species. Australian Journal of Agricultural Research 20: 809-818.

Ozanne PG, Kirkton DJ, Shaw TC (1961) The loss of phosphorus from sandy soils. Australian Journal of Agricultural Research 12: 409-423.

Pang J, Ryan MH, Tibbett M, Cawthray GR, Siddique KHM, Bolland MDA, Denton MD, Lambers H (2010a) Variation in morphological and physiological parameters in herbaceous perennial legumes in response to phosphorus supply. Plant and Soil 331: 241-255.

Pang J, Tibbett M, Denton MD, Lambers H, Siddique KHM, Bolland MDA, Revell CK, Ryan MH (2010b) Variation in seedling growth of 11 perennial legumes in response to phosphorus supply. Plant and Soil 328: 133-143.

Pang JY, Yang JY, Lambers H, Tibbett M, Siddique KHM, Ryan MH (2015) Physiological and morphological adaptations of herbaceous perennial legumes allow differential access to sources of varyingly soluble phosphate. Physiologia Plantarum 154: 511-525.

Paynter BH (1990) Comparative phosphate requirements of yellow serradella (*Ornithopus compressus*), burr medic (*Medicago polymorpha Var brevispina*) and subterranean clover (*Trifolium subterraneum*). Australian Journal of Experimental Agriculture 30: 507-514.

Paynter BH (1992) Comparison of the phosphate requirements of burr medic and yellow serradella with subterranean clover in the low rainfall wheat belt of Western Australia. Australian Journal of Experimental Agriculture 32: 1077-1086.

Pearse SJ, Veneklaas EJ, Cawthray GR, Bolland MDA, Lambers H (2006) Carboxylate release of wheat, canola and 11 grain legume species as affected by phosphorus status. Plant and Soil 288: 127-139.

Pinkerton A, Randall PJ (1994) Internal phosphorus requirements of six legumes and two grasses. Australian Journal of Experimental Agriculture 34: 373-379.

Pinkerton A, Smith F, Lewis D (1997) Pasture species. In: DJ Reuter, JB Robinson (eds) Plant Analysis - an Interpretation Manual. Inkata Press, Melbourne.

Pires N. DL (2011) Origin and Diversification of Basic-Helix-Loop-Helix Proteins in Plants. Molecular Biology and Evolution 27: 862-874.

Poorter H, Buhler J, van Dusschoten D, Climent J, Postma JA (2012) Pot size matters: a meta-analysis of the effects of rooting volume on plant growth. Functional Plant Biology 39: 839-850.

Qiu LJ, Chen PY, Liu ZX, Li YH, Guan RX, Wang LH, Chang RZ (2011) The worldwide utilization of the Chinese soybean germplasm collection. Plant Genet Resour-Charact Util 9: 109-122.

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rasband WS (1997-2014) ImageJ. U. S. National Institutes of Health, Bethesda, Maryland, USA.

Rayment GE, Higginson FR (1992) Australian Laboratory Handbook of Soil and Water Chemical Methods. Inkata Press, Melbourne.

Rayment GE, Lyons D (2011) Soil Chemical Methods - Australasia. CSIRO Publishing Melbourne, Australia.
Reijnders L (2014) Phosphorus resources, their depletion and conservation, a review. Resource Conservationand Recycling 93: 32-49.
Reuter DJ, Dyson CB, Elliott DE, Lewis DC, Rudd CL (1995) An appraisal of soil phosphorus testing data for crops and pastures in South Australia. Australian Journal of Experimental Agriculture 35: 979-995.
Rice JA (2007) Mathematical statistics and data analysis. Duxbury Press., Belmont, USA.
Richardson AE, Lynch JP, Ryan PR, Delhaize E, Smith FA, Smith SE, Harvey PR, Ryan MH, Veneklaas EJ, Lambers H, Oberson A, Culvenor RA, Simpson RJ (2011) Plant and microbial strategies to improve the phosphorus efficiency of agriculture. Plant and Soil 349: 121-156.
Roelofs RFR, Rengel Z, Cawthray GR, Dixon KW, Lambers H (2001) Exudation of carboxylates in Australian Proteaceae: chemical composition. Plant Cell and Environment 24: 891-903.
Rose TJ, Mori A, Julia CC, Wissuwa M (2016) Screening for internal phosphorus utilisation efficiency: comparison of genotypes at equal shoot P content is critical. Plant and Soil 401: 79–91.
Rossiter RC (1952) Phosphorus toxicity in subterranean clover and oats grown on Muchea sand, and the modifying effects of lime and nitrate nitrogen. Australian Journal of Agricultural Research 3: 227-243.
Russell JS (1960) Soil fertility changes in the long-term experimental plots at Kybybolite, South Australia II. Changes in phosphorus. Australian Journal of Agricultural Research 11: 902-947.
Ryan MH, Kirkegaard JA (2012) The agronomic relevance of arbuscular mycorrhizas in the fertility of Australian extensive cropping systems. Agriculture, Ecosystems and Environment 163: 37-53.
Ryan MH, McCully ME, Huang CX (2007) Relative amounts of soluble and insoluble forms of phosphorus and other elements in intraradical hyphae and arbuscules of arbuscular mycorrhizas. Functional Plant Biology 34: 457-464.
Ryan MH, Tibbett M, Edmonds-Tibbett T, Suriyagoda LDB, Lambers H, Cawthray GR, Pang J (2012) Carbon trading for phosphorus gain: the balance between rhizosphere carboxylates and arbuscular mycorrhizal symbiosis in plant phosphorus acquisition. Plant, Cell and Environment 35: 2170-2180.
Ryan MH, van Herwaarden AF, Angus JF, Kirkegaard JA (2005) Reduced growth of autumn-sown wheat in a low P soil is associated with high colonisation by arbuscular mycorrhizal fungi. Plant and Soil 270: 275-286.
Ryan PR, James RA, Weligama C, Delhaize E, Rattey A, Lewis DC, Bovill WD, McDonald G, Rathjen TM, Wang E, Fettell NA, Richardson AE (2014) Can citrate efflux from roots improve phosphorus uptake by plants? Testing the hypothesis with near-isogenic lines of wheat. Physiologia Plantarum 151: 230-242.
Ryser P, Eek L (2000) Consequences of phenotypic plasticity vs. interspecific differences in leaf and root traits for acquisition of aboveground and belowground resources. Am J Bot 87: 402-411.
Sainz MJ, Arines J (1988) Effect of indegenous and introduced vesicular-arbuscular mycorrhizal fungi on growth and phosphorus uptake of *Trifoilum-pratense* and on inorganic phosphorus fractions in a cambisol Biology and Fertility of Soils 6: 55-60.
Scholz RW, Ulrich AE, Eilittä M, Roy A (2013) Sustainable use of phosphorus: A finite resource. Science of the Total Environment 461–462: 799-803.
Schweiger PF, Robson AD, Barrow NJ (1995) Root hair length determines beneficial effect of a *Glomus* species on shoot growth of some pasture species. New Phytologist 131: 247-254.
Searle PL (1984) The berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. Analyst 109: 549-568.
Sheng M, Tang M, Chen H, Yang BW, Zhang FF, Huang YH (2009) Influence of arbuscular mycorrhizae on the root system of maize plants under salt stress. Can J Microbiol 55: 879-886.
Simpson R GP, Davies L, Zurcher E (2009) Five easy steps to ensure you are making money from superphosphate, CSIRO & Industry and Investment NSW, Australia, 39 pp. Farm advisory booklet and computer decision-support tool, (ISBN 978-0-643-10084-8). http://www.mla.com.au
Simpson R, Stefanski A, Marshall D, Moore A, Richardson A (2015) Management of soil phosphorus fertility determines the phosphorus budget of a temperate grazing system and is the key to improving phosphorus-balance efficiency. Agriculture Ecosystems & Environment 212: 263–277.
Simpson RJ, Oberson A, Culvenor RA, Ryan MH, Veneklaas EJ, Lambers H, Lynch JP, Ryan PR, Delhaize E, Smith FA, Smith SE, Harvey PR, Richardson AE (2011a) Strategies and

agronomic interventions to improve the phosphorus-use efficiency of farming systems. Plant and Soil 349: 89–120.
Simpson RJ, Richardson AE, Nichols SN, Crush JR (2014) Pasture plants and soil fertility management to improve the efficiency of phosphorus fertiliser use in temperate grassland systems. Crop and Pasture Science 65: 556-575.
Simpson RJ, Richardson AE, Riley IT, McKay AC, McKay SF, Ballard RA, Ophel-Keller K, Hartley D, O'Rourke TA, Li H, Sivasithamparam K, Ryan MH, Barbetti MJ (2011b) Damage to roots of *Trifolium subterraneum* L. (subterranean clover), failure of seedlings to establish and the presence of root pathogens during autumn-winter. Grass and Forage Science 66: 585-605.
Smith FA, Grace EJ, Smith SE (2009) More than a carbon economy: nutrient trade and ecological sustainability in facultative arbuscular mycorrhizal symbioses. New Phytologist 182: 347-358.
Smith FA, Smith SE (2011) What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? Plant and Soil 348: 63-79.
Sun XG, Tang M (2013) Effect of arbuscular mycorrhizal fungi inoculation on root traits and root volatile organic compound emissions of *Sorghum bicolor*. South African Journal of Botany 88: 373-379.
Suriyagoda LB, Lambers H, Renton M, Ryan M (2012) Growth, carboxylate exudates and nutrient dynamics in three herbaceous perennial plant species under low, moderate and high phosphorus supply. Plant and Soil 358: 105-117.
Sutton M.A. BA, Howard C.M., Bekunda M., Grizzetti B., de Vries W., van Grinsven H.J.M., Abrol Y.P., Adhya T.K., Billen G., Davidson E.A., Datta A., Diaz R., Erisman J.W., Liu X.J., Oenema O., Palm C., Raghuram N., Reis S., Scholz R.W., Sims T., Westhoek H., Zhang F.S., (2013) Our Nutrient World: the challenge to produce more food and energy with less pollution. Global Overview of Nutrient Management. Centre for Ecology and Hydrology, Edinburgh on behalf of the Global Partnership on Nutrient Management and the International, Edinburgh
Syers JK, Johnston AE, Curtin D (2008) Efficiency of soil and fertilizer phosphorus use: Reconciling changing concepts of soil phosphorus behaviour with agronomic information. FAO, Rome.
Tang C, Fang RY, Raphael C (1998) Factors affecting soil acidification under legumes II. Effect of phosphorus supply. Australian Journal of Agricultural Research 49: 657-664.
Tawaraya K, Tokairin K, Wagatsuma T (2001) Dependence of *Allium fistulosum* cultivars on the arbuscular mycorrhizal fungus, *Glomus fasciculatum*. Applied Soil Ecology 17: 119-124.
Thippayarugs S, Bansal M, Abbott LK (1999) Morphology and infectivity of fine endophyte in a mediterranean environment. Mycological Research 103: 1369-1379.
Thomas HM, Morgan WG, Humphreys MW (2003) Designing grasses with a future - combining the attributes of Lolium and Festuca. Euphytica 133: 19-26.
Thompson JP, Wildermuth GB (1989) Colonisation of crop and pasture species with vesicular-arbuscular mycorrhizal fungi and infection by *Bipolaris sorokiniana*. Canadian Journal of Botany 67: 687-693.
Tibbett M, Ryan MH, Barker SJ, Chen YL, Denton MD, Edmonds-Tibbett T, Walker C (2008) The diversity of arbuscular mycorrhizas of selected Australian Fabaceae. Plant Biosystems 142: 420-427.
Tinker PB (1975) Soil chemistry of phosphorus and mycorrhizal effects on plant growth. In: S F.E, M B, T P.B (eds) Endomycorrhizas. Academic Press, New York.
Turner BL, Cade-Menun BJ, Condron LM, Newman S (2005) Extraction of soil organic phosphorus. Talanta 66: 294-306.
Uloth MB, You MP, Cawthray G, Barbetti MJ (2015) Temperature adaptation in isolates of *Sclerotinia sclerotiorum* affects their ability to infect *Brassica carinata*. Plant Pathology 64: 1140-1148.
Upadhyaya HD, Ortiz R (2001) A mini core subset for capturing diversity and promoting utilization of chickpea genetic resources in crop improvement. Theoretical and Applied Genetics 102: 1292-1298.
van den Berg M NK, Vuuren DP, Bouwman AF, Kram T, Bakkes J (2016) Exploring resource efficiency for energy, land and phosphorus use: Implications for resource scarcity and the global environment. . Global Environmental Change 36: 21-34.
Van Kauwenbergh SJ (2010) 'World phosphate rock reserves and resources'. (International Fertilizer Development Centre, Muscle Shoals, Alabama, USA).
Veneklaas EJ LH, Bragg J, Finnegan PM, Lovelock CE, Plaxton WC, Price CA, , Scheible W SM, White PJ, Raven JA (2012) Opportunities for improving phosphorus-use efficiency in crop plants. . New Phytologist 195: 306-320.

Veneklaas EJ, Stevens J, Cawthray GR, Turner S, Grigg AM, Lambers H (2003) Chickpea and white lupin rhizosphere carboxylates vary with soil properties and enhance phosphorus uptake. Plant and Soil 248: 187-197.

Vierheilig H, Coughlan AP, Wyss U, Piché Y (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Applied Environmental Microbiology 64: 5004–5007.

Virgona JM, Dear BS (1996) Comparative performance of caucasian clover (*Trifolium ambiguum* cv Monaro) after 11 years under low-input conditions in south-eastern Australia. New Zealand Journal of Agricultural Research 39: 245-253.

Vu DT, Armstrong RD, Newton PJ, Tang C (2011) Long-term changes in phosphorus fractions in growers' paddocks in the northern Victorian grain belt. Nutrient Cycling in Agroecosystems 89: 351-362.

Wahl S, Ryser P, Edwards PJ (2001) Phenotypic plasticity of grass root anatomy in response to light intensity and nutrient supply. Annals of Botany 88: 1071-1078.

Walkley A, Black IA (1934) An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Science 37: 29-38.

Weaver DM, Reed AEG (1998) Patterns of nutrient status and fertiliser practice on soils of the south coast of Western Australia. Agriculture, Ecosystems and Environment 67: 37-53.

Weaver DM, Wong MTF (2011) Scope to improve phosphorus (P) management and balance efficiency of crop and pasture soils with contrasting P status and buffering indices. Plant and Soil 349: 37-54.

Weir RG, Cresswell GC (1994) Plant Nutrient Disorders 4: Pastures and Field Crops. Inkata, Melbourne.

Williams PH, Haynes R (1992) Balance sheet of phosphorus, sulphur and potassium in a long-term grazed pasture supplied with superphosphate. Fertilizer Research 31: 51-60.

Williams W.M. VIM, Ansari H.A., Ellison N.W., Hussain S.W., Widdup K.H., Nichols S.N., Williamson M.L., Naeem M., Ullah I. (2010) Splitting the clover genome: radical new clovers for agriculture. Proceedings of the New Zealand Grassland Association.

Williams W.M. VIM, Hussain S.W., Ansari H.A., Widdup K.H., Ellison N.W., Nichols S.N. (2013) Widening the adaptation of white clover by incorporation of valuable new traits from wild clover species. In 'Revitalising grasslands to sustain our communities' Proceedings of the 22nd International Grassland Congress pp 287-289 http://wwwigc2013com .

Wong DH, Barbetti MJ, Sivasithamparam K (1984) Effects of soil temperature and moisture on the pathogenicity of fungi associated with root-rot of subterranean clover. Australian Journal of Agricultural Research 35: 675-684.

Yang Z, Culvenor R, Haling R, Stefanski A, Ryan M, Sandral G, Kidd D, Lambers H, Simpson R (2015) Variation in root traits associated with nutrient foraging amongst temperate pasture legumes and grasses. Grass and Forage Science *In Press*. doi: 10.1111/gfs.12199.

Yi K, Menand B, Bell E, Dolan L (2010) A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. Nature Genetics 42: 264-U108.

Yucel C, Ozkan H, Ortas I, Yagbasanlar T (2009) Screening of wild emmer wheat accessions (*Triticum turgidum* subsp. *dicoccoides*) for mycorrhizal dependency. Turkish Journal of Agriculture and Forestry 33: 513-523.

Zhang CC, Postma JA, York LM, Lynch JP (2014) Root foraging elicits niche complementarity-dependent yield advantage in the ancient 'three sisters' (maize/bean/squash) polyculture. Annals of Botany 114: 1719-1733.

Zimmer W, Mendel R (1999) Molybdenum metabolism in plants. Plant Biology 1: 160-168.