Root morphological traits that determine phosphorus-acquisition efficiency and critical external phosphorus requirement in pasture species

Rebecca E. Haling^{A,D}, Zongjian Yang^A, Natalie Shadwell^A, Richard A. Culvenor^A, Adam Stefanski^A, Megan H. Ryan^B, Graeme A. Sandral^C, Daniel R. Kidd^B, Hans Lambers^B and Richard J. Simpson^A

^ACSIRO Agriculture, GPO Box 1600, Canberra, ACT 2601, Australia.

^BSchool of Plant Biology and Institute of Agriculture, The University of Western Australia, Crawley, WA 6009, Australia.

^CDepartment of Primary Industries, Wagga Wagga Agricultural Institute, Pine Gully Road, Wagga Wagga, NSW 2650, Australia.

^DCorresponding author. Email: rebecca.haling@csiro.au

Annual pasture legume species can vary more than 3-fold in their critical external phosphorus (P) requirement (i.e. P required for 90% of maximum yield). In this work we investigated the link between root morphology, P acquisition and critical external P requirement among pasture species. The root morphology acclimation of five annual pasture legumes and one grass species to low soil P availability was assessed in a controlled-environment study. The critical external P requirement of the species was low (*Dactylis glomerata* L., *Ornithopus compressus* L., *Ornithopus sativus* Brot.), intermediate (*Biserrula pelecinus* L., *Trifolium hirtum* All.) or high (*Trifolium subterraneum* L.). Root hair cylinder volumes (a function of root length, root hair length and average root diameter) were estimated in order to assess soil exploration and its impact on P uptake. Most species increased soil exploration in response to rates of P supply near or below their critical external P requirement. The legumes differed in how they achieved their maximum root hair cylinder volume. The main variables were high root length density, long root hairs and/or high specific root length. However, total P uptake per unit surface area of the root hair cylinder was similar for all species at rates of P supply below critical P. Species that maximised soil exploration by root morphology acclimation were able to prolong access to P in moderately P-deficient soil. However, among the species studied, it was those with an intrinsic capacity for a high root-hair-cylinder surface area (i.e. long roots and long root hairs) that achieved the lowest critical P requirement.

Additional keywords: cocksfoot, orchard grass, phosphorus deficiency, phosphorus-use efficiency, rose clover, serradella, subterranean clover.

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 because the accumulation of sparingly-available P in fertilised soil and the risk of P loss to the wider environment is reduced (Simpson et al. 2014, 2015). In a previous study, we demonstrated that annual pasture legume species can vary more than 3-fold in their critical external P requirement (Haling et al. 2016). *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* L. and *Ornithopus sativus* Brot., 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 *Trifolium hirtum* All. 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 (Haling et al. 2016).

The legume species used by Halting et al. (2016) were initially selected based on their potentially superior root traits for nutrient foraging relative to *T. subterraneum*. Yang et al. (2015) 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. In Halting et al. (2016), 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) (Halting et al. 2016). 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 (Halting et al. 2016). 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 (Halting et al. 2016). 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).

Halting et al. (2016) surmised that differences in P uptake per unit root dry matter among the pasture species they examined were likely to be a consequence of differences in root morphology and acclimation of root morphology to low P supply. Here we tested this hypothesis and demonstrate that the root morphology of some legume species allows them to achieve external critical P requirements that are low enough to underpin development of more P-efficient grazing systems.

Materials and methods

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 (Halting et al. 2016). 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; *Ornithopus sativus* Brot. (French serradella or pink serradella) cv. Margurita; *Biserrula pelecinus* L. (biserrula) cv. Mauro; and *Trifolium hirtum* All. (rose clover) cv. Hykon.

Plant growth conditions and experimental design

The experiment was conducted as described by Halting et al. (2016). The soil, a sandy loam (Yellow Chromosol; Isbell 1996) had a low concentration of extractable P (8.3 mg kg⁻¹ P; Colwell 1963) (method 9b; Rayment and Lyons 2011) and a phosphorus buffering index of 52 (0–15 cm) (Burkitt et al. 2002, 2008). It was pasteurised to reduce levels of disease inoculum, sieved to <5 mm and mixed with lime (1.06 g CaCO₃ kg⁻¹) to raise the pH (1 : 5 w/v, 0.01 M CaCl₂) to 5.5. All essential nutrients other than P were then mixed into the soil to ensure that only P and, for the legumes, N were in low supply. Six P-fertilised soil treatments were established by mixing KH₂PO₄ with subsamples of the amended soil at rates of 0, 15, 30, 70, 135 and 250 mg P kg⁻¹ soil. The Colwell-extractable P concentrations of the soil after all soil amendment treatments had been applied were: 8.3, 19.2, 30.9,

58.4, 111 and 203 mg kg⁻¹ respectively (Colwell 1963). The CaCl₂-extractable P concentrations of the soil after all soil amendment treatments had been applied were: 0.08, 0.14, 0.29, 0.92, 2.83 and 8.80 mg kg⁻¹ respectively (method 9F2; Rayment and Lyons 2011). Pots (cylindrical PVC; 87 mm internal diameter, 190 mm soil height) 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). 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 (Haling et al. 2016). An additional 21 mg N pot⁻¹ (as NH₄NO₃) was applied to the grass, 32 days after sowing. This was intended to mimic field conditions in which grasses use available soil N and legumes rely predominantly on fixation of atmospheric N₂.

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 μ mol 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 75–80% of field capacity by daily watering and fully wet up once per week.

Harvest and measurements

Plants were harvested 6 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 photographed using a Leica MZFLIII Fluorescence microscope (Leica Microsystems, Sydney, NSW) fitted with a Zeiss AxioCam camera (Zeiss, Sydney, NSW). The length of 10 root hairs per replicate was measured using ImageJ (US National Institutes of Health, Bethesda, MA, USA). 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 then total dry mass of roots in each soil layer

was calculated; this included adjusting for loss of mass from the subsamples that were stored in ethanol (Haling et al. 2016).

Shoot and root samples (not stored in ethanol) were milled and a 25–50 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 (Fig. 1):

$$\text{RHCV} = \pi \times ((\text{ARD}/2) + \text{RHL})^2 \times \text{RL}, (1)$$

where ARD is the average root diameter, RHL is the average root hair length measured in the topsoil and RL is the 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 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.

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 6 week period used in the original experiment. This was used to assess the validity of some of the assumptions made when demonstrating the link between root morphology, P uptake and critical external P requirement. *T. 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 3, 4, 5 and 6 weeks after sowing. Five replicates were grown per treatment. The experiment 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.

Statistical analyses

As described by Haling et al. (2016), a Mitscherlich curve was fitted using R (R Development Core Team, R Foundation for Statistical Computing: Vienna, Austria.) to the shoot dry matter data in response to application of P. 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 edn (VSN International, Hemel Hempstead, UK). Root length density (root length divided by volume of soil) 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. A 5% level of significance was used.

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 of diffusion; 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.

Results

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. 2). *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. 2). 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. 2a–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.

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. 3a–e) and was 486 m g⁻¹ for the grass (Fig. 3f). 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. 4). 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.

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. 5). 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).

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. 6a). 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.).

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. 6b, 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 Fig. 7a, were mostly similar among species (Table 1). Total plant P uptake was analysed in response to the product of the total surface area of the root hair cylinder and CaCl₂-extractable soil P concentration (for rates of applied P less than or approximately equivalent to the critical P requirement of each species; Fig. 7b). 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 CaCl₂-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.

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. 8). 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.

Discussion

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 (Haling et al. 2016). 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). 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). 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 (Haling et al. 2016).

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 (Haling et al. 2016). 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.

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 (Haling et al. 2016). However, 90% of maximum yield for each species was achieved at an essentially similar amount of total P uptake per sward (Fig. 7a; Table 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:

$$J = K \times dC/dX, (2)$$

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) (dC/dX 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 6b, 7a) 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 and Nielsen 1997, 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. 8). 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 dX (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 dC), P uptake per unit surface area of the root hair cylinder volume would be similar for all of the legume species: i.e. $J = P \text{ uptake} / \text{SARHC} = K \times dC / dX$; where P uptake is total P content of shoots and roots, SARHC is the surface area of the root hair cylinder and K and dC/dX 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. 7b). 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 dC/dX : 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. 6c). However, the species in this study do not accumulate carboxylates per unit root length in the high amounts required by species such as *Lupinus* spp. to access ‘sparingly-available’ forms of P (Kidd et al. 2016). 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 $dC \times \text{SARHC}$: i.e. $P \text{ uptake} = (K/dX) \times (dC \times \text{SARHC})$. In this case, K/dX 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 $\times$ SARHC for each species were linear (Fig. 7b), 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 dX (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. 7b).

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.

Conclusion

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.

Acknowledgements

The authors thank Branka Culvenor for technical assistance and Brent Henderson for statistical advice. The work was supported by Meat and Livestock Australia and Australian Wool Innovation Limited (B.PUE.0104), CSIRO as part of a Summer Research Studentship awarded to N.S. and an ARC Future Fellowship (FT140100103) awarded to M.R.

References

- Barber SA (1984) 'Soil nutrient bioavailability.' (Wiley: New York)
- Blair GJ, Cordero S (1978) Phosphorus efficiency of three annual legumes. *Plant and Soil* 50, 387–398. doi:10.1007/BF02107187
- 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. doi:10.1007/BF01064224
- Brouwer R (1962) Distribution of dry matter in the plant. *Netherlands Journal of Agricultural Science* 10, 361–376.
- Burkitt LL, Moody PW, Gourley CJP, Hannah MC (2002) A simple phosphorus buffering index for Australian soils. *Australian Journal of Soil Research* 40, 497–513. doi:10.1071/SR01050
- Burkitt LL, Sale PWG, Gourley CJP (2008) Soil phosphorus buffering measures should not be adjusted for current phosphorus fertility. *Australian Journal of Soil Research* 46, 676–685. doi:10.1071/SR06126

- 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. doi:10.1071/EA9630190
- Evans PS (1977) Comparative root morphology of some pasture grasses and clovers. *New Zealand Journal of Agricultural Research* 20, 331–335. doi:10.1080/00288233.1977.10427343
- Föhse 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. doi:10.1007/BF00010407
- Gahoonia TS, Nielsen NE (1997) Variation in root hairs of barley cultivars doubled soil phosphorus uptake. *Euphytica* 98, 177–182. doi:10.1023/A:1003113131989
- 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 & Environment* 26, 1759–1766. doi:10.1046/j.1365-3040.2003.01093.x
- 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. doi:10.1002/jpln.201400590
- Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist* 84, 489–500. doi:10.1111/j.1469-8137.1980.tb04556.x
- Haling RE, Yang Z, Shadwell N, Culvenor RA, Stefanski A, Ryan MH, Sandral GA, Kidd DR, Lambers H, Simpson RJ (2016) Growth and root dry matter allocation by plants with contrasting external critical phosphorus requirements. *Plant and Soil* doi:10.1007/s11104-016-2808-2.
- 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. doi:10.1007/s11104-006-0014-3
- 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. doi:10.1080/00103629009368377
- Isbell RF (1996) ‘The Australian soil classification.’ (CSIRO Publishing: Melbourne)
- Kidd DR, Ryan MH, Halting RE, Lambers H, Sandral GA, Yang Z, Culvenor RA, Cawthray GR, Stefanski A, Simpson RJ (2016) Rhizosphere carboxylates and morphological root traits in pasture legumes and grasses. *Plant and Soil* doi:10.1007/s11104-015-2770-4.
- Lambers H, Plaxton WC (2015) Phosphorus: back to the roots. In ‘Annual plant reviews. Vol. 48: Phosphorus metabolism in plants’. pp. 3–22. (Wiley-Blackwell Publishing: Chichester, UK)
- Lynch JP, Brown KM (2001) Topsoil foraging – an architectural adaptation of plants to low phosphorus availability. *Plant and Soil* 237, 225–237. doi:10.1023/A:1013324727040
- 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. doi:10.1023/A:1012728819326
- Ozanne PG, Keay J, Biddiscombe EF (1969) Comparative applied phosphate requirements of eight annual pasture species. *Australian Journal of Agricultural Research* 20, 809–818. doi:10.1071/AR9690809
- 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. doi:10.1071/AR9760479

- 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. doi:10.1071/EA9900507
- Rayment GE, Lyons DJ (2011) ‘Soil chemical methods – Australasia.’ (CSIRO Publishing: Melbourne)
- Ryser P, Eek L (2000) Consequences of phenotypic plasticity vs. interspecific differences in leaf and root traits for acquisition of aboveground and belowground resources. *American Journal of Botany* 87, 402–411. doi:10.2307/2656636
- 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. doi:10.1111/j.1469-8137.1995.tb05726.x
- 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. doi:10.1071/CP13395
- 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. doi:10.1016/j.agee.2015.06.026
- Vierheilig H, Coughlan AP, Wyss U, Piché Y (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied and Environmental Microbiology* 64, 5004–5007.
- 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. doi:10.1006/anbo.2001.1551
- 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* doi:10.1111/gfs.12199. Manuscript received 28 January 2016, accepted 20 April 2016

Table 1. Critical total plant P uptake determined as total plant P uptake at 90% of relative yield from a fitted Mitscherlich response ($y = a - b(e^{-cx})$; where y is relative yield; x is total plant P uptake and a is maximum yield (see Fig. 7a)

Values $\pm$ s.e. Different letters within columns denote significant differences ($P < 0.05$; $n = 5$)

Species	Critical total plant P uptake (mg P pot ⁻¹)	Intercept	Parameter	
			A	B
<i>Ornithopus compressus</i>	8.0 $\pm$ 0.3 a	-0.30 $\pm$ 0.08 a	1.01 $\pm$ 0.01 a	-6.52
<i>Ornithopus sativus</i>	9.4 $\pm$ 0.3 b	-0.09 $\pm$ 0.04 b	1.00 $\pm$ 0.02 a	-4.93
<i>Trifolium subterraneum</i>	9.8 $\pm$ 0.3 b	0.05 $\pm$ 0.01 c	0.99 $\pm$ 0.01 ab	-1.31
<i>Biserrula pelecinus</i>	11.0 $\pm$ 0.4 c	0.02 $\pm$ 0.03 bc	1.05 $\pm$ 0.03 b	-2.08
<i>Trifolium hirtum</i>	9.4 $\pm$ 0. b	0.06 $\pm$ 0.03 c	1.06 $\pm$ 0.04 a	-1.47

508

509

510 Fig. 1 Schematic diagram of the root hair cylinder i.e. the cylinder of soil encompassing the
511 root and root-hair zone.

513
514

515 Fig. 2 Root length density in topsoil and subsoil in response to phosphorus (P) applied to the
516 topsoil for (a) *Trifolium subterraneum* (b) *Trifolium hirtum* (c) *Ornithopus compressus* (d)
517 *Ornithopus sativus* (e) *Biserrula pelecinus* and (f) *Dactylis glomerata*. Bar shows LSD for
518 three-way interaction of P x species x soil depth ($P<0.001$; $n=5$). Dashed line shows critical
519 external P requirement for each species.

520
521
522

523
524

525
526 Fig. 3 Specific root length in topsoil and subsoil in response to phosphorus (P) applied to the
527 topsoil for (a) *Trifolium subterraneum* (b) *Trifolium hirtum* (c) *Ornithopus compressus* (d) *O.*
528 *sativus* (e) *Biserrula pelecinus* and (f) *Dactylis glomerata*. Bar shows LSD for three-way
529 interaction of P x species x soil depth ($P<0.05$; $n=5$). Dashed line shows critical external P
530 requirement for each species.

531

Fig. 4 Average root diameter in (a) topsoil and (b) subsoil for one grass and five legume species in response to phosphorus (P) applied to the topsoil. Bar shows LSD for three-way interaction of P x species x soil depth ($P<0.05$; $n=5$)

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

Fig. 6 (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 the 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.

Fig. 7 (a) Total plant phosphorus (P) uptake versus relative yield for each legume species. Values for critical total plant P uptake of the species in (a) are shown in Table 1. (b) Regressions fitted between product of $\text{CaCl}_2\text{-extractable P}$ 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. Gradient *Ornithopus compressus* > *O. sativus* = *Biserrula pelecinus* = *Trifolium hirtum* = *T. subterraneum*. Intercepts of species did not differ significantly except for *O. sativus* > *T. subterraneum* = *O. compressus* and *B. pelecinus* > *O. compressus* ($P < 0.05$).

562

563 Fig. 8 (a) Shoot dry mass (b) root dry mass in topsoil and (c) root length density in topsoil of
564 *Trifolium subterraneum* grown at four rates of phosphorus (P) applied to the topsoil and
565 harvested at four times after sowing. Bars show ± 1 standard error (n=5).

566

567

568

569

570

Supplementary Figure: (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 ($P < 0.05$; $n = 3$). The co-efficients of determination (R^2) for specific root length and average root diameter between plants grown in a sward or as single plants was 0.62 and 0.61, respectively.