

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

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19 **Summary text for table of contents**

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

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32 ABSTRACT

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

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

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

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

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

53 **Abbreviations:** Arbuscular mycorrhizal (AM), specific root length (SRL), total root length
54 (TRL), root tissue density (RTD), average root diameter (ARD), average root hair length
55 (ARHL), root mass fraction (RMF), dry mass (DM) and 15, 60 mg P kg⁻¹ dry soil (P15 and
56 P60).

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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.* 2016a, 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; *Claroideoglossum 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

124 bicarbonate-extractable P (8 mg P kg⁻¹ dry soil) (Colwell 1965), a high P-buffering index
125 (PBI = 348), a pH in CaCl₂ of 5.2 and 7 mg kg⁻¹ nitrate-N, 12 mg kg⁻¹ ammonium-N and 118
126 mg kg⁻¹ bicarbonate-extractable K.

127 Cylindrical free-draining PVC pots (90 mm diameter × 200 mm depth) were filled with 700 g
128 of the oven-dried soil, which was then wet with 200 ml of deionised water; 300 g of dry soil
129 were then added on top of the wetted soil. Two P supply treatments were established prior to
130 sowing. In order to mimic the stratification of P in topsoil that occurs under pastures
131 (McLaughlin *et al.* 2011) an 80 ml solution of KH₂PO₄, sufficient to saturate the top 300 g of
132 soil, was applied at either 15 or 60 mg P kg⁻¹ dry soil (P15 or P60). Total K was balanced to
133 100 mg kg⁻¹ among P treatments with KCl (Pang *et al.* 2010) and all essential nutrients
134 except P were provided at final concentrations of (mg kg⁻¹ dry soil): N 30, S 50, Ca 45, Mg
135 10, Cu 0.5, Zn 7, Mn 4, B 0.12, Mo 0.4 and Fe 5. Nitrogen was added as a mixture of
136 NH₄NO₃ and Ca(NO₃)₂ with a molar ratio of 1 to provide an initial supply after germination,
137 prior to nodulation.

138 *Experimental design and canopy treatments*

139 Ten seeds per pot were sown and thinned to six seedlings following emergence; hence a
140 micro-sward was established in each pot. *Trifolium subterraneum* and *O. compressus* seeds
141 were inoculated with a slurry of peat-based inoculum of Group C or S¹ rhizobia (Becker
142 Underwood, Somersby, New South Wales, Australia), respectively. The soil surface was
143 covered with a thin layer of white alkathene beads to minimise evaporation. Half of the pots
144 of each species and P-treatment combination were sheathed with sleeves with a reflective
145 inner surface. Sleeve height was adjusted to that of the micro-sward daily with the intention
146 of constraining canopy spread of the micro-sward, as would occur in a large area of dense
147 pasture sward in the field (Rossiter 1974; Hill *et al.* 2005). There were five replicates of each
148 treatment combination.

149 Plants were grown for eight weeks from 3 August 2015 in a glasshouse maintained between
150 16°C and 23°C at The University of Western Australia, Perth, Australia (S 31° 98' – E 115°
151 81'). Pots were watered by weight to 80% of pot capacity, defined as the water content of a
152 saturated pot that has ceased draining, with deionised water three times a week and rotated
153 randomly within replicates weekly to minimise the effects of temperature and light gradients
154 within the glasshouse (Poorter *et al.* 2012a). Pot weights recorded during the final two weeks
155 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 CaCl₂ 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 al.*
177 *et 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 HNO₃:HClO₄ 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

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

225 RESULTS

226 *Plant growth*

227 Although *T. subterraneum* had greater shoot DM than did *O. compressus*, the impact of
228 constraining the canopy on plant growth and root morphology was similar for both species.
229 Shoot DM of constrained canopy micro-swards was less, relative to that of unconstrained
230 swards, by 10-17% for *T. subterraneum* and 21-28% for *O. compressus* (Fig. 1a). Shoot DM
231 was greater (53-103%) at P60 than at P15, for all treatments. Root DM of constrained canopy
232 micro-swards of both species was less, relative to that of unconstrained micro-swards, by 39-
233 59% (Fig. 1b). Root DM also varied less among constrained canopy micro-swards (0.22-0.33
234 g DM) than among unconstrained micro-swards (0.37-0.79 g), and was greater (51-61% for
235 unconstrained and 2-35% for constrained micro-swards) at P60 than at P15. The RMF of
236 constrained canopy micro-swards was 18-46% less than that of unconstrained micro-swards;
237 this difference was greatest for *T. subterraneum* at P60 and <23% for all other treatments
238 (Fig. 2a). For *T. subterraneum* and *O. compressus* (respectively), RMF was less at P60 than
239 at P15 to a greater extent for constrained (41% and 9% less) than for unconstrained (16% and
240 4% less) canopy micro-swards.

241 *Root morphology*

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

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

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

255 There was little variation in ARD (0.37-0.45 mm) among all treatment combinations (Fig.
256 2e). Constraining the canopy did not affect the ARD of *O. compressus* at P60, but at P15
257 resulted in it being 2.6% less than that of unconstrained canopy micro-swards. For *T.*
258 *subterraneum*, ARD was 11.1% less at P15 and 2.4% less at P60 when the canopy was
259 constrained. There was no significant effect of P supply on ARD.

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

263 *Plant phosphorus content*

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

269 *Rhizosphere soil carboxylates*

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

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

278 The total amount of all rhizosphere carboxylates measured at a detectable level was low (2.3-
279 3.7 nmol cm⁻¹ RL) (16.9-21.2 μmol g⁻¹ root DM) for all unconstrained canopy micro-swards
280 of both species (Fig. 4b). However, the total amount of all rhizosphere carboxylates, per
281 micro-sward and per unit root length, was 202-648% and 348-1200% greater, respectively,
282 when the canopy was constrained (12-48 nmol cm⁻¹ RL; 117-214 μmol g⁻¹ root DM). Impacts
283 of species and P supply were relatively small or absent.

284 *Water use*

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

290 *Colonisation of roots by arbuscular mycorrhizal fungi*

291 At P15, colonisation by AM fungi was greater for *O. compressus* (70-75%) than for *T.*
292 *subterraneum* (48-51%) (Fig. 5b). However, AM fungal colonisation at P60 was similar (16-
293 23%) for both species. There was no effect of constraining the shoots.

294 *Shoot element concentrations*

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

301 The shoot Al concentration (860-1760 μg g⁻¹ DM) was high (Osborne *et al.* 1980; Bouma *et*
302 *al.* 1981) for all micro-swards, with no consistent trend and no significant effect of any
303 treatment. Shoot Mo concentration was two- to three-fold greater for *O. compressus* than for
304 *T. subterraneum* and was independent of canopy and P supply for both species, with the
305 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

337 effect on ARD and ARHL. The reduced RMF and SRL are a reflection of shoot and root DM,
338 which both decreased when canopies were constrained, especially at P60 when plants were
339 taller. This is consistent with lower rates of photosynthesis in shaded leaves causing
340 decreased carbon allocation to the roots (Poorter *et al.* 2012b). For *T. subterraneum*, our
341 findings are consistent with previous research where reduced light intensity resulting from
342 decreased irradiance or increased plant density decreased root DM, root/shoot ratio and
343 initiation of lateral roots (Stern 1965; Tester *et al.* 1986; Demotes-Mainard and Pellerin
344 1992). Therefore, our results provide support for the increase in SRL and decrease in RTD
345 with increased P supply observed in other studies of constrained micro-swards of *T.*
346 *subterraneum* (Haling *et al.* 2016b; Jeffery *et al.* 2016) being a result of larger plants at high
347 P supply causing a greater proportion of leaves within the micro-sward canopy to be shaded.

348 The ARHL of *T. subterraneum* (0.16-0.18 mm) and *O. compressus* (0.28-0.33 mm) was also
349 comparable with other studies; 0.15-0.27 and 0.48 mm for unconstrained canopy micro-
350 swards of *T. subterraneum* and *O. compressus*, respectively (Evans 1977; Schweiger *et al.*
351 1995; Hill *et al.* 2010; Yang *et al.* 2015; Ryan *et al.* 2016). Longer ARHL (0.75 mm) has
352 been measured for *O. compressus* grown for four weeks in a controlled environment cabinet,
353 although the ARD of *T. subterraneum* (0.23 mm) in the same study was similar to our
354 observations (Haling *et al.* 2015). Constraining the canopy had no impact on ARHL,
355 indicating that the assessment of pasture legumes for root hair length can be undertaken
356 without constraining the canopy. Therefore values of ARHL measured for unconstrained
357 canopy micro-swards in the glasshouse may differ to those in field conditions as a result of
358 differences in P supply or growth media, but not canopy constraint.

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

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

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

369 to decreased irradiance has often been reported for *T. subterraneum* (Tester *et al.* 1985;
370 Tester *et al.* 1986) and many other plant species (Hayman 1974; Daft and El-Giahmi 1978;
371 Graham *et al.* 1982). However, there are exceptions; Facelli *et al.* (1999) found no impact of
372 decreased (by >50%) light intensity, and a negative impact of plant density independent of
373 light, on AM fungal colonisation of *T. subterraneum*. Similarly, Stonor *et al.* (2014) reported
374 that shade-induced (72 to 262 $\mu\text{mol m}^{-2} \text{s}^{-1}$) reductions in photosynthetic carbon availability
375 had no effect on AM fungal colonisation, arbuscule development or mycorrhizal growth
376 response for *Triticum aestivum* compared to that of unshaded (325 to 1025 $\mu\text{mol m}^{-2} \text{s}^{-1}$)
377 conditions. These contrasting results might reflect the light intensity in the control or shading
378 treatments, which may have been mild or strong, respectively. However, considering that the
379 >50% reduction in light intensity (400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ unshaded and 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ shaded)
380 in Facelli *et al.* (1999) was similar to the intensity (from 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ unshaded and 100
381 $\mu\text{mol m}^{-2} \text{s}^{-1}$ shaded) which decreased AM fungal colonisation for *T. subterraneum* in
382 previous studies (Tester *et al.* 1985; Tester *et al.* 1986) this seems unlikely. An alternative
383 explanation is that the effect of shading is less for less carbon-demanding AM fungi (van der
384 Heijden *et al.* 2015; Argüello *et al.* 2016).

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

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

391 In the constrained treatments, the total amount of carboxylates in the rhizosphere soil was
392 unexpectedly high, especially for *T. subterraneum* (20-45 $\text{nmol cm}^{-1} \text{RL}$ and 17-214 $\mu\text{mol g}^{-1}$
393 root DM), which was previously reported to have 10-20 $\mu\text{mol g}^{-1}$ root DM in unconstrained
394 (Nazeri *et al.* 2014; Kidd *et al.* 2016) and <1.6 $\text{nmol cm}^{-1} \text{RL}$ in constrained (Jeffery *et al.*
395 2016) canopy micro-swards. *Ornithopus compressus* was previously reported to have 4 nmol
396 $\text{cm}^{-1} \text{RL}$ total carboxylates in unconstrained canopy micro-swards (Kidd *et al.* 2016).

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

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

408 We found that constraining the canopy decreased root DM by up to 59%, but had no effect on
409 shoot P content (Fig. 3), even at P15, when P availability was limiting plant growth. Total P
410 content of shoots and whole plants was shown to increase with increased P supply for *T.*
411 *subterraneum* and *O. compressus* (Caradus 1980; Paynter 1990). Therefore our results
412 suggest that the increase in rhizosphere carboxylates in the constrained canopy treatment may
413 have solubilised P from soil that was previously unavailable to plant roots (Veneklaas *et al.*
414 2003; Lambers *et al.* 2015b) and compensated for the reduction in root biomass and length.
415 This scenario is supported by shoot P concentration and content following a similar trend to
416 total rhizosphere carboxylates. It also appears the most likely explanation for the lack of
417 change in P uptake, as there was no change in AM fungal colonisation and daily water-use
418 was greater for unconstrained micro-swards than constrained, suggesting that movement of P
419 to roots by mass flow would also have been decreased in the constrained canopy micro-
420 swards. The decreased shoot P concentration at P60 compared with P15 for all unconstrained
421 canopy micro-swards (Table 2) is presumably due to dilution of P within the greater shoot
422 DM.

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

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

433 2016). This could be tested in future studies by measuring carboxylate exudation from the
434 intact root system into a trap solution, after the rhizosphere is washed from the roots
435 (Nannipieri *et al.* 2008).

436 Overall, our results indicate that when the canopy was constrained a large increase in amount
437 of rhizosphere carboxylates acted as a substitute for root length and root DM in terms of plant
438 ability to access P. This most likely accounted for the lack of difference in shoot P content
439 and concentration between constrained and unconstrained micro-swards. This novel finding
440 suggests that future research is required to further our understanding of the carbon dynamics
441 and trade-offs among physiological, morphological and symbiotic root traits. It also
442 illustrates the huge effects on plant growth that seemingly small variation in experimental
443 methodology can cause, and the importance these details hold for designing experiments with
444 the aim of simulating the behaviour of plants and root systems under field conditions (Black
445 1961). Finally, we suggest that it is likely that constrained canopy micro-swards should be
446 routinely used in future experiments focussed on roots when data are required to be relevant
447 to pasture swards in the field.

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

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

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

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

467 CONCLUSIONS

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

484

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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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Element	<i>Trifolium subterraneum</i> L.				<i>Ornithopus compressus</i> L.			
	P15		P60		P15		P60	
	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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687 FIGURE CAPTIONS

688 **Fig. 1** The impact of unconstrained and constrained shoot canopy micro-swards on shoot dry
689 mass (DM) (a) and root DM (b) for *Trifolium subterraneum* cv. Riverina (*T*) and *Ornithopus*
690 *compressus* cv. Santorini (*O*) grown with 15 and 60 mg kg soil⁻¹ phosphorus (P) supply for
691 eight weeks in a low-P field soil (mean ± s.e., n=5); statistical outcomes are reported in Table
692 1.

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

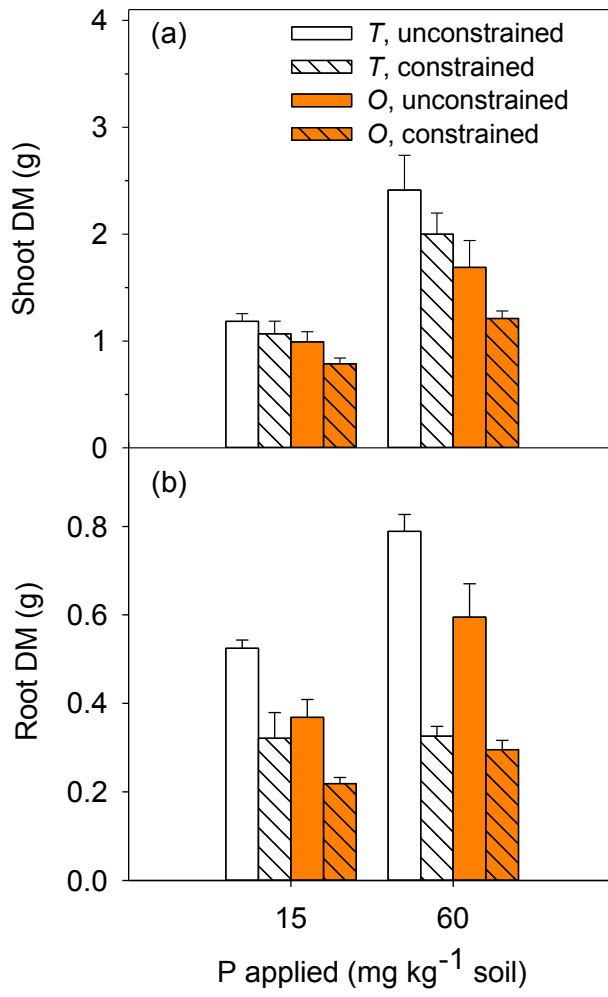
699 **Fig. 3** The impact of unconstrained and constrained canopy micro-swards on shoot
700 phosphorus (P) content for *Trifolium subterraneum* L. (*T*) and *Ornithopus compressus* L. (*O*)
701 grown with 15 and 60 mg kg soil⁻¹ P supply for eight weeks in a low-P field soil (mean ± s.e.,
702 n=5): statistical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

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

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

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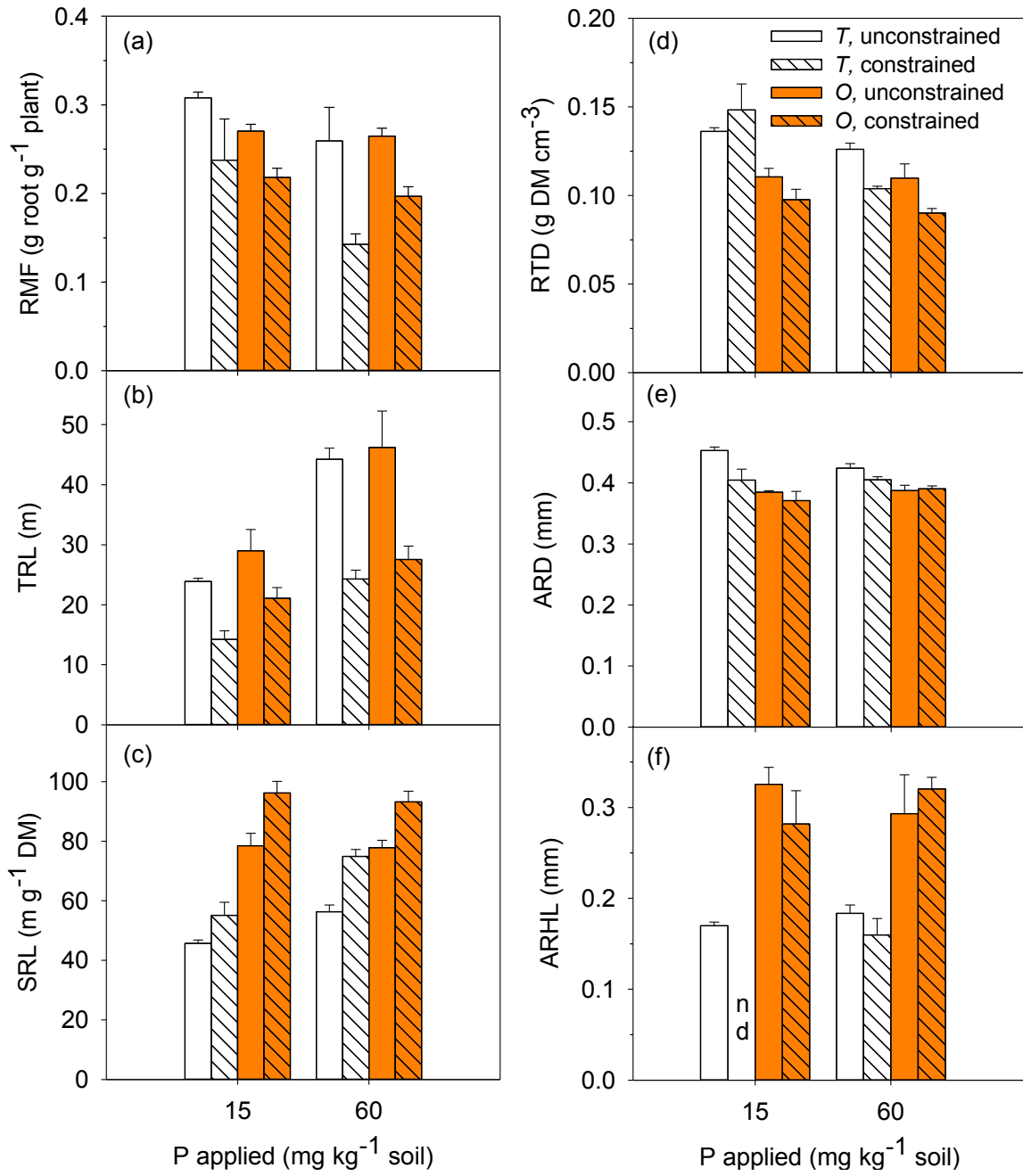


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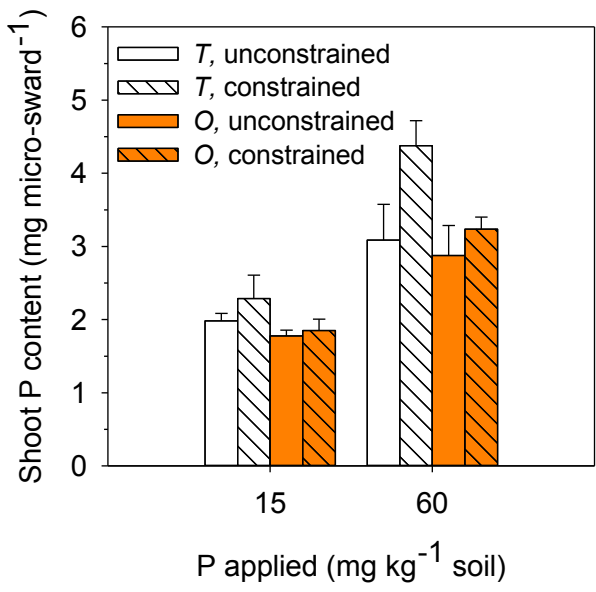
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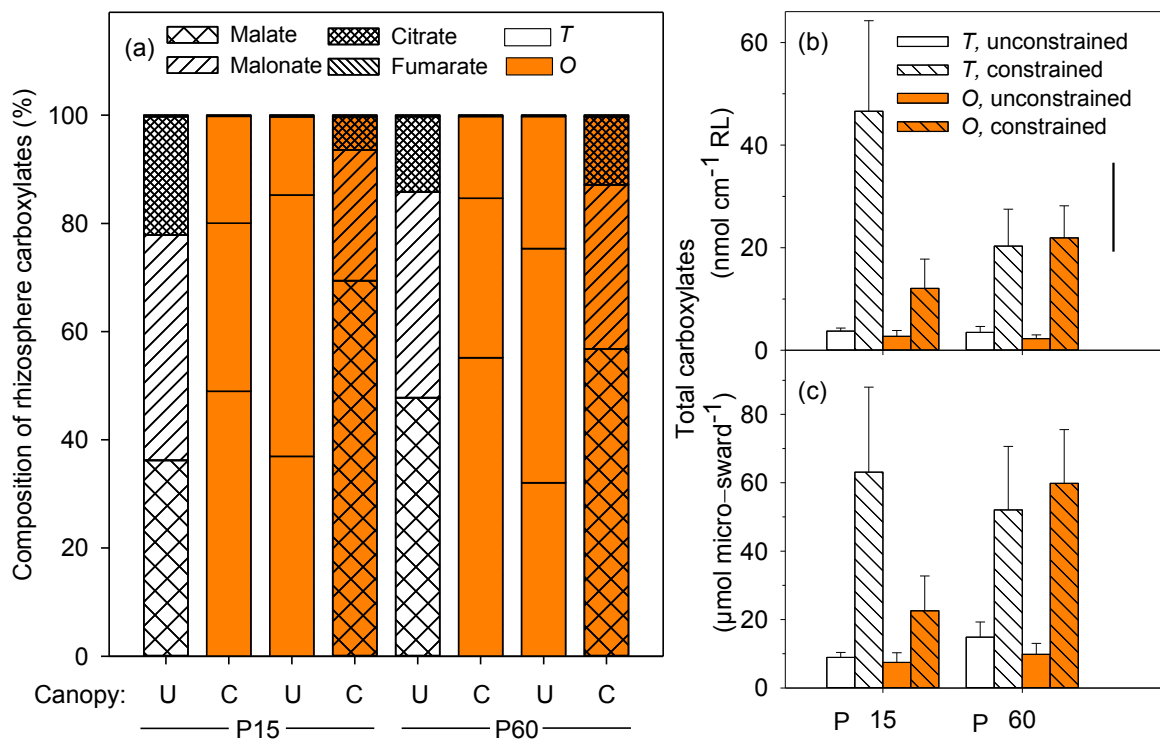
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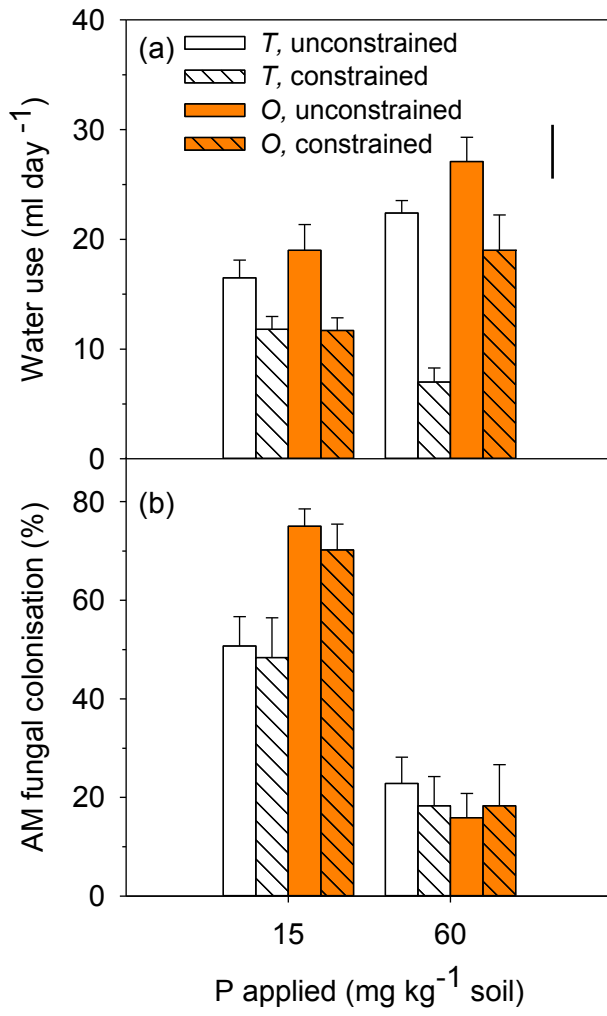
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