Differences in nutrient foraging among *Trifolium subterraneum* cultivars deliver improved P-acquisition efficiency

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19 **Abstract**

20 Background and Aims

21 The growth and root morphology responses to soil phosphorus (P) fertility by five cultivars of
22 *Trifolium subterraneum* (a temperate annual pasture legume) were examined to assess whether
23 differences in root morphology and/or root acclimation to P stress influenced P-acquisition by
24 the clover varieties, or their critical P requirements (i.e. the rate of P supply needed for
25 maximum shoot yield).

26 Methods

27 The clovers were grown as microwards in soil with P stratified in a topsoil layer to mimic
28 growth conditions and soil P availability in a pasture. Yield and P content of shoots, and roots
29 from the topsoil and subsoil layers was determined after 5 weeks growth in a controlled-
30 environment cabinet. The lengths, diameters, and root hair lengths of nutrient foraging roots
31 from the topsoil layer were quantified.

32 Results

33 The shoot yield of the cultivars was similar when grown with high soil P fertility. However,
34 the cultivars varied up to 1.5-fold in their ability to yield at low levels of soil P supply, and by
35 1.6-fold in their critical P requirements. All cultivars acclimated to low P soil by increasing
36 root length density in the topsoil but those that yielded relatively well did so by maintaining a
37 large root hair cylinder volume (i.e. they explored more soil) under low soil P conditions. This
38 was associated with maintenance of dry matter allocations to topsoil roots and higher specific
39 root lengths. Both factors assisted development of high root length density for nutrient
40 foraging. Root acclimation responses to P were compared among the cultivars at equivalent
41 relative shoot yields to assess the influence of plant P stress as a trigger for nutrient foraging.

42 The least P-efficient cultivars slowed their allocation of dry matter to foraging roots at lower
43 levels of P stress.

44 Conclusions

45 The results suggest variation within *T. subterraneum* for root proliferation and specific root
46 length could be targeted in breeding for improved P-acquisition efficiency.

47

48 **Additional keywords:** critical phosphorus requirement, root diameter, root hair length,
49 subterranean clover

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Introduction

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 N₂-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) (Ozanne et al. 1969, 1976; Hill et al. 2006; Haling et al. 2016a). The P-balance efficiency of temperate pastures in Australia is low (i.e. P outputs expressed as a percentage of P inputs). 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. 2014; Simpson et al. 2015) are associated with soil when it is managed with high concentrations of plant-available P.

Recent studies 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 (Haling et al. 2016ab; Yang et al. 2017). *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) (Haling et al. 2016b; Kidd et al. 2016), which allow them to maximise soil exploration and uptake of P in low-P soil (Haling et al. 2016b). 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 (Haling et al. 2016b; Kidd et al. 2016). The relatively low specific root length of *T. subterraneum* roots also means that nutrient foraging incurs a relatively large carbon cost (Haling et al. 2016b; Kidd et al. 2016).

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. Root proliferation in response to P stress may also be an important trait for P-acquisition efficiency in *T. subterraneum*, but

little is known of how intra-specific variation in the root morphology traits of *T. subterraneum* (Jeffery et al. 2016; Kidd et al. 2016; Ryan et al. 2016) influences P acquisition efficiency.

This paper describes experiments that quantify 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 morphology traits for P acquisition in low-P soil.

Materials and Methods

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 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 CaCO₃ kg⁻¹) to raise the pH (1:5 w/v, 0.01 M CaCl₂) to 5.2 and thereby reduce concentrations of extractable Al³⁺ to negligible levels. Nutrients were mixed into the soil at rates of 41 mg kg⁻¹ soil MgSO₄·7H₂O, 43 mg kg⁻¹ CaSO₄·2H₂O, 169 mg kg⁻¹ KNO₃, 28 mg kg⁻¹ (NH₄)₂SO₄, 17 mg kg⁻¹ NH₄NO₃, 119 µg kg⁻¹ H₃BO₃, 759 µg kg⁻¹ MnCl₂·4H₂O, 359 µg kg⁻¹ ZnSO₄·7H₂O, 33 µg kg⁻¹ CuSO₄·5H₂O, 72 µg kg⁻¹ (NH₄)₂MoO₄, 20 µg kg⁻¹ CoCl₂·6H₂O and 1530 µg kg⁻¹ Fe-EDTA. Seven P-fertilised soil treatments were established (0, 10, 20, 40, 60, 80 and 250 mg P kg⁻¹ soil) by spraying solutions of KH₂PO₄ onto subsamples of the amended soil whilst the soil was being mixed in a rotating barrel. The P application rate of 250 mg P kg⁻¹ was known to support the maximum clover growth rate (Haling et al. 2016a). The Colwell-extractable P concentrations of the soil after all soil amendment treatments had been applied were: 7.1, 11.5, 17.3, 23.5, 43.8, 53.0 and 141 mg kg⁻¹ (method 9b in Rayment and Lyons 2011). The CaCl₂-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; 200 mm 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; 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 cultivar by P treatment were established. Plants were grown in a controlled-environment growth cabinet [12 h light (620 μ mol quanta m⁻² s⁻¹)/ 12 h dark; 20/15°C]. Pots were arranged in a randomised complete block design and rotated weekly within blocks to minimise any potential effect of light gradients within the growth cabinet. 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 watering 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.

Harvest and measurements

Plants were harvested five weeks after sowing. Shoots were cut at the soil surface, dried at 70°C for 48 hours 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 ($\frac{4}{3} * \text{the combined dry mass of roots from the scanned quarter and the un-scanned half segments}$).

Root hairs were measured on five lengths (~3-4 mm) of lateral root selected at random from the roots that had been washed from the fertilised topsoil (0, 10, 20, 60 and 250 mg P kg⁻¹ treatments) in each pot. Each root subsample was photographed using a Leica MZFLIII Fluorescence microscope (Leica Microsystems, Sydney, Australia) fitted with a Zeiss AxioCam camera (Zeiss, Sydney, Australia). The lengths of 15 root hairs per replicate were measured using ImageJ (Rasband 1997–2014); root hairs perpendicular to the line of vision were selected randomly for measurement. Root hair length of *T. subterraneum* is not adjusted in response to soil P supply (Haling et al. 2016b, see also Fig. 5b) so, when required for root hair cylinder volume calculations, the length of root hairs in the 40 and 80 mg P kg⁻¹ treatments was estimated as the average of root hair lengths in adjacent P treatments.

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.

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

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 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.

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 (Haling et al. 2016a). 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. This was analysed by means of a broken stick linear regression with the ‘right side’ regression forced to be a horizontal line (Equation [2]) using the package R2LINES (A.W.A. Murray & J.T. Wood) in Genstat 16th Edition (VSN International, UK). This yielded the form:

$$y = \begin{cases} mx + c, & x \leq \text{Breakpoint} \\ k, & x \geq \text{Breakpoint} \end{cases} \quad \text{Equation [2]}$$

Where y is the shoot dry mass, x is the P application, k is the maximum shoot dry mass, c is shoot dry mass at 0 mg P kg⁻¹ and m is the gradient.

The Breakpoint of the broken stick model (i.e. the critical P requirement) has a distribution which is approximately an F distribution on 1 and n-3 degrees of freedom. Utilising this, error estimates and confidence intervals for the Breakpoint were formed and generated in R2LINES. To test whether the breakpoints were pair-wise significantly different, a difference of unpaired means t test was used.

Relative shoot yield was calculated as shoot yield relative to the 250 mg P kg⁻¹ treatment and was considered a surrogate measure of P stress.

Curvilinear responses between shoot P concentration and rate of P addition were fitted between 10 and 250 mg P kg⁻¹ for each cultivar. Critical internal P concentration was then determined for each cultivar as the shoot P concentration corresponding to the critical external P-application rate of the cultivar (Pinkerton and Randall 1994). A Taylor series approximation was used to generate standard errors and 95% confidence intervals for the critical internal P concentration and used in t-tests to test pair-wise significant differences between cultivars.

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 to normalise the data.

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

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). 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 microwards 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 remaining treatments, total root length in the topsoil section was too large to be analysed in a single scan and due to time constraints only a sub-section of the topsoil was scanned. 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).

Results

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 from 10 to 80 mg P kg⁻¹ (Fig. 1). Maximum shoot yields (i.e. shoot dry mass at 250 mg P kg⁻¹) mostly did not differ significantly among the five cultivars. However, the cultivars differed in their critical external requirement for P (i.e. the P application rate required for maximum yield) (Table 1). This reflected the range (up to a 1.5-fold) in the ability of the cultivars to yield at levels of P supply between 40 and 80 mg P kg⁻¹.

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 1).

Root dry matter and root mass fraction

All cultivars developed 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. 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 topsoil 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.

The cultivars also differed in how their root dry matter in the subsoil responded to P supply in the topsoil (Fig. 2b). Napier and Riverina demonstrated a gradual increase in subsoil 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 (≤ 80 mg P kg⁻¹) than did other cultivars. In contrast, root dry matter of Leura was less when P supply was lower than 40 mg P kg⁻¹, 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. 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; calculated as shoot yield relative to the 250 mg P kg⁻¹ treatment) (Fig. 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 (i.e. ≤ 20 mg P kg⁻¹; Fig. 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. 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. The trends for each cultivar were also reflected in the examination of subsoil root mass fraction in relation to relative yield (Fig. 3f).

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 (60 or 80 mg P kg⁻¹ depending upon the cultivar) in the topsoil (Fig. 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 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. 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. 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% in soil with no P applied. 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 ($R = 0.87$ to 0.99) between specific root length and average root diameter (Table 2) among the

five cultivars (relationships not shown) indicating that high specific root length was associated with smaller average root diameter.

Among the cultivars, average root hair length (across the P treatments) 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.

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. 4c) followed similar trends to that of root length density (Fig. 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. 4d). Losa achieved the largest maximum root hair cylinder volume (80 cm³ 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 cm³; 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. Surface area of the root hair cylinder (i.e. the effective interface for P uptake by the root system) was negatively correlated with critical external P requirement (Fig. 6). This relationship was strongest at lower levels of applied P.

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 mostly greater when the plants were grown with P-addition rates above 20 mg P kg⁻¹ (Fig. 7a). 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 (i.e. 0 - 20 mg P kg⁻¹) 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. 7b). 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 (Haling et al. 2016b). 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. 7b).

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

In most but not all 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. 8a). 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. 8b). 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). Losa had significantly higher specific root lengths than did Napier (Table 3).

At each P application rate, the root mass fractions (total, topsoil and subsoil) of each cultivar were generally not significantly different when grown in pots of 63 to 134 mm diameter. However, in the smallest pots (51 mm diameter), Losa had significantly higher total root mass fractions at both rates of applied P, and Napier had significantly higher total root mass fraction at 250 mg P kg⁻¹. This was associated with significant increases in root mass fractions in both the topsoil and subsoil.

Discussion

Pot size and root proliferation

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 (51-134 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 confirms 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.

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 (i.e. <250 mg P kg⁻¹) and hence a lower critical external P requirement were associated with development of high root length density and consequently 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.

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 associated with 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 (Bates and Lynch 1996; Datta et al. 2015; Haling et al. 2016b). 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 appeared to be predominantly dependent on the characteristic 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 low 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 by Losa was a consequence of it allocating root mass to nutrient foraging combined with a relatively high specific root length. In contrast, Napier was the poorest performing cultivar in low P soil. It 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 at “intermediate” levels of P supply (40-80 mg P kg⁻¹). This was associated with low specific root length and an apparent failure to continue to allocate root mass 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) relative to other species (e.g. *Ornithopus* spp.; Haling et al. 2016b) 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 (Haling et al. 2016b; Kidd et al. 2016; Yang et al. 2017). 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.

Acclimation of roots to low P supply

The soil P thresholds for root mass (and consequently root length) allocation to nutrient foraging in the topsoil 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. 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. 3d). The superior cultivars (e.g. Losa, Leura) benefited 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).

Previously it was shown that *T. subterraneum* (cv. Leura) acclimates to low P soil mainly by adjusting root length in the P-enriched topsoil (Haling et al. 2016b). It was argued that P acquisition by cv. Leura in that experiment could largely be explained by diffusion of P to the surface of the root hair cylinder in the topsoil layer. This initially appeared to also be a reasonable assumption for all five of the clover cultivars in the present experiment because surface area of the root hair cylinder, the effective (putative) interface between their roots and the topsoil, was highly correlated with the critical P requirements of the cultivars. We examined this further by calculating P uptake per unit surface area of the root hair cylinder. If P diffusion to the root-soil interface is limiting P uptake, it is expected that P uptake per unit surface area

of the root hair cylinder will be similar among the genotypes: i.e. differences among them in P acquisition from low P soil will be due to the size of the root hair cylinder that they develop. Interestingly, the estimate of P uptake per unit surface area of root hair cylinder for Napier and Riverina at the three lowest rates of P application was greater than that of the other three cultivars (Fig. 7a). 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 of the topsoil layer alone (Fig. 7b). As outlined in the Results section, this quotient should be approximately constant and similar for all cultivars if P diffusion is limiting their P uptake. Indeed, this was observed for all cultivars when subject to moderate P-deficiency (i.e. relative yields between 0.4 and 1.0). However, 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 growing in very low P soil and were extremely P-stressed (i.e. relative yields 0.3-0.4; Fig. 7b). We concluded that the apparent elevation in the rate of P acquisition was more likely to be a result of the plants relying increasingly on P acquired from the subsoil (i.e. a breakdown in the assumptions underpinning the P uptake calculation), but enhanced P diffusion due to an increase in root exudates could not be ruled out. Either way, the apparent enhancement in P uptake rate under these circumstances was of limited value for the P-stressed plants as it did not alleviate the impact of severe P deficiency.

Collectively, the results indicate that there is potential to select among existing cultivars of *T. subterraneum* cultivars to achieve maximum productivity at lower concentrations of available soil P. For example, Losa required 50% less applied P to achieve the same maximum yield as Napier. Likewise, selection among existing cultivars could also enable higher productivity when growing in low-P soils (e.g. Losa could achieve 50% more shoot biomass than Napier when growing in low-P soils). However, the level of benefit appears to be dependent upon the degree of P stress experienced by the plant. When the cultivars were very P stressed (i.e. soil P availability producing relative yields less than ~0.3-0.4) there was little obvious difference between the cultivars. The relatively large differences in P acquisition efficiency were only detected when the plants were growing in soils that permitted relative yields greater than ~0.4. Further work is required to verify differences in the ability of these cultivars to yield on low-P soils in the field.

510

511 *Conclusions*

512 This experiment demonstrated that dry matter partitioning to nutrient foraging roots and the
513 specific root length of these roots were major factors determining the differential ability of five
514 cultivars of *T. subterraneum* to acquire P and yield well in soil with low concentrations of
515 plant-available P. The primary result of these root system attributes was the development of a
516 high root length density in the P-enriched zone of the soil profile. However, the cultivars that
517 were most effective at acquiring P also exhibited a superior ability to maintain high root length
518 density when soil P fertility levels were very low. Further work, with a much broader range of
519 *T. subterraneum* genotypes, is required to understand the extent of intra-specific variation in
520 these key nutrient foraging traits. Short root hairs on *T. subterraneum* are a constraint to more
521 effective nutrient foraging and an assessment of whether there is useful variation in root hair
522 length within this species is also needed. However, the results from this study already indicate
523 that there is potentially useful variation within *T. subterraneum* for root proliferation and
524 specific root length that could be used to breed cultivars with improved P-acquisition
525 efficiency.

526

527

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Table 1 Critical external requirement for phosphorus (P) and critical internal requirement for P for five cultivars of *Trifolium subterraneum*. Different letters denote significant differences.

Cultivar	Critical external P requirement (mg P kg ⁻¹)	Critical internal P requirement (%)
cv. Leura	120.3 b	0.34 a
cv. Losa	100.9 a	0.36 ab
cv. Napier	168.9 d	0.40 b
cv. Riverina	136.2 c	0.38 ab
cv. Seaton Park	114.8 ab	0.37 ab

Table 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. Values $\pm$ standard error (n=5). Interaction of P applied x Cultivar was not significant for $P<0.05$.

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

Table 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 (m g ⁻¹)		Total root mass fraction		Topsoil root mass fraction		Subsoil root mass fraction	
	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 (<i>P</i> =0.05)	n.s.		0.027		0.019		0.023

Fig. 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 $\times$ 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.

Fig. 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.

Fig. 3 The response of the root mass fraction for the (a) total root system (c) topsoil roots and (e) 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) total root system (d) topsoil roots and (f) 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 $\pm$ standard error.

Fig. 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 $\pm$ standard error.

Fig. 5 (a) Specific root length in topsoil and (b) root hair length in topsoil for five cultivars of *Trifolium subterraneum* grown in response to 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.

Fig. 6 Relationship between surface area of root hair cylinder (m²) and critical external phosphorus (P) requirement for five cultivars of *Trifolium subterraneum* grown in soils with P applied to the topsoil of the pot at rates of (a) 0, 10, 20, 40 and (b) 60, 80 and 250 mg kg⁻¹. Each of the five cultivars is represented within each P treatment.

Fig. 7 (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 $\pm$ standard error.

Fig. 8 (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). Bar in (a) shows LSD ($P=0.05$) for 3-way interaction for Cultivar x P applied x Pot size. Error bars in (b) show $\pm$ standard error; 3-way interaction was not significant ($P>0.05$).