Plants in constrained canopy micro-swards compensate for decreased root biomass and soil exploration with increased amounts of rhizosphere carboxylates.

Robert P. Jeffery[1], Richard J. Simpson[1,2], Hans Lambers[1], Daniel R. Kidd[1], Megan H. Ryan[1]

[1] School of Plant Biology, and Institute of Agriculture, The University of Western Australia, 35 Stirling Hwy, Crawley (Perth), WA 6009, Australia

[2] CSIRO Agriculture, GPO Box 1600, Canberra, ACT 2601, Australia

Author for correspondence

Mr Robert P Jeffery

Ph +61 0 420513107

Email: robert.jeffery@research.uwa.edu.au

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Annual pasture legumes with a superior ability to acquire soil phosphorus (P) through specialised root systems could lower P-fertiliser requirements. Root traits related to P-acquisition are commonly examined for plants in pots grown in glasshouses, however we found that root length was greatly decreased and root exudates greatly increased when shoots of these plants were constrained from spreading laterally, as would also occur in dense pasture swards in the field. We suggest that canopy constraint should be routinely used when screening plants in the glasshouse for root traits likely to improve P-acquisition under field conditions.

ABSTRACT

Background and Aims Root traits related to phosphorus (P) acquisition are used to make inferences about a species' P-foraging ability under glasshouse conditions. However, the effect on such root traits of constrained canopy spread, as occurs in dense pasture swards, is unknown.

Methods We grew micro-swards of *Trifolium subterraneum* L. and *Ornithopus compressus* L. at 15 and 60 mg kg^{-1} soil P in a glasshouse. Shoots either spread beyond the pot perimeter or were constrained by a cylindrical sleeve adjusted to canopy height. After eight weeks, shoot and root dry mass (DM), shoot tissue P concentration, rhizosphere carboxylates, arbuscular mycorrhizal (AM) fungal colonisation, total and specific root length (TRL and SRL, respectively), average root diameter (ARD) and average root hair length (ARHL) were measured.

Results In all species and treatments, constrained canopy spread decreased root DM (39-59%), TRL (27-45%) and shoot DM (10-28%), and increased SRL (20-33%), but did not affect ARD, ARHL and AM fungal colonisation. However, shoot P concentration and content increased, and rhizosphere carboxylates increased 3.5 to 12-fold per unit RL and 2 to 6.5-fold per micro-sward.

Conclusions Greater amounts of rhizosphere carboxylates when canopy spread was constrained appeared to compensate for reduced root growth enabling shoot P content to be maintained.

Keywords: reflective sleeves, shading, subterranean clover, light, yellow serradella,

Abbreviations: Arbuscular mycorrhizal (AM), specific root length (SRL), total root length (TRL), root tissue density (RTD), average root diameter (ARD), average root hair length (ARHL), root mass fraction (RMF), dry mass (DM) and 15, 60 mg P kg^{-1} dry soil (P15 and P60).

61 INTRODUCTION

62 A common plant response to a limiting P supply is to increase specific root length (SRL) and
63 decrease root tissue density (RTD) (Lynch and Brown 2001; Hill *et al.* 2006). However, the
64 opposite response, that is, decreased SRL and increased RTD in response to decreased P
65 supply, has been reported for micro-swards of *Trifolium subterraneum* (Haling *et al.* 2016b;
66 Jeffery *et al.* 2016). In both these experiments, shoot canopy was constrained, to mimic self-
67 shading conditions in a dense pasture sward in the field, by reflective sleeves placed around
68 pots and adjusted daily to canopy height (Haling *et al.* 2016b; Jeffery *et al.* 2016). The
69 authors of these studies attributed their unexpected results to increased self–shading and
70 reduced carbon availability to roots of constrained micro-swards at high P supply when shoot
71 growth was greatest. If the impact of constraining canopy on root morphological acclimation
72 to P deficiency is a result of decreased carbon availability, it seems likely that other carbon-
73 costly traits related to P acquisition, such as arbuscular mycorrhizal (AM) fungal colonisation
74 and the amount of rhizosphere carboxylates (Ryan *et al.* 2012), would also be decreased in
75 constrained micro-swards.

76 Two pasture legume species commonly sown in Australia and previously examined in
77 constrained canopy micro-swards are *T. subterraneum* and *Ornithopus compressus* (Haling *et*
78 *al.* 2015; Haling *et al.* 2016*a*, *b*; Jeffery *et al.* 2016). These species differ in their P-
79 acquisition efficiency and morphological root acclimation to low P supply, with greater root
80 biomass allocation by *T. subterraneum* than by *O. compressus* (Haling *et al.* 2016a).
81 Therefore, we envisage that the impact of constraining the canopy on roots would differ
82 between these two species.

83 Compared with field experiments, pot experiments increase the ease and accuracy of
84 scanning intact root systems and of sampling roots and rhizosphere soil for assessment of AM
85 fungi and carboxylates. Thus, root traits related to P acquisition of *T. subterraneum* and *O.*
86 *compressus* are most commonly examined for plants grown in unconstrained canopy micro-
87 swards in glasshouse experiments (Nazeri *et al.* 2014; Kidd *et al.* 2016; Ryan *et al.* 2016).
88 The objective of many of these previous studies was to contribute towards the identification
89 and development of species with improved P-acquisition efficiency to increase agricultural
90 productivity in P-deficient soils (Simpson *et al.* 2011). However, it is not known whether the
91 competition for light experienced by individual plants in a dense pasture sward in the field
92 results in differences in root traits compared with plants grown in unconstrained canopy

93 micro-swards in pots, where shoots are able to expand outside the confines of the pot. It is
94 vital to examine this issue, as it is important that results from glasshouse studies are
95 applicable to pasture swards in the field.

96 The purpose of this study was, therefore, to examine the impact of constraining the canopy
97 spread of micro-swards of annual pasture legumes (*T. subterraneum* and *O. compressus*) on
98 root traits related to P-uptake at low and high P supply. We hypothesised that due to
99 increased shoot shading and less carbon being available for transport to roots, constraining
100 the canopy would: 1) decrease root and shoot dry mass (DM), 2) decrease AM fungal
101 colonisation and rhizosphere carboxylates, and 3) increase specific root length (SRL) and
102 decrease root tissue density (RTD). We further hypothesised that 4) the effects of
103 constraining the canopy would vary between species.

104 MATERIALS and METHODS

105 *Experimental design*

106 Morphological, physiological and symbiotic root traits related to P acquisition were examined
107 for two annual pasture legume species, *Trifolium subterraneum* L. ssp. *yannicum* cv. Riverina
108 and *Ornithopus compressus* L. cv. Santorini, grown in a glasshouse with two rates of soil P
109 application (15 and 60 mg P kg^{-1} dry soil) and the canopy either unconstrained or constrained
110 by reflective sleeves. These species were selected because they had been included in previous
111 P-response experiments with the canopy constrained (Haling *et al.* 2016b; Jeffery *et al.*
112 2016).

113 *Soil and phosphorus (P) treatments*

114 An unfertilised, sandy loam at 0–40 cm depth was collected from the University of Western
115 Australia Future Farm, Pingelly, Western Australia (S 32° 30’ 23” – E 116° 59’ 31”) on 13
116 April 2015. The soil was sieved through a 5-mm grid, pasteurised at 63°C for 90 minutes on
117 two consecutive days, oven-dried at 40°C for seven days and mixed thoroughly with a
118 commercial AM fungal inoculum (Microbe Smart start-up super VAM inoculum) stated to
119 contain four species; *Claroideoglomus etunicatum*, *Funneliformis mosseae*, *F. coronatum* and
120 *Rhizophagus irregularis*. Pasteurised soil was analysed by CSBP analytical laboratories
121 (Bibra Lake, Western Australia) and methods, unless otherwise specified, followed those of
122 Rayment and Lyons (2011); refer to Jeffery *et al.* (2016) for codes from this reference. The
123 soil was a sandy loam (8% clay, 82% sand and 10% silt) with low plant-available

bicarbonate-extractable P (8 mg P kg^{-1} dry soil) (Colwell 1965), a high P-buffering index (PBI = 348), a pH in $CaCl_2$ of 5.2 and 7 mg kg^{-1} nitrate-N, 12 mg kg^{-1} ammonium-N and 118 mg kg^{-1} bicarbonate-extractable K.

Cylindrical free-draining PVC pots (90 mm diameter × 200 mm depth) were filled with 700 g of the oven-dried soil, which was then wet with 200 ml of deionised water; 300 g of dry soil were then added on top of the wetted soil. Two P supply treatments were established prior to sowing. In order to mimic the stratification of P in topsoil that occurs under pastures (McLaughlin *et al.* 2011) an 80 ml solution of KH_2PO_4, sufficient to saturate the top 300 g of soil, was applied at either 15 or 60 mg P kg^{-1} dry soil (P15 or P60). Total K was balanced to 100 mg kg^{-1} among P treatments with KCl (Pang *et al.* 2010) and all essential nutrients except P were provided at final concentrations of (mg kg^{-1} dry soil): N 30, S 50, Ca 45, Mg 10, Cu 0.5, Zn 7, Mn 4, B 0.12, Mo 0.4 and Fe 5. Nitrogen was added as a mixture of NH_4NO_3 and $Ca(NO_3)_2$ with a molar ratio of 1 to provide an initial supply after germination, prior to nodulation.

Experimental design and canopy treatments

Ten seeds per pot were sown and thinned to six seedlings following emergence; hence a micro-sward was established in each pot. *Trifolium subterraneum* and *O. compressus* seeds were inoculated with a slurry of peat-based inoculum of Group C or S^1 rhizobia (Becker Underwood, Somersby, New South Wales, Australia), respectively. The soil surface was covered with a thin layer of white alkathene beads to minimise evaporation. Half of the pots of each species and P-treatment combination were sheathed with sleeves with a reflective inner surface. Sleeve height was adjusted to that of the micro-sward daily with the intention of constraining canopy spread of the micro-sward, as would occur in a large area of dense pasture sward in the field (Rossiter 1974; Hill *et al.* 2005). There were five replicates of each treatment combination.

Plants were grown for eight weeks from 3 August 2015 in a glasshouse maintained between 16°C and 23°C at The University of Western Australia, Perth, Australia (S 31° 98' – E 115° 81'). Pots were watered by weight to 80% of pot capacity, defined as the water content of a saturated pot that has ceased draining, with deionised water three times a week and rotated randomly within replicates weekly to minimise the effects of temperature and light gradients within the glasshouse (Poorter *et al.* 2012a). Pot weights recorded during the final two weeks were used to estimate daily water use of micro-swards.

156 *Harvest*

157 On 12 October 2015, plants were removed from pots and separated into roots and shoots.
158 Individual plants within the micro-sward established in each pot were not separated and
159 measurements are the sum of the six plants in each micro-sward. Shoots were oven-dried at
160 70°C for 72 hours then weighed.

161 *Rhizosphere carboxylates*

162 Intact root systems were gently shaken to remove loosely adhered soil. The roots and
163 remaining soil, which was considered to constitute the rhizosphere, were then immersed in 50
164 ml of 0.2 mM $CaCl_2$ and further shaken. A filtered subsample of the rhizosphere extract was
165 stored in 1 ml Waters high-performance liquid chromatography (HPLC) vials containing 25
166 µl of orthophosphoric acid at -20°C and later analysed for low–molecular-weight
167 carboxylates by HPLC on an Alltima C-18 reverse-phase column (Cawthray 2003), as
168 described previously by Jeffery *et al.* (2016). The carboxylates examined and corresponding
169 limits of detection (µmol) were fumarate 0.06; citrate 5; malate 7; malonate 8; lactate 13;
170 acetate 24; maleate 0.05; succinate 15; cis-aconitate 0.1; and trans-aconitate 0.1. Carboxylate
171 quantities below the limit of detection were changed to zero to enable statistical analysis
172 (Cawthray 2003). The total amount of rhizosphere carboxylates, relative to root length (nmol
173 cm^{-1}) and per pot (µmol micro-$sward^{-1}$), and the percentage of total carboxylates comprised
174 by individual carboxylates were calculated.

175 *Colonisation by arbuscular mycorrhizal fungi*

176 The genetic identities of the arbuscular root-colonising fungi were examined by Orchard *et*
177 *al.* (2017) for *T. subterraneum* with unconstrained shoots at 15 mg P kg^{-1} dry soil based on
178 ~260 base pair fragments of the 18S rRNA gene. More than 60% of the sequences
179 were identified as *R. irregularis*, and 22% were identified as other species of AM fungi
180 (Glomeromycota). A further ~15% of the sequences were identified as fine root
181 endophytes, arbuscule-forming root-colonising fungi recently found to be related to the
182 subphylum Mucoromycotina (Orchard *et al*. 2017). For assessment of the percentage of root
183 length colonised by AM fungi, subsamples (~250 mg) of roots were cleared in 10% (w/v)
184 KOH for five days at room temperature (~25°C), stained in a 5% (v/v) Schaeffer blue ink and
185 vinegar solution for 1 h and stored in lacto-glycerol (1:1:2 (v/v/v) lactic acid, deionised water,
186 glycerol) (Vierheilig *et al.* 1998). The percentage of root length colonised by AM fungi was

187 calculated using the gridline intersect method for a minimum of 100 intersections per sample
188 (Giovannetti and Mosse 1980). Note that colonisation by fine root endophyte was frequently
189 observed, but was not assessed and is not included in the AM fungal colonisation
190 measurement.

191 *Root morphological traits*

192 After being subsampled for AM fungi, root systems were then washed, refrigerated at 4°C
193 and later scanned to determine root length and diameter using an Epson 1680 scanner and
194 WinRHIZO version 4.1c (Regent Instructions, Quebec, Canada), as described previously
195 (Jeffery *et al.* 2016). Root systems were assessed for disease symptoms and nodulation, with
196 no visible disease symptoms and nodules present on all plants. The remaining roots were
197 dried, weighed and the total root dry mass (DM) calculated. Root mass fraction (RMF) was
198 calculated as the proportion of root DM relative to total plant DM. Specific root length (SRL)
199 was calculated as the root length per unit DM and root tissue density (RTD) as the ratio of
200 root DM to root volume.

201 Average root hair length (RHL) was assessed using the same stained root subsamples used to
202 assess AM fungal colonisation, which were photographed at 40× magnification using an
203 Olympus microscope (BX51) and camera (DP72). The length of ten root hairs in each of ten
204 ~10 mm length root sections, taken at least 20 mm from the root tip, were measured for each
205 sample with Olympus DP2-BSW software. The RHL of only three of the five replicate pots
206 for each treatment combination were measured, except for *T. subterraneum* with shoots
207 constrained at P15 which was not assessed for RHL as samples had been sent for molecular
208 analysis of AM fungal communities (Orchard *et al*. 2017).

209 *Shoot nutrient analysis*

210 The shoot tissue concentration of P and other elements in ~0.1 g subsamples of ground shoot
211 material digested in a 3:1 HNO_3:$HClO_4$ solution (Motomizu *et al.* 1983) was measured by
212 inductively coupled plasma (ICP) atomic absorption with a Perkin Elmer Optima 5300 DV
213 optical emission spectrometer (OES; Shelton, CT, USA).

214 *Statistical analyses*

215 Data were analysed by a three-way general analysis of variance (ANOVA) using GenStat
216 15.2 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK, 2012) with species

(*T. subterraneum, O. compressus*), canopy (constrained, unconstrained) and P supply level (P15, P60) and their interactions as factors. Normality was checked and no transformations were needed. Outcomes of the ANOVAS are presented in Table 1 and the three-way interaction of species, canopy and P supply is presented for all parameters either graphically or as a table: if significant, the the l.s.d. at P=0.05 for this interaction is also supplied. Data are expressed per micro-sward (i.e. the total of all six plants in each pot) and not per individual plant. Less than three outliers were removed from each parameter to ensure the data met the assumption of normality.

RESULTS

Plant growth

Although *T. subterraneum* had greater shoot DM than did *O. compressus*, the impact of constraining the canopy on plant growth and root morphology was similar for both species. Shoot DM of constrained canopy micro-swards was less, relative to that of unconstrained swards, by 10-17% for *T. subterraneum* and 21-28% for *O. compressus* (Fig. 1a). Shoot DM was greater (53-103%) at P60 than at P15, for all treatments. Root DM of constrained canopy micro-swards of both species was less, relative to that of unconstrained micro-swards, by 39-59% (Fig. 1b). Root DM also varied less among constrained canopy micro-swards (0.22-0.33 g DM) than among unconstrained micro-swards (0.37-0.79 g), and was greater (51-61% for unconstrained and 2-35% for constrained micro-swards) at P60 than at P15. The RMF of constrained canopy micro-swards was 18-46% less than that of unconstrained micro-swards; this difference was greatest for *T. subterraneum* at P60 and <23% for all other treatments (Fig. 2a). For *T. subterraneum* and *O. compressus* (respectively), RMF was less at P60 than at P15 to a greater extent for constrained (41% and 9% less) than for unconstrained (16% and 4% less) canopy micro-swards.

Root morphology

The TRL was 27-40% shorter for constrained, relative to unconstrained, canopy micro-swards for all treatments (Fig. 2b). Total root length was greater at P60 than at P15, to a lesser extent in constrained canopy micro-swards (71% and 31%) than in unconstrained micro-swards (85% and 59%) for *T. subterraneum* and *O. compressus*, respectively.

Specific root length was greater, relative to unconstrained micro-swards, by 20-33% when the canopy was constrained. For *T. subterraneum*, SRL was greater (23-34%) at P60 than at P15, whereas *O. compressus* exhibited similar (<3.1% difference) SRL at both P levels (Fig. 2c).

Root tissue density was 9-23% less when the canopy was constrained, relative to that of unconstrained canopy micro-swards, with the exception of *T. subterraneum* at P15 (Fig. 2d). For *O. compressus*, RTD was 10% less at greater P supply for constrained canopy micro-swards and independent of P supply for unconstrained canopy micro-swards. The RTD of *T. subterraneum* at P60, relative to P15, was 33% and 7% less for constrained and unconstrained canopy micro-swards, respectively.

There was little variation in ARD (0.37-0.45 mm) among all treatment combinations (Fig. 2e). Constraining the canopy did not affect the ARD of *O. compressus* at P60, but at P15 resulted in it being 2.6% less than that of unconstrained canopy micro-swards. For *T. subterraneum*, ARD was 11.1% less at P15 and 2.4% less at P60 when the canopy was constrained. There was no significant effect of P supply on ARD.

There was no significant effect of constraining the canopy or P supply on ARHL (Fig. 2f), but *T. subterraneum* had significantly shorter root hairs (0.16-0.18 mm) than did *O. compressus* (0.28-0.33 mm).

Plant phosphorus content

Shoot P content per micro-sward was 4-36% greater when the canopy was constrained, relative to that of unconstrained canopy micro-swards, and 57-84% greater with increased P supply for both species (Fig. 3). There was no significant effect of P supply on shoot P concentration, which was 26-65% greater for *T. subterraneum* and 28-57% greater for *O. compressus* when the canopy was constrained than when it was unconstrained (Table. 2).

Rhizosphere soil carboxylates

Malate, malonate and citrate were the most abundant carboxylates detected in the rhizosphere soil (Fig. 4a). Constraining the canopy, relative to unconstrained canopy micro-swards, increased the proportion of total carboxylates comprised of malate. The proportion of total carboxylates comprised of malonate for constrained canopy micro-swards was smaller than that of unconstrained micro-swards. The percentage of total carboxylates which consisted of citrate was smaller for constrained than for unconstrained canopy micro-swards of *O.*

compressus, but this difference was not evident for *T. subterraneum*. Fumarate was the only other carboxylate present in quantities greater than its limit of detection.

The total amount of all rhizosphere carboxylates measured at a detectable level was low (2.3-3.7 nmol cm^{-1} RL) (16.9-21.2 μmol g^{-1} root DM) for all unconstrained canopy micro-swards of both species (Fig. 4b). However, the total amount of all rhizosphere carboxylates, per micro-sward and per unit root length, was 202-648% and 348-1200% greater, respectively, when the canopy was constrained (12-48 nmol cm^{-1} RL; 117-214 μmol g^{-1} root DM). Impacts of species and P supply were relatively small or absent.

Water use

Constraining shoots reduced water use by *T. subterraneum* and *O. compressus* during the final two weeks of the experiment by 28-69% and 30-38%, respectively (Fig. 5a). Water use at P60 was 36-62% greater than at P15 for all treatments except *T. subterraneum* with the canopy constrained, which used 41% less water at greater P supply (note that in this treatment the RMF was lowest).

Colonisation of roots by arbuscular mycorrhizal fungi

At P15, colonisation by AM fungi was greater for *O. compressus* (70-75%) than for *T. subterraneum* (48-51%) (Fig. 5b). However, AM fungal colonisation at P60 was similar (16-23%) for both species. There was no effect of constraining the shoots.

Shoot element concentrations

The shoot concentrations of P, K, S, Ca, Mg, Na and Cu were generally greater for constrained than for unconstrained canopy micro-swards (Table. 2); this trend was particularly marked for P, Na and Cu (26-65%, 31-78% and 8-55% greater concentration, respectively). Shoot concentrations of the other measured elements (Zn, Mn, Mo, Al, Co and Fe) were affected by canopy constraint and P supply treatments, but in a variable manner different to that of shoot P.

The shoot Al concentration (860-1760 μg g^{-1} DM) was high (Osborne *et al.* 1980; Bouma *et al.* 1981) for all micro-swards, with no consistent trend and no significant effect of any treatment. Shoot Mo concentration was two- to three-fold greater for *O. compressus* than for *T. subterraneum* and was independent of canopy and P supply for both species, with the exception of constrained canopy micro-swards of *O. compressus* at P60.

306 DISCUSSION

307 We examined the impact of constraining the shoot canopy, as would occur within a dense
308 pasture sward, on root morphology, rhizosphere carboxylates and colonisation by AM fungi
309 for two pasture legume species at low and high P supply in a glasshouse. Both species (*T.*
310 *subterraneum* and *O. compressus*) responded similarly when their canopy was constrained.

311 Constraining canopies reduced root DM and TRL by up to 59% and 45%, respectively, but
312 had no negative impact on shoot P content, even under P limitation. This supports our first
313 hypothesis that constraining the canopy would reduce shoot and root DM of micro-swards,
314 although the lack of a reduction in shoot P concentration for constrained canopy micro-
315 swards was unexpected. Canopy constraint had no effect on ARHL or the percentage of root
316 length colonised by AM fungi. However, the amount of rhizosphere carboxylates (per micro-
317 sward) was up to seven-fold greater. Therefore, our second hypothesis, that constraining the
318 canopy would decrease AM fungal colonisation and rhizosphere carboxylates due to limited
319 carbon availability, was rejected.

320 These results suggest that the greater amount of rhizosphere carboxylates when canopy
321 spread was constrained by reflective sleeves likely substituted for the reduction in TRL and
322 thus explains why shoot P content did not decrease. The fact that constraining canopy spread
323 had no impact on AM fungal colonisation, which often decreases in response to shading
324 (Konvalinková and Jansa 2016), suggests that the reduction in carbon availability responsible
325 for the decreased root DM in the presence of sleeves was not great enough to affect AM
326 fungal colonisation. These results and their implications are discussed in detail below.

327 *1) Impact of constrained canopy micro-swards on root morphology*

328 To our knowledge this is the first study to examine the impact of constraining canopy spread
329 on the root growth and morphology of *T. subterraneum* and *O. compressus*. However, the
330 values for individual traits reported by previous studies of the same species grown in
331 unconstrained (Schweiger *et al.* 1995; Nazeri *et al.* 2013; Kidd *et al.* 2016; Ryan *et al.* 2016)
332 or constrained canopy micro-swards (Haling *et al.* 2015; Haling *et al.* 2016a; Haling *et al.*
333 2016b; Jeffery *et al.* 2016) were generally similar to those in the corresponding canopy
334 treatment of the present study.

335 For both species, the constrained canopy decreased root DM (by 39-39%), TRL (by 27-45%),
336 RMF and RTD (with the exception of *T. subterraneum* at P15), increased SRL and had no

effect on ARD and ARHL. The reduced RMF and SRL are a reflection of shoot and root DM, which both decreased when canopies were constrained, especially at P60 when plants were taller. This is consistent with lower rates of photosynthesis in shaded leaves causing decreased carbon allocation to the roots (Poorter *et al.* 2012b). For *T. subterraneum*, our findings are consistent with previous research where reduced light intensity resulting from decreased irradiance or increased plant density decreased root DM, root/shoot ratio and initiation of lateral roots (Stern 1965; Tester *et al.* 1986; Demotes-Mainard and Pellerin 1992). Therefore, our results provide support for the increase in SRL and decrease in RTD with increased P supply observed in other studies of constrained micro-swards of *T. subterraneum* (Haling *et al.* 2016b; Jeffery *et al.* 2016) being a result of larger plants at high P supply causing a greater proportion of leaves within the micro-sward canopy to be shaded.

The ARHL of *T. subterraneum* (0.16-0.18 mm) and *O. compressus* (0.28-0.33 mm) was also comparable with other studies; 0.15-0.27 and 0.48 mm for unconstrained canopy micro-swards of *T. subterraneum* and *O. compressus*, respectively (Evans 1977; Schweiger *et al.* 1995; Hill *et al.* 2010; Yang *et al.* 2015; Ryan *et al.* 2016). Longer ARHL (0.75 mm) has been measured for *O. compressus* grown for four weeks in a controlled environment cabinet, although the ARD of *T. subterraneum* (0.23 mm) in the same study was similar to our observations (Haling *et al.* 2015). Constraining the canopy had no impact on ARHL, indicating that the assessment of pasture legumes for root hair length can be undertaken without constraining the canopy. Therefore values of ARHL measured for unconstrained canopy micro-swards in the glasshouse may differ to those in field conditions as a result of differences in P supply or growth media, but not canopy constraint.

2) *No effect of constraining canopy on arbuscular mycorrhizal fungi*

Root colonisation was dominated by AM fungi and while fine root endophyte were also present, the extent of their colonisation was not assessed. The percentage of root length colonised by AM fungi decreased with increased P supply and was similar to that in other studies of the same species grown in constrained (Hill *et al.* 2010; Jeffery *et al.* 2016; Waddell *et al.* 2016) and unconstrained (Bolan *et al.* 1984; Schweiger *et al.* 1995; Nazeri *et al.* 2013; Nazeri *et al.* 2014) canopy micro-swards.

However, there was no negative effect of canopy constraint on the AM fungal colonisation. We had hypothesised that colonisation would be reduced due to increased shading of plants within constrained micro-swards, as decreased colonisation of roots by AM fungi in response

to decreased irradiance has often been reported for *T. subterraneum* (Tester *et al.* 1985; Tester *et al.* 1986) and many other plant species (Hayman 1974; Daft and El-Giahmi 1978; Graham *et al.* 1982). However, there are exceptions; Facelli *et al.* (1999) found no impact of decreased (by >50%) light intensity, and a negative impact of plant density independent of light, on AM fungal colonisation of *T. subterraneum*. Similarly, Stonor *et al.* (2014) reported that shade-induced (72 to 262 μmol m^{-2} s^{-1}) reductions in photosynthetic carbon availability had no effect on AM fungal colonisation, arbuscule development or mycorrhizal growth response for *Triticum aestivum* compared to that of unshaded (325 to 1025 μmol m^{-2} s^{-1}) conditions. These contrasting results might reflect the light intensity in the control or shading treatments, which may have been mild or strong, respectively. However, considering that the >50% reduction in light intensity (400 μmol m^{-2} s^{-1} unshaded and 180 μmol m^{-2} s^{-1} shaded) in Facelli *et al.* (1999) was similar to the intensity (from 450 μmol m^{-2} s^{-1} unshaded and 100 μmol m^{-2} s^{-1} shaded) which decreased AM fungal colonisation for *T. subterraneum* in previous studies (Tester *et al.* 1985; Tester *et al.* 1986) this seems unlikely. An alternative explanation is that the effect of shading is less for less carbon-demanding AM fungi (van der Heijden *et al.* 2015; Argüello *et al.* 2016).

In view of the above, we conclude that the reduction in root DM when shoots were constrained was most likely due to limited carbon availability, but that carbon availability did not become low enough to reduce the level of colonisation by AM fungi. This is perhaps consistent with shading in our study only affecting leaves low in the canopy, whereas leaves at the top of the canopy still experienced full sunlight.

4) *Did increased rhizosphere carboxylates substitute for decreased root growth for P uptake?*

In the constrained treatments, the total amount of carboxylates in the rhizosphere soil was unexpectedly high, especially for *T. subterraneum* (20-45 nmol cm^{-1} RL and 17-214 μmol g^{-1} root DM), which was previously reported to have 10-20 μmol g^{-1} root DM in unconstrained (Nazeri *et al.* 2014; Kidd *et al.* 2016) and <1.6 nmol cm^{-1} RL in constrained (Jeffery *et al.* 2016) canopy micro-swards. *Ornithopus compressus* was previously reported to have 4 nmol cm^{-1} RL total carboxylates in unconstrained canopy micro-swards (Kidd *et al.* 2016).

The effects of constraining the canopy, or shading, on the amount of carboxylates in rhizosphere soil have not been examined previously. Our finding of up to 5–fold more carboxylates in the rhizosphere in constrained canopy micro-swards compared with that in unconstrained micro-swards was unexpected, especially as previous studies suggest that

carboxylate exudation may be quite sensitive to reduced carbon availability due to competing sinks or reduced photosynthesis (Rovira 1959; Ryan *et al.* 2012; Nazeri *et al.* 2014). For instance, inoculation with AM fungi was reported to decrease rhizosphere carboxylates, relative to uncolonised plants, by up to 53% for ten *Kennedia* and five annual pasture legume species (Ryan *et al.* 2012; Nazeri *et al.* 2014). Moreover, exudation of amino acids, mainly glutamic acid and serine, by *T. subterraneum* also decreased by 100-200% in response to decreased light intensity (Rovira 1959).

We found that constraining the canopy decreased root DM by up to 59%, but had no effect on shoot P content (Fig. 3), even at P15, when P availability was limiting plant growth. Total P content of shoots and whole plants was shown to increase with increased P supply for *T. subterraneum* and *O. compressus* (Caradus 1980; Paynter 1990). Therefore our results suggest that the increase in rhizosphere carboxylates in the constrained canopy treatment may have solubilised P from soil that was previously unavailable to plant roots (Veneklaas *et al.* 2003; Lambers *et al.* 2015b) and compensated for the reduction in root biomass and length. This scenario is supported by shoot P concentration and content following a similar trend to total rhizosphere carboxylates. It also appears the most likely explanation for the lack of change in P uptake, as there was no change in AM fungal colonisation and daily water-use was greater for unconstrained micro-swards than constrained, suggesting that movement of P to roots by mass flow would also have been decreased in the constrained canopy micro-swards. The decreased shoot P concentration at P60 compared with P15 for all unconstrained canopy micro-swards (Table 2) is presumably due to dilution of P within the greater shoot DM.

Malate comprised a greater proportion of total rhizosphere carboxylates in constrained canopy micro-swards than in unconstrained canopy micro-swards due to a reduction in the proportion of carboxylates that consisted of citrate and malonate. This could have significance for the effectiveness of carboxylates at mobilising P in the rhizosphere, as citrate and malonate have a greater reported impact on P availability in the soil than malate (Oburger *et al*. 2009; Ryan *et al*. 2012).

Even though the field soil used in our experiment was pasteurised prior to the experiment commencing, it is possible that the amount of carboxylates originally exuded could be much larger than the amounts measured in the rhizosphere extracts due to the rapid microbial degradation of carboxylates in the rhizosphere (Martin *et al*. 2016; Menezes-Blackburn *et al.*

2016). This could be tested in future studies by measuring carboxylate exudation from the intact root system into a trap solution, after the rhizosphere is washed from the roots (Nannipieri *et al.* 2008).

Overall, our results indicate that when the canopy was constrained a large increase in amount of rhizosphere carboxylates acted as a substitute for root length and root DM in terms of plant ability to access P. This most likely accounted for the lack of difference in shoot P content and concentration between constrained and unconstrained micro-swards. This novel finding suggests that future research is required to further our understanding of the carbon dynamics and trade-offs among physiological, morphological and symbiotic root traits. It also illustrates the huge effects on plant growth that seemingly small variation in experimental methodology can cause, and the importance these details hold for designing experiments with the aim of simulating the behaviour of plants and root systems under field conditions (Black 1961). Finally, we suggest that it is likely that constrained canopy micro-swards should be routinely used in future experiments focussed on roots when data are required to be relevant to pasture swards in the field.

5) *Impacts of constrained canopy on shoot tissue element concentrations*

Constraining the canopy of micro-swards resulted in increased concentrations in shoot tissue of several macro and micro-nutrients, most notably P (discussed above), S, K, Na and Cu. It is possible that the increase in some of these nutrients was due to the increase in rhizosphere carboxylates. For instance, it has been suggested that carboxylates, mainly citrate and malate, could enhance mobilisation of Zn, Fe, Cu and Mn in some soils and circumstances (Marschner *et al.* 1987; Yang *et al.* 1994). However, there is no evidence of this in our study and no consistent reports of any correlation between the shoot concentration of any of these nutrients, except Mn and P?, and carboxylate exudation. Therefore, our understanding of the impact of increased rhizosphere carboxylates in constrained canopy micro-swards on shoot concentration of nutrients other than P remains limited.

It has been suggested that leaf tissue concentrations of Mn could be a proxy for carboxylate exudation and, therefore, be used as a relatively quick and simple means to rank plant genotypes for P-acquisition efficiency in low-P soils (Lambers *et al.* 2015a). However, there was no support for this suggestion in our experiment, as shoot Mn concentrations in *T. subterraneum* and *O. compressus* were not greater in the constrained canopy treatments,

where very large amounts of rhizosphere carboxylates were present. Perhaps plant Mn nutrition was greatly affected by the presence of AM fungi (Nazeri *et al.* 2014) and this masked a direct effect of carboxylate release on plant Mn uptake.

CONCLUSIONS

Constraining the canopy spread of micro-swards of *T. subterraneum* and *O. compressus* resulted in decreased root and shoot biomass and less dense roots with increased SRL. These effects are consistent with decreased carbon allocation to roots due to reduced rates of photosynthesis in the lower canopy due to shading. This supports our first hypothesis and provides an explanation for the increase in SRL and decrease in RTD previously observed in response to increased P supply in experiments where plants were grown in constrained canopy micro-swards (Jeffery *et al.* 2016) and larger plants at high P supply had a greater proportion of leaves shaded. In addition, a large increase in rhizosphere carboxylates when the canopy was constrained appears to have compensated for reduced root growth and enabled shoot P content to be maintained. This has not been observed previously and deserves further investigation, as it has implications for the field relevance of glasshouse experiments. Overall, our results support the routine use of constrained canopy micro-swards in glasshouse experiments which examine root traits of pasture legumes, as allowing plant shoots to expand beyond the confines of the pot changed root traits related to P acquisition in a low-P soil in a manner likely inconsistent with what would occur in a dense pasture sward in the field.

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494 REFERENCES

495 Argüello A, O'Brien MJ, Van der Heijden MGA, Wiemken A, Schmid B, Niklaus PA (2016)
496 Options of partners improve carbon for phosphorus trade in the arbuscular
497 mycorrhizal mutualism. *Ecology Letters* **19**, 648-656.

498 Black J (1961) Border and orientation effects and their elimination in experimental swards of
499 subterranean clover (*Trifolium subterraneum* L.). *Australian Journal of Agricultural*
500 *Research* **12**, 203-211.

501 Bolan NS, Robson AD, Barrow NJ (1984) Increasing phosphorus supply can increase the
502 infection of plant roots by vesicular-arbuscular mycorrhizal fungi. *Soil Biology and*
503 *Biochemistry* **16**, 419-420.

504 Bouma D, Dowling E, David D (1981) Relations between plant aluminium content and the
505 growth of lucerne and subterranean clover: their usefulness in the detection of
506 aluminium toxicities. *Australian Journal of Experimental Agriculture* **21**, 311-317.

507 Caradus JR (1980) Distinguishing between grass and legume species for efficiency of
508 phosphorus use. *New Zealand Journal of Agricultural Research* **23**, 75-81.

509 Cawthray GR (2003) An improved reversed-phase liquid chromatographic method for the
510 analysis of low-molecular mass organic acids in plant root exudates. *Journal of*
511 *Chromatography A* **1011**, 233-240.

512 Colwell J (1965) An automatic procedure for determination of phosphorus in sodium
513 hydrogen carbonate extracts of soils. Soc Chemical Industry London, England

514 Daft MJ, El-Giahmi AA (1978) Effect of arbuscular mycorrhiza on plant growth. *New*
515 *Phytologist* **80**, 365-372.

516 Demotes-Mainard S, Pellerin S (1992) Effect of mutual shading on the emergence of nodal
517 roots and the root/shoot ratio of maize. *Plant and Soil* **147**, 87-93.

518 Evans PS (1977) Comparative root morphology of some pasture grasses and clovers. *New*
519 *Zealand Journal of Agricultural Research* **20**, 331-335.

520 Facelli E, Facelli JM, Smith SE, McLaughlin MJ (1999) Interactive effects of arbuscular
521 mycorrhizal symbiosis, intraspecific competition and resource availability on
522 *Trifolium subterraneum* cv. Mt. Barker. *New Phytologist* **141**, 535-547.

523 Giovannetti M, Mosse B (1980) Evaluation of techniques for measuring vesicular arbuscular
524 mycorrhizal infection in roots. *New Phytologist* **84**, 489-500.

525 Graham JH, Leonard RT, Menge JA (1982) Interaction of light intensity and soil temperature
526 with phosphorus inhibition of vesicular-arbuscular mycorrhiza formation. *New*
527 *Phytologist* **91**, 683-690.
528 Haling RE, Yang Z, Shadwell N, Culvenor RA, Stefanski A, Ryan MH, Sandral GA, Kidd
529 DR, Lambers H, Simpson RJ (2016a) Growth and root dry matter allocation by
530 pasture legumes and a grass with contrasting external critical phosphorus
531 requirements. *Plant and Soil* 1-13. doi: 10.1007/s11104-016-2808-2
532 Haling RE, Yang Z, Shadwell N, Culvenor RA, Stefanski A, Ryan MH, Sandral GA, Kidd
533 DR, Lambers H, Simpson RJ (2016b) Root morphological traits that determine
534 phosphorus-acquisition efficiency and critical external phosphorus requirement in
535 pasture species. *Functional Plant Biology* **43**, 815-826.
536 Haling RE, Yang Z, Shadwell N, Simpson RJ, Culvenor RA, Stefanski A, Sandral GA, Kidd
537 D, Lambers H, Ryan M (2015) Phosphorus-efficient pastures: legume root traits for
538 improved nutrient foraging. In '*Building Productive, Diverse and Sustainable*
539 *Landscapes : Proceedings of the 17th Australian Agronomy Conference 2015, 21-24*
540 *September 2015, Hobart Tasmania*. (Eds T Acuña, C Moeller, D Parsons, M
541 Harrison) (Agronomy Australia: Warragul)
542 Hayman DS (1974) Plant growth responses to vesicular-arbuscular mycorrhiza. VI. Effect of
543 light and temperature. *The New Phytologist* **73**, 71-80.
544 Hill JO, Simpson RJ, Moore AD, Chapman DF (2006) Morphology and response of roots of
545 pasture species to phosphorus and nitrogen nutrition. *Plant and Soil* **286**, 7-19.
546 Hill JO, Simpson RJ, Ryan MH, Chapman DF (2010) Root hair morphology and mycorrhizal
547 colonisation of pasture species in response to phosphorus and nitrogen nutrition. *Crop*
548 *and Pasture Science* **61**, 122-131.
549 Hill JO, Simpson RJ, Wood JT, Moore AD, Chapman DF (2005) The phosphorus and
550 nitrogen requirements of temperate pasture species and their influence on grassland
551 botanical composition. *Australian Journal of Agricultural Research* **56**, 1027-1039.
552 Jeffery RP, Simpson RJ, Lambers H, Kidd DR, Ryan MH (2016) Root morphology
553 acclimation to phosphorus supply by six cultivars of *Trifolium subterraneum* L. *Plant*
554 *and Soil* 1-14. doi: 10.1007/s11104-016-2869-2
555 Kidd DR, Ryan MH, Haling RE, Haling R, Lambers H, Sandral GA, Yang Z, Culvenor RA,
556 Cawthray GR, Stefanski A, Simpson RJ (2016) Rhizosphere carboxylates and
557 morphological root traits in pasture legumes and grasses. *Plant and Soil* **402**, 77-89.

558 Konvalinková T, Jansa J (2016) Lights off for arbuscular mycorrhiza: on its symbiotic
559 functioning under light deprivation. *Frontiers in Plant Science* **7:782**, 1-11. Doi:
560 10.3389/fpls.2016.00782
561 Lambers H, Hayes PE, Laliberte E, Oliveira RS, Turner BL (2015a) Leaf manganese
562 accumulation and phosphorus-acquisition efficiency. *Trends in Plant Science* **20**, 83-
563 90.
564 Lambers H, Martinoia E, Renton M (2015b) Plant adaptations to severely phosphorus-
565 impoverished soils. *Current Opinion in Plant Biology* **25**, 23-31.
566 Lynch JP, Brown KM (2001) Topsoil foraging - an architectural adaptation of plants to low
567 phosphorus availability. *Plant and Soil* **237**, 225-237.
568 Marschner H, Römheld V, Cakmak I (1987) Root-induced changes of nutrient availability in
569 the rhizosphere. *Journal of Plant Nutrition* **10**, 1175-1184.
570 Martin BC, George SJ, Price CA, Shahsavari E, Ball AS, Tibbett M, Ryan MH (2016) Citrate
571 and malonate increase microbial activity and alter microbial community composition
572 in uncontaminated and diesel-contaminated soil microcosms. *Soil* **2**, 487-498.
573 McLaughlin MJ, McBeath TM, Smernik R, Stacey SP, Ajiboye B, Guppy C (2011) The
574 chemical nature of P accumulation in agricultural soils - implications for fertiliser
575 management and design: an Australian perspective. *Plant and Soil* **349**, 69-87.
576 Menezes-Blackburn D, Paredes C, Zhang H, Giles CD, Darch T, Stutter M, Wendler R
577 (2016) Organic acids regulation of chemical–microbial phosphorus transformations in
578 soils. *Environmental Science & Technology* **50**, 11521-11531.
579 Motomizu S, Wakimoto T, Toei K (1983) Spectrophotometric determination of phosphate in
580 river waters with molybdate and malachite green. *Analyst* **108**, 361-367.
581 Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G, Valori F (2008)
582 Effects of root exudates in microbial diversity and activity in rhizosphere soils. In
583 *Molecular mechanisms of plant and microbe coexistence*. Nautiyal and Dion P,
584 Springer Berlin Heidelberg **15**, 339-365.
585 Nazeri NK, Lambers H, Tibbett M, Ryan MH (2013) Do arbuscular mycorrhizas or
586 heterotrophic soil microbes contribute toward plant acquisition of a pulse of mineral
587 phosphate? *Plant and Soil* **373**, 699-710.
588 Nazeri NK, Lambers H, Tibbett M, Ryan MH (2014) Moderating mycorrhizas: arbuscular
589 mycorrhizas modify rhizosphere chemistry and maintain plant phosphorus status
590 within narrow boundaries. *Plant, Cell and Environment* **37**, 911-921.

591 Oburger E, Kirk GJD, Wenzel WW, Puschenreiter M, Jones DL (2009) Interactive effects of
592 organic acids in the rhizosphere. *Soil Biology & Biochemistry* **41**, 449-457.
593 Orchard S, Hilton S, Bending G, Dickie I, Standish R, Gleeson D, Jeffery RP, Powell J,
594 Walker C, Bass D, Monk J, Simonin A, Ryan MH (2017). Fine endophytes (*Glomus*
595 *tenue*) are related to Mucoromycotina, not Glomeromycota. *New Phytologist* **213**,
596 481–486.
597 Osborne GJ, Pratley JE, Stewart WP (1980) The tolerance of subterranean clover (*Trifolium*
598 *subterreneum* L.) to aluminium and manganese. *Field Crops Research* **3**, 347-358.
599 Pang JY, Tibbett M, Denton MD, Lambers H, Siddique KHM, Bolland MDA, Revell CK,
600 Ryan MH (2010) Variation in seedling growth of 11 perennial legumes in response to
601 phosphorus supply. *Plant and Soil* **328**, 133-143.
602 Paynter BH (1990) Comparative phosphate requirements of yellow serradella (*Ornithopus-*
603 *Compressus*), burr medic (*Medicago polymorpha* Var *brevispina*) and subterranean
604 clover (*Trifolium subterraneum*). *Australian Journal of Experimental Agriculture* **30**,
605 507-514.
606 Poorter H, Fiorani F, Stitt M, Schurr U, Finck A, Gibon Y, Usadel B, Munns R, Atkin OK,
607 Tardieu F, Pons TL (2012*a*) The art of growing plants for experimental purposes: a
608 practical guide for the plant biologist. *Functional Plant Biology* **39**, 821-838.
609 Poorter H, Niklas KJ, Reich PB, Oleksyn J, Poot P, Mommer L (2012*b*) Biomass allocation
610 to leaves, stems and roots: meta-analyses of interspecific variation and environmental
611 control. *New Phytologist* **193**, 30-50.
612 Rayment GE, Lyons DJ (2011) 'Soil chemical methods : Australasia (Series: Australian soil
613 and land survey handbook; v. 3.).' (CSIRO Publishing: Collingwood, Victoria)
614 Rossiter RC (1974) The relative success of strains of *Trifolium subterraneum* L. in binary
615 mixtures under field conditions. *Crop and Pasture Science* **25**, 757-766.
616 Rovira A (1959) Root excretions in relation to the rhizosphere effect. *Plant and Soil* **11**, 53-
617 64.
618 Ryan MH, Kidd DR, Sandral GA, Yang Z, Lambers H, Culvenor RA, Stefanski A, Nichols
619 PGH, Haling RE, Simpson RJ (2016) High variation in the percentage of root length
620 colonised by arbuscular mycorrhizal fungi among 139 lines representing the species
621 subterranean clover (*Trifolium subterraneum*). *Applied Soil Ecology* **98**, 221-232.
622 Ryan MH, Tibbett M, Edmonds-Tibbett T, Suriyagoda LDB, Lambers H, Cawthray GR,
623 Pang J (2012) Carbon trading for phosphorus gain: the balance between rhizosphere

624 carboxylates and arbuscular mycorrhizal symbiosis in plant phosphorus acquisition.
625 *Plant, Cell and Environment* **35**, 2170-2180.
626 Schweiger PF, Robson AD, Barrow NJ (1995) Root hair length determines beneficial effect
627 of a *Glomus* species on shoot growth of some pasture species. *New Phytologist* **131**,
628 247-254.
629 Simpson RJ, Oberson A, Culvenor RA, Ryan MH, Veneklaas EJ, Lambers H, Lynch JP,
630 Ryan PR, Delhaize E, Smith FA, Smith SE, Harvey PR, Richardson AE (2011)
631 Strategies and agronomic interventions to improve the phosphorus-use efficiency of
632 farming systems. *Plant and Soil* **349**, 89-120.
633 Stern W (1965) The effect of density on the performance of individual plants in subterranean
634 clover swards. *Australian Journal of Agricultural Research* **16**, 541-555.
635 Stonor RN, Smith SE, Manjarrez M, Facelli E, Smith FA (2014) Mycorrhizal responses in
636 wheat: shading decreases growth but does not lower the contribution of the fungal
637 phosphate uptake pathway. *Mycorrhiza* **24**, 465-472.
638 Tester M, Smith FA, Smith SE (1985) Phosphate inflow into *Trifolium subterraneum* L.:
639 Effects of photon irradiance and mycorrhizal infection. *Soil Biology and Biochemistry*
640 **17**, 807-810.
641 Tester M, Smith SE, Smith FA, Walker NA (1986) Effects of photon irradiance on the
642 growth of shoots and roots, on the rate of initiation of mycorrhizal infection and on
643 the growth of infection units in *Trifolium subterraneum* L. *New Phytologist* **103**, 375-
644 390.
645 van der Heijden MGA, Martin FM, Selosse M-A, Sanders IR (2015) Mycorrhizal ecology
646 and evolution: the past, the present, and the future. *New Phytologist* **205**, 1406-1423.
647 Veneklaas EJ, Stevens J, Cawthray GR, Turner S, Grigg AM, Lambers H (2003) Chickpea
648 and white lupin rhizosphere carboxylates vary with soil properties and enhance
649 phosphorus uptake. *Plant and Soil* **248**, 187-197.
650 Vierheilig H, Coughlan AP, Wyss U, Piche Y (1998) Ink and vinegar, a simple staining
651 technique for arbuscular-mycorrhizal fungi. *Applied and Environmental Microbiology*
652 **64**, 5004-5007.
653 Waddell HA, Simpson RJ, Ryan MH, Lambers H, Garden DL, Richardson AE (2016) Root
654 morphology and its contribution to a large root system for phosphorus uptake by
655 Rytidosperma. *Plant and Soil* 1-13. doi: 10.1007/s11104-016-2933-y

Yang X, Römheld V, Marschner H (1994) Effect of bicarbonate on root growth and accumulation of organic acids in Zn-inefficient and Zn-efficient rice cultivars (*Oryza sativa* L.). *Plant and Soil* **164**, 1-7.

Yang Z, Culvenor RA, Haling RE, Stefanski A, Ryan MH, Sandral GA, Kidd DR, Lambers H, Simpson RJ (2015) Variation in root traits associated with nutrient foraging among temperate pasture legumes and grasses. *Grass and Forage Science*. doi: 10.1111/gfs.12199

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669 **Table. 1.** Probability values of treatment effects and interactions (*P=0.05,* three-way
670 ANOVA) of all parameters measured for unconstrained and constrained canopy micro-
671 swards of *Trifolium subterraneum* L. and *Ornithopus compressus* L. grown with 15 and 60
672 mg kg soil^{-1} phosphorus (P) supply for eight weeks in a low-P field soil. Note - l.s.d. only
673 provided where significant.

	C	S	P	C×S	C×P	S×P	C×S×P	l.s.d.
Shoot DM	0.019	<.001	<.001	ns	ns	0.042	ns	
Root DM	<.001	<.001	<.001	ns	0.002	ns	ns	
RMF	<.001	ns	0.011	ns	ns	ns	ns	
TRL	<.001	0.047	<.001	ns	0.017	ns	ns	
SRL	<.001	<.001	0.004	ns	ns	<.001	ns	
RTD	0.01	<.001	<.001	ns	0.020	0.009	ns	
ARD	0.004	<.001	ns	0.031	0.076	ns	ns	
ARHL	ns	<.001	ns	ns	ns	ns	ns	
Shoot P content	0.009	0.009	<.001	ns	ns	ns	ns	
Total carboxylates								
(cm^{-1} RL)	<.001	0.040	ns	ns	ns	0.036	0.035	17.6
(micro-sward^{-1})	<.001	ns	ns	ns	ns	ns	ns	
Water use	<.001	<.001	0.002	0.332	0.022	0.006	0.047	4.9
AM fungal colonisation	ns	0.022	<.001	ns	ns	<.001	ns	
Shoot concentration								
P	<.001	0.013	ns	ns	0.005	ns	ns	
K	<.001	<.001	ns	ns	<.001	ns	ns	
S	<.001	<.001	0.023	ns	ns	ns	ns	
Ca	<.001	<.001	<.001	ns	ns	ns	ns	
Mg	0.003	<.001	0.005	ns	ns	ns	ns	
Cu	<.001	ns	<.001	ns	0.014	ns	ns	
Zn	0.003	<.001	<.001	ns	ns	ns	ns	
Mn	ns	ns	ns	ns	ns	ns	ns	
Mo	0.013	<.001	ns	0.046	ns	ns	ns	
Al	ns	ns	ns	ns	ns	ns	ns	
Na	<.001	0.025	ns	ns	<.001	0.002	ns	
Co	ns	ns	0.012	ns	ns	ns	ns	
Fe	ns	ns	ns	ns	ns	ns	ns	

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679 **Table. 2.** The mean shoot element concentrations of unconstrained (UC) and constrained
680 (CC) shoot canopy micro-swards for *Trifolium subterraneum* L. and *Ornithopus compressus*
681 L. grown with 15 and 60 mg kg soil^{-1} phosphorus (P) supply (P15 and P60) for eight weeks in
682 a low-P field soil (mean, n=5). No more than three outliers removed from each parameter.

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	Trifolium subterraneum L.				*Ornithopus compressus* L.			
	P15		P60		P15		P60	
Element	**UC**	**CC**	**UC**	**CC**	**UC**	**CC**	**UC**	**CC**
Macro (mg g^{-1} shoot DM)								
P	1.68	2.12	1.48	2.45	1.83	2.34	1.72	2.70
K	27.56	35.88	24.50	39.51	30.07	40.45	27.40	42.58
S	2.36	2.64	2.12	2.61	3.48	3.76	3.01	3.73
Ca	9.77	10.97	8.89	10.11	10.93	13.07	10.18	10.72
Mg	4.23	4.65	3.93	4.09	4.78	5.49	4.51	4.99
Micro (μg g^{-1} shoot DM)								
Cu	6.8	7.7	4.2	6.5	7.2	7.7	4.3	6.6
Zn	42	53	52	62	56	69	75	74
Mn	188	227	186	205	212	229	215	172
Mo	10	10	10	10	20	20	20	30
Al	990	860	1120	1090	1160	1760	1430	1110
Na	4280	5720	3380	6030	3650	4770	3630	6060
Co	0.5	0.6	0.8	1.0	0.6	0.6	0.7	0.6
Fe	1080	530	770	1030	730	1000	790	660

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FIGURE CAPTIONS

Fig. 1 The impact of unconstrained and constrained shoot canopy micro-swards on shoot dry mass (DM) (a) and root DM (b) for *Trifolium subterraneum* cv. Riverina (*T*) and *Ornithopus compressus* cv. Santorini (*O*) grown with 15 and 60 mg kg soil^{-1} phosphorus (P) supply for eight weeks in a low-P field soil (mean ± s.e., n=5); statistical outcomes are reported in Table 1.

Fig. 2 The impact of unconstrained and constrained canopy micro-swards on root mass fraction (RMF) (a), total root length (TRL) (b), specific root length (SRL) (c), root tissue density (RTD) (d), average root diameter (ARD) (e) and average root hair length (ARHL) (f) for *Trifolium subterraneum* L. (*T*) and *Ornithopus compressus* L. (*O*) grown with 15 and 60 mg kg soil^{-1} phosphorus (P) supply for eight weeks in a low-P field soil (mean ± s.e., n=5): statistical outcomes are reported in Table 1. One outlier was removed from (c) and (d).

Fig. 3 The impact of unconstrained and constrained canopy micro-swards on shoot phosphorus (P) content for *Trifolium subterraneum* L. (*T*) and *Ornithopus compressus* L. (*O*) grown with 15 and 60 mg kg soil^{-1} P supply for eight weeks in a low-P field soil (mean ± s.e., n=5): statistical outcomes are reported in Table 1. Two outliers were removed.

Fig. 4 The impact of unconstrained and constrained canopy micro-swards on the composition of total rhizosphere carboxylates (a), total amount of rhizosphere carboxylates per unit root length (b) and total amount of carboxylates per micro-sward/pot (c) for *Trifolium subterraneum* L. (*T*) and *Ornithopus compressus* L. (*O*) grown with 15 and 60 mg kg soil^{-1} phosphorus (P) application (P15, P60), for eight weeks in a low-P field soil (mean ± s.e., n=5, l.s.d. at P=0.05): statistical outcomes are reported in Table 1.

Fig. 5 The impact of unconstrained and constrained canopy micro-swards on water use in the two weeks prior to harvest (per microsward) (a) and the percentage of root length colonised by arbuscular mycorrhizal (AM) fungi (b) for *Trifolium subterraneum* L. (*T*) and *Ornithopus compressus* L. (*O*) grown with 15 and 60 mg kg soil^{-1} phosphorus (P) application for eight weeks in a low-P field soil (mean ± s.e., n=5): statistical outcomes are reported in Table 1. Two outliers were removed from (b).

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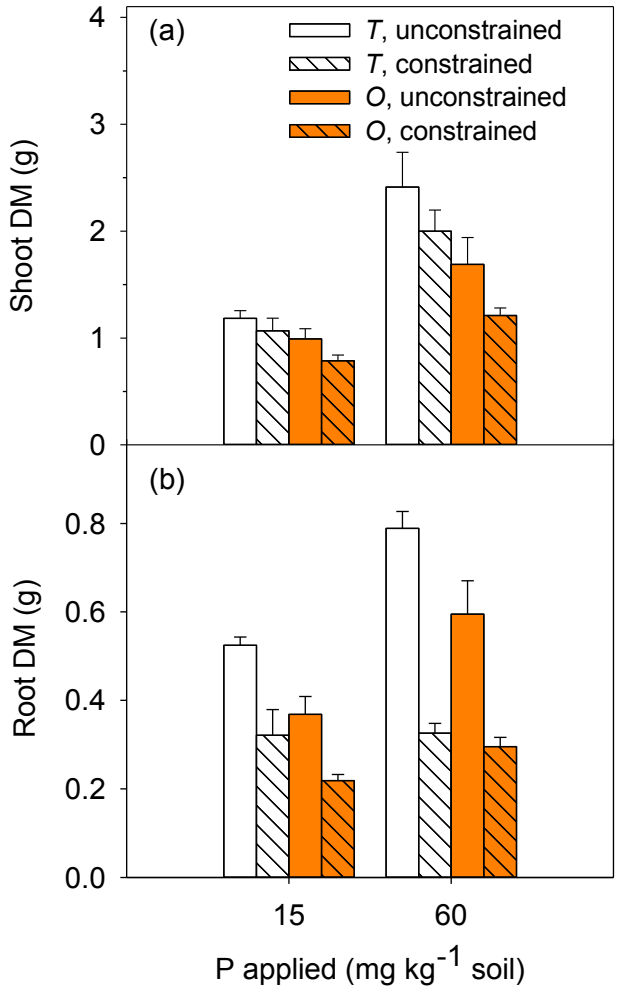

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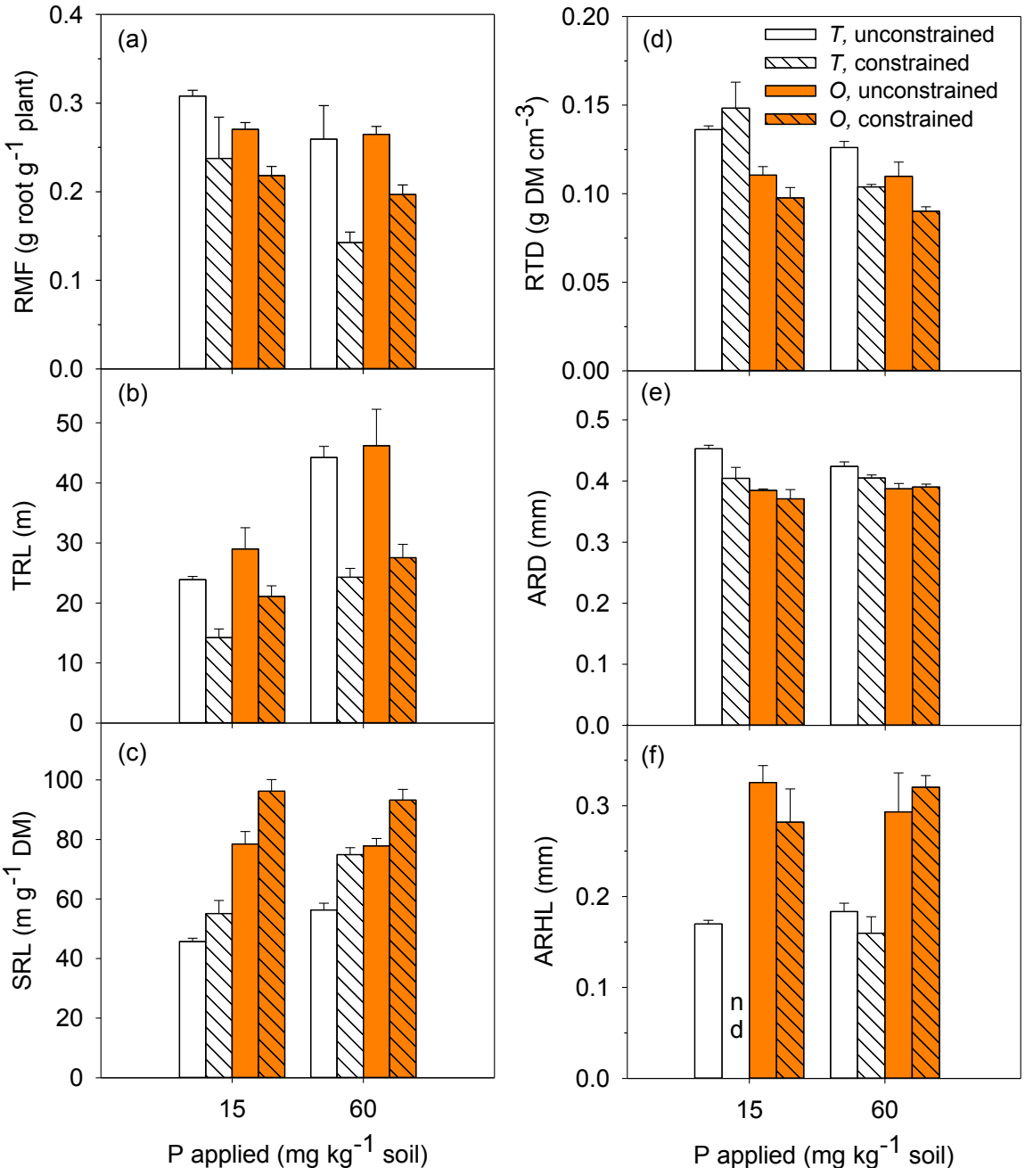
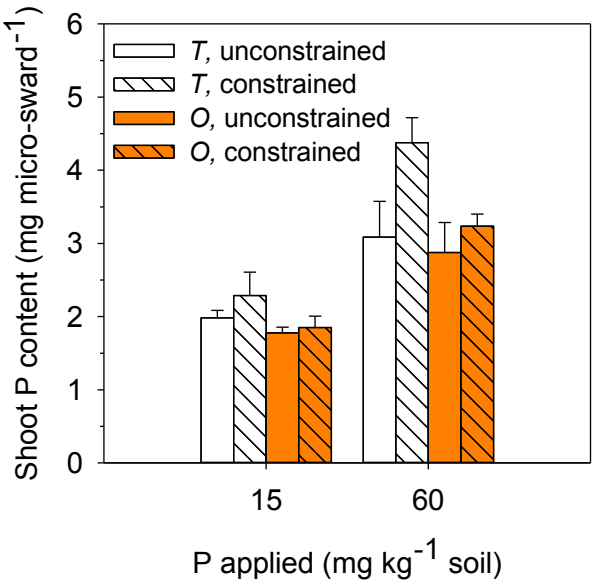

(a)
RMF (g root g-1 plant)
0.4
0.3
0.2
0.1
0.0
(b)
TRL (m)
50
40
30
20
10
0
(c)
SRL (m g-1 DM)
100
80
60
40
20
0
15
60
P applied (mg kg-1 soil)
(d)
RTD (g DM cm-3)
0.20
0.15
0.10
0.05
0.00
T, unconstrained
T, constrained
O, unconstrained
O, constrained
(e)
ARD (mm)
0.5
0.4
0.3
0.2
0.1
0.0
(f)
ARHL (mm)
0.3
0.2
0.1
0.0
n
d
15
60
P applied (mg kg-1 soil)

722

723

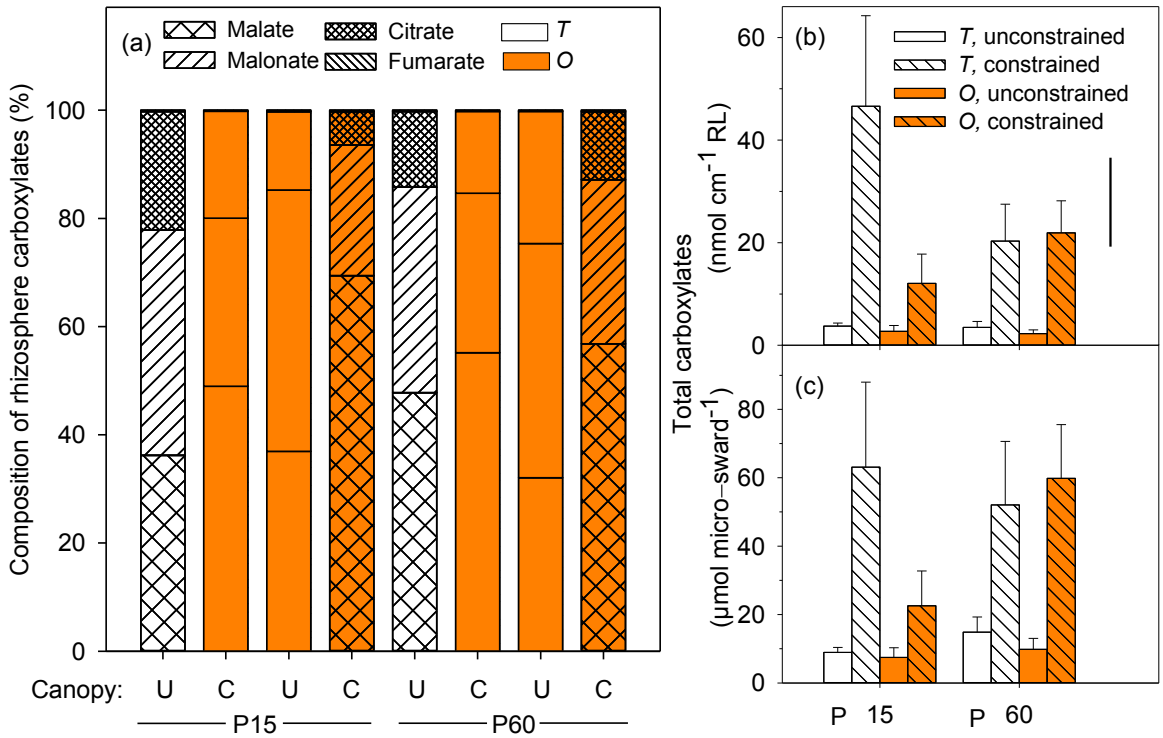
724


Shoot P content (mg micro-sward-1)
6
5
4
3
2
1
0
T, unconstrained
T, constrained
O, unconstrained
O, constrained
15
60
P applied (mg kg-1 soil)

725

726

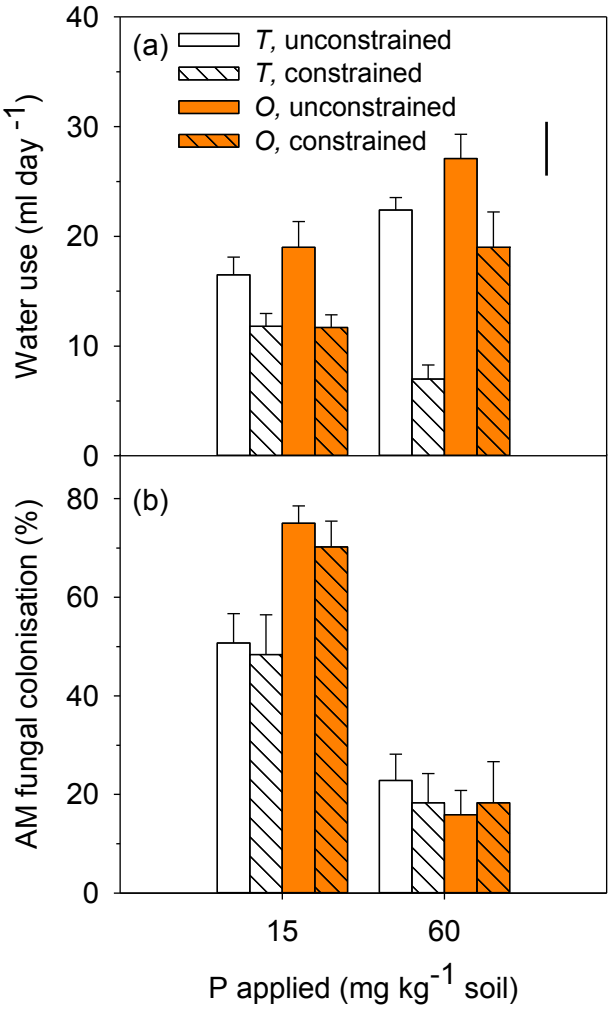
727


(a)
Malate
Malonate
Citrate
Fumarate
T
O
Composition of rhizosphere carboxylates (%)
100
80
60
40
20
0
Canopy: U C U C U C U C
P15
P60
(b)
T, unconstrained
T, constrained
O, unconstrained
O, constrained
Total carboxylates (nmol cm-1 RL)
60
40
20
0
(c)
(µmol micro–sward-1)
80
60
40
20
0
P 15
P 60

728

729

730



731

732

733

734