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Rebecca E. Miller, Roslyn M. Gleadow and Timothy R. Cavagnaro

Age versus stage: does ontogeny modify the effect of phosphorus and arbuscular mycorrhizas on above- and below-ground defence in forage sorghum?

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2 Age versus stage: does ontogeny modify the effect of phosphorus 3 and arbuscular mycorrhizas on above- and below-ground defence 4 in forage sorghum?

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6 REBECCA E. MILLER*, ROSLYN M. GLEADOW AND TIMOTHY R. CAVAGNARO

7

8 School of Biological Sciences, Monash University, Clayton, Vic, 3800, Australia

9

10 * Corresponding author: Rebecca Miller, rebecca.miller@monash.edu Tel: +61 3 99055217

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12

13 RUNNING HEAD

14 Effect of P and AM on defence

15

16 ## ABSTRACT

17

18 Arbuscular mycorrhizas (AM) can increase plant acquisition of P and N. No published studies
19 have investigated the impact of P and AM on the allocation of N to the plant defence,
20 cyanogenic glucosides. We investigated the effects of soil P and AM on cyanogenic glucoside
21 (dhurrin) concentration in roots and shoots of two forage sorghum lines differing in
22 cyanogenic potential (HCNp). Two harvest times allowed plants grown at high and low P to
23 be compared at the same age and the same size, to take account of known ontogenetic changes
24 in shoot HCNp. P responses were dependent on ontogeny and tissue type. At the same age, P-
25 limited plants were smaller and had higher shoot HCNp but lower root HCNp.
26 Ontogenetically controlled comparisons showed a P effect of lesser magnitude, and that there
27 was also an increase in the allocation of N to dhurrin in shoots of P-limited plants.
28 Colonisation by AM had little effect on shoot HCNp, but increased root HCNp and the
29 allocation of N to dhurrin in roots. Divergent responses of roots and shoots to P, AM and with
30 ontogeny demonstrate the importance of broadening the predominantly foliar focus of plant
31 defence studies/theory, and of ontogenetically controlled comparisons.

32

33

34

35 ## KEYWORDS

36 arbuscular mycorrhizas (AM), cyanogenesis, dhurrin, ontogeny, phenotypic plasticity,
37 phosphorus, plant defence, roots, resource allocation, sorghum

38 ## INTRODUCTION

39 Central to the field of plant defence are theories that attempt to explain and predict the
40 allocation of resources to chemical defence in response to environmental variation, both
41 among and within species. Intra-specifically, phenotypic variation in concentrations of
42 different chemical defence compounds is predicted in response to variation in the availability
43 of resources such as light, nutrients and water, either due to direct effects on biosynthetic
44 pathways, or indirect effects via changes to plant growth rate and physiology. Such changes in
45 growth and physiology may modify the trade-off in allocation of resources between primary
46 and secondary metabolism (e.g. Coley, Bryant & Chapin, 1985, Herms & Mattson, 1992,
47 Neilson *et al.*, 2013, Stamp, 2003). The vast majority of studies testing these phenotypic
48 theories and documenting changes in chemical defences in response to environmental
49 variation compare plants grown under different conditions at a common time/age (see also
50 Barton & Koricheva, 2010, Boege & Marquis, 2005, Moles *et al.*, 2013). We know, however,
51 that many plant traits, ranging from biomass allocation to defence chemistry, vary not only
52 with genotype and environment, but also with plant size and developmental stage (Boege &
53 Marquis, 2005, Elger *et al.*, 2009, McCarthy & Enquist, 2007, McConnaughay & Coleman,
54 1999, Moriuchi & Winn, 2005). Same age comparisons may therefore obscure both the
55 developmental basis of a defence phenotype as well as true phenotypic plasticity in response
56 to environmental variability. Plants grown under conditions that slow growth, for example,
57 may simply be at an earlier point along the same, fixed developmental trajectory compared
58 with those with higher growth rates (Coleman, McConnaughay & Ackerly, 1994, Moriuchi &
59 Winn, 2005), or it may be that the shape of the trajectory is affected by different conditions. A
60 meta-analysis by Barton and Koricheva (2010) examined ontogenetic patterns in herbivory
61 and defence, and found that ontogenetic patterns in defence vary with plant form (woody,
62 herbaceous), with herbivore type, and type of defence. It further highlighted that among plant
63 forms, there is a paucity of information about grasses, and that ontogenetic variability in

64 defence strategies is poorly integrated into current studies and theories of plant defence (see
65 also Boege & Marquis, 2005).

66 Among environmental variables, the effect of soil P on chemical defences has been little
67 explored (e.g. Bryant *et al.*, 1993). This is surprising because P is important for
68 photosynthesis and growth and consequently has the potential to affect the trade-off in
69 resources between primary and secondary metabolism implicit in most defence theories (e.g.
70 Sampedro *et al.*, 2010, Sampedro, Moreira & Zas, 2011). The potential impact on plant
71 defence of below-ground associations such as arbuscular mycorrhizal (AM) associations, an
72 important pathway for the uptake of P and other nutrients in plants (Smith & Read, 2008), has
73 similarly been neglected (see also Bezemer & van Dam, 2005, Cavagnaro, Gleadow & Miller,
74 2011, Erb *et al.*, 2009, Schloter & Matyssek, 2009). There are few studies of the effect of P
75 and/or AM on chemical defence and none, to our knowledge, on a possible link with
76 cyanogenic glycosides, an important class of constitutive N-containing allelochemicals found
77 in over 5% of all species, including many crops (Jones, 1998). Cyanogenic glycosides are safe
78 when stored *in planta*, but release toxic hydrogen cyanide (HCN) when plant tissues are
79 crushed or chewed, and high concentrations can have significant impacts on human and
80 animal health (Cliff *et al.*, 1985, Wheeler *et al.*, 1990).

81 Here we use the cyanogenic species *Sorghum bicolor* (L.) Moench – one of the top five crops
82 in terms of area, with over 300 million ha planted worldwide in 2011 (FAOSTAT, 2011) – as
83 a model to address several questions about phenotypic plasticity in chemical defence in
84 response to P and AM. Sorghum is an appropriate model for this study as it is capable of
85 forming AM (Rillig *et al.*, 2001), and owing to the agronomic significance of understanding
86 factors affecting forage toxicity, has been the subject of the only previous work on the effects
87 of P on cyanogenesis. Understanding P effects on plants (in particular crops), is of growing
88 importance given peak P, the point where the maximum global phosphorus production rate is
89 reached, is predicted to occur in about 2030 (Cordell, Drangert & White, 2009). Data in the

90 literature report a significant negative association between P supply and cyanogenic potential
91 (HCNp) in sorghum shoots (e.g. Harms & Tucker, 1973, Patel & Wright, 1958, Wheeler *et*
92 *al.*, 1990) and significant stimulation of HCNp by N supply (e.g. Busk & Møller, 2002,
93 McBee & Miller, 1980, Patel & Wright, 1958). Cyanogenic potential (HCNp) is the amount
94 of cyanide released from all cyanogenic compounds present in plant tissue. The majority of
95 these studies failed to effectively take account of plant size and variations in growth rate and
96 developmental stage – several not reporting biomass data, and none comparing plants at an
97 equivalent size or developmental stage. This is important because the HCNp of sorghum is
98 known to change significantly with ontogeny: HCNp reaches a maximum 4 days after
99 germination where dhurrin content in the young seedling tips may account for 6% of the dry
100 weight, and decreases more than 5-fold by about 40 days old (Akazawa, Miljanich & Conn,
101 1960, Busk & Møller, 2002, Halkier & Møller, 1989, Loyd & Gray, 1970). Against such
102 significant ontogenetic drift in cyanogenic capacity, any interpretation of the effects of
103 nutrients on plant defence phenotype are likely confounded by differences in plant size and
104 development. This is illustrated by the study of Patel and Wright (1958) in which sorghum
105 shoot HCNp of same aged plants differed by a maximum of ~450 ppm across nutrient
106 treatments, while within treatments, shoot HCNp varied by ~400 ppm with ontogeny. Despite
107 significant developmental changes in HCNp, biomass data was not presented in this study so
108 no comparisons of same-sized plants could be made (Patel and Wright 1958). While
109 comparing plants at the same time point may be appropriate for some studies of defensive
110 traits, such as simultaneous comparisons of chemical composition in relation to herbivore
111 abundance (Coleman *et al.*, 1994), size dependent comparisons may be more appropriate for
112 studies focused on assessing phenotypic plasticity and the costs of resource allocation to
113 chemical defence in tests of plant defence theories (Barton & Koricheva, 2010, Goodger,
114 Gleadow & Woodrow, 2006, Simon, Gleadow & Woodrow, 2010).

115 We sought to investigate (1) the effect of phosphorus supply and, given the importance of
116 AMF for plant P and N nutrition (Ames *et al.*, 1983), (2) the effect of AM on resource
117 allocation to a N-based chemical defence in above- and below-ground tissues. In so doing, we
118 aimed to not only to resolve the uncertainty about the effect of P on the cyanogenic capacity
119 of sorghum, but also to explore the interplay between ontogenetic and environmental controls
120 on resource allocation to a constitutive, N-based chemical defence at the whole plant level. In
121 order to investigate the mycorrhizal and P effects on growth, nutrition and cyanogenesis in
122 shoots and roots, we grew two lines of forage sorghum known to differ in HCNp in soil at two
123 levels of phosphate, either with or without arbuscular mycorrhizal fungi (AMF). In order to
124 account for the potentially confounding effects of different ontogenies on HCNp, plants were
125 harvested at two time points: when plants were the same chronological age; and again when
126 plants from the low P treatment were at the same developmental stage as the high P-grown
127 plants. The first harvest time was chosen to be well after the initial peak in seedling HCNp,
128 reached in the first week after germination (e.g. Busk & Møller, 2002). Based on the
129 conceptual framework proposed by Cavagnaro, Gleadow & Miller (2011), we hypothesised
130 that improved P and N nutrition, either by higher P supply or AM association, will be
131 associated with increased HCNp in above- and below-ground tissues, but that such changes
132 can only be appropriately quantified when ontogenetically controlled comparisons are made.

133

134 **MATERIALS AND METHODS**

135 **Soils, plant material and nutrient treatments**

136 A soil:sand mix (18:82 w/w on a dry weight basis) was used in all experiments. This mixture
137 was comprised of coarse washed sand and collected from the 0-15 cm soil layer at the
138 Wallenjoe Swamp State Game Reserve, northern Victoria, Australia. Soil from this site has
139 low plant available (Colwell) P (10.2 mg g^{-1} dry soil) and a high AM inoculum potential (see

140 Cavagnaro & Martin, 2011, Watts-Williams & Cavagnaro, 2012); this soil:sand mix is
141 referred to as 'soil' hereafter. Non-mycorrhizal treatments were established following Smith
142 & Smith (1981) and others (Asghari & Cavagnaro, 2011, Lovelock & Miller, 2002).
143
144 The forage sorghum cultivars used here are hybrids between grain sorghum, *Sorghum bicolor*
145 (L.) Moench ssp. *bicolor* and sudan grass (*S. bicolor* (L.) Moench ssp. *drummondii* (Nees ex
146 Steud.) de Wet & Harlan; sensu syn. *S. sudanense).* Experiments were performed using two
147 genetically related hybrids (cv. A and cv. B) known to differ in cyanogenic potential, and
148 therefore potentially also in the allocation of N to cyanogenesis. Seeds were surface sterilised
149 by immersion for 10 min in an aerated 3% sodium hypochlorite solution, rinsed with reverse
150 osmosis (RO) water to remove any trace of NaOCl, and them imbibed in aerated RO water for
151 20 min. Seeds were then directly sown into seedling trays, containing sterilised (twice
152 autoclaved, as above) seed raising mix (Debco, Australia). After 7 days, seedlings were
153 carefully washed from the seed raising mix with RO water and seedlings of a uniform size
154 were transplanted into 1L pots containing the soil:sand mix. Ten additional seedlings of each
155 genotype (equivalent to those used in the experiments) were oven dried to determine an initial
156 biomass for relative growth rate (RGR) determination.
157
158 Plants were grown in a glasshouse with supplemental lighting (MK-1 Just-a-shade, Ablite
159 Australia) on the Monash University Clayton campus in April 2009, with a 16/8 h day/night
160 photoperiod. Air temperature and relative humidity were logged as 5 min averages throughout
161 the experiment using a Veriteq Spectrum SP-2000-20R logger (Veriteq, Richmond, BC,
162 Canada) while photosynthetic photon flux density (PPFD) was logged for a two week period
163 as 15 min averages using two visible light sensors (LI-190SA, Li-Cor Inc., Lincoln, NE,
164 USA). Conditions in the glasshouse were as follows: mean day time temperature 22.1^{o}C (min
165 18.3^{o}C/ max 25.9^{o}C); mean night time temperature 20.1^{o}C (min 17.3^{o}C/max, 22.6^{o}C); mean

166 light intensity 144.2 ± 30.3 μmol quanta m^{-2} s^{-1}; mean daily photon load 495.1 ± 108.6 mol
167 quanta m^{-2} and mean relative humidity 44 ± 9 %. Plants were watered three times weekly with
168 20% modified Long Ashton solution minus P (Cavagnaro & Martin, 2011, Cavagnaro *et al.*,
169 2001) with a total of 8 mM nitrogen supplied as 4 mM $NaNO_3$ and 2 mM $(NH_4)_2SO_4$. Plants
170 were also flushed with RO water once a week to prevent salt accumulation over the course of
171 the experiment. Plants within treatments and treatments were randomised spatially in the
172 glasshouse weekly.
173

174 **Experimental design**

175 ***Experiment 1**:* In order identify the optimal amount of P to be added to the soil in the main
176 experiment, five P-addition treatments were established by mixing $CaHPO_4.2H_2O$, with the
177 soil:sand mix at rates of 0, 0.025, 0.125, 0.25 and 0.4 g kg^{-1} dry soil. Seedlings of cv. A were
178 used, and only mycorrhizal treatments were included in this preliminary experiment to
179 confirm the high inoculum potential of the soil. Plants were harvested when they were at the
180 *ca.* 5-leaf stage, 23-24 days after transplanting (or 27 days after sowing).
181

182 ***Experiment 2*:** Based on the results of the first experiment described above, P was added to
183 the soil at 0.025 and 0.125g $CaHPO_4.2H_2O$ kg^{-1} dry soil (as above) to establish low and high
184 P addition treatments. To investigate chronological and ontogenetic patterns in plant
185 responses, there were two harvests. The first harvest (Time 1) was 24 days after transplanting
186 (31 days after sowing), when plants are approx. at 5-6 leaf stage. Because of the known
187 ontogenetic variation in sorghum cyanogenesis (Loyd & Gray, 1970, McBee & Miller, 1980,
188 Patel & Wright, 1958), a second harvest (Time 2) was included to enable comparison of
189 plants at the same size (developmental stage); that is, 63-64 days after transplanting the low P
190 plants had reached the same size (on the basis of leaf number, and biomass, see results) of the
191 high P plants harvested at Time 1. The first harvest time was chosen as it is well after the

192 maximum seedling HCNp, which occurs within a week of germination (Busk & Møller, 2002,
193 Loyd & Gray, 1970).
194

195 **Plant biomass sampling and analysis**

196 Plants were destructively harvested by carefully washing the plants free from the pots and soil
197 with RO water. The roots and shoots were separated and fresh weights determined. Plant
198 height, leaf number, and leaf area was measured for the leaf blade above the ligule. Stem, leaf
199 and root samples were snap frozen in liquid N_2 and freeze dried to determine shoot and root
200 dry weights. Freeze dried samples were ground to a fine powder using an IKA Labortechnic
201 A10 microgrinder (Janke & Kunkel, KG, Staufen, Germany), with above ground material
202 pooled (stem and leaf), and roots ground separately for chemical analysis. A weighed sub-
203 sample of roots collected prior to freezing was stored in 70% EtOH and used for staining and
204 assessment of mycorrhizal colonisation. Roots were cleared with KOH (10% w/w) and
205 stained with Trypan Blue and colonisation of roots was determined using a modification of
206 the gridline intersect method (Cavagnaro *et al.*, 2006).
207

208 **Leaf nitrogen and carbon**

209 Freeze dried and ground root and shoot material (5-10 mg) was analysed for CHN
210 composition by using either a LECO CHN 2000 (LECO, Australia) using EDTA as a standard
211 or a Carlos Erba NA 1500 Series 2 NCS Analyzer and AS-200 Autosampler (Fisons
212 Instruments, Milan, Italy), calibrated using the standard atropine (Fisons Instruments #338
213 24400).
214

215 **Cyanogenic glucoside concentration**

216 The concentration of cyanogenic glucosides was measured by trapping the cyanide (CN),
217 liberated following hydrolysis of the glucoside, in a 1M NaOH well (Brinker & Seigler, 1989)

218 as modified by (Miller, Gleadow & Woodrow, 2004). Freeze-dried, ground plant tissue (10-
219 15 mg dwt) was incubated for 24 h at 37ºC with 1 mL of 0.1 M citrate–HCl (pH 5.5). β-
220 glucosidase emulsin from *Prunus amygdalis* Batsch (EC 3.2.1.21, Sigma G-0395) was added
221 to the buffer at the rate of 1.12 units mL^{-1}. Cyanide in the NaOH well was adapted for use
222 with a photometric microplate reader (Labsystems Multiskan ® Ascent, with incubator,
223 Labsystems, Helsinki, Finland). The absorbance was measured at 595nm with NaCN as the
224 standard.
225

226 **Statistical Analyses**

227 The effects of P supply on biomass and tissue chemistry in Experiment 1 were analysed using
228 1-way Analysis of Variance (ANOVA). Data from Experiment 2 were analysed in two
229 separate 2-factor general linear models (GLMs). The first assessed the impacts of P and AM
230 treatments on plants of the same age (i.e. harvested at Time 1). The second assessed the
231 impacts of P and AM treatments on plants of the same size (i.e Low P-grown plants harvested
232 at Time 2 and High-P grown plants harvested at Time 1). In order to satisfy the assumptions
233 of ANOVA, several variables were transformed. Significant differences between treatments at
234 the P=0.05 level were assessed using Tukey's HSD post-hoc analysis. Some additional 2-
235 factor GLM analyses were conducted to investigate changes in shoot and root N
236 concentrations over time in low P treated plants. All analyses were conducted using JMP v.9
237 (SAS Institute Inc.). In order to compare the extent of mycorrhizal colonisation across
238 genotypes and treatments, a 3-way GLM was conducted. This analysis also included harvest
239 2, high P plants to compare the effect of P on the extent of colonisation at this longer time
240 period and to compare the colonisation of genotypes. GLM results for data relating to Figures
241 2 and 3 are presented in the Supplemental Data Table.

242

243 **RESULTS**

244 **Experiment 1: Growth and HCNp at a range of P concentrations**

245 In the initial experiment, sorghum was grown at five different levels of P and harvested 27
246 days after sowing. Shoot and root biomass of seedlings increased with increasing soil P
247 addition, up to an addition of 0.125g $CaHPO_4.2H_2O$ kg^{-1} dry soil, after which further P
248 addition had no detectable effect on growth (Fig. 1a). Leaf area increased, while root:shoot
249 decreased with increasing P supply (see Supplementary Data Table). Whereas the
250 concentrations of cyanogenic glucosides in the shoots of plants decreased significantly (by
251 65%) with P addition, root cyanogenic glucoside concentrations increased by 88% (Fig. 1b).
252 These divergent responses of shoot and root cyanogenic glucoside concentration are despite
253 similar declines in root and shoot N with increasing soil P supply (data not shown). On the
254 basis of these results, P addition treatments selected for inclusion in the main experiments
255 were 0.025 and 0.125g $CaHPO_4.2H_2O$ kg^{-1} dry soil. In addition, colonisation of roots by AMF
256 was detected (but not quantified) in plants at the time of harvest, indicating that the soil used
257 contained appropriate inoculum.

258

259 **Experiment 2: Mycorrhizal colonisation and plant growth**

260 In the main experiment plants were grown supplied with two concentrations of P (low P, high
261 P), in either sterilised soil or soil containing AM inoculum. Plants were harvested 27 days
262 after planting, when high P plants had 5-6 leaves (Time 1). A second set of low P plants was
263 harvested after 63-64 days when they attained the same developmental stage as the high P
264 plants at the first harvest (Time 2). This combination of harvests provided the opportunity to
265 directly compare plants of the same size (ontogeny), as well as at the same age (chronology).
266 Overall, total biomass of low P plants harvested at Time 2 was not significantly different from

267 the biomass of high P plants harvested at Time 1, validating the use of leaf number as a
268 determinant of plant size (Table 1 and 2).
269
270 ***Colonisation rates.*** Significant differences in per cent root colonisation were detected with
271 genotype ($F_{7,47}$=60.4; *P*=0.0045), harvest time (*P*<0.0001) and P treatment (*P*<0.0001). Plants
272 in the non-mycorrhizal treatment were either not colonised by AMF (cv. A) or showed only
273 very low levels of colonisation (<2%; cv. B) (Table 2). Rates of colonisation increased
274 significantly with time from 2.2-12.8% at Time 1 to 13-61% at Time 2, depending on the
275 cultivar and the treatment (Table 1 and 2). Root colonisation was approximately 5-fold greater
276 in low P plants compared to high P plants and roots of plants of cv. B were significantly
277 (*P*=0.004) more colonised than those of cv. A.
278
279 ***Biomass and biomass partitioning.*** When plants of the same age, grown under different P
280 regimes were compared, total biomass of both genotypes was significantly higher in the high
281 P treatment, irrespective of AM treatment (Table 1 and 2). For both genotypes, significant
282 effects of AM colonisation on total biomass were detected at Time 1, when plants were the
283 same age, but in opposite directions. Specifically, for cv. B, which had higher levels of AMF
284 colonisation, the AM treatment was associated with increased total biomass, an effect most
285 evident at low P, where there was a 28% increase in total biomass (Table 2). Conversely, for
286 cv. A there was a significant, albeit relatively small, growth depression in the mycorrhizal
287 plants at Time 1, despite low levels of AM colonisation in the high P plants. When both
288 genotypes were harvested at the same ontogenetic stage, there was no detectable effect of AM
289 colonisation on total biomass, unlike in the same age comparison. Consequently, high P plants
290 had a higher relative growth rate (RGR) than low P plants in both genotypes, with 1.5-1.6 fold
291 increase when compared at that stage age (Time 1), and 2.2-2.3-fold increase when compared
292 at the same stage. AM colonisation was only associated with enhanced RGR in cv. B in the

293 same age comparison (by 2.2% and 7.0% in high P and low P treatments, respectively) but
294 not same size analysis (Table 1 and 2).
295
296 Treatment effects on biomass partitioning differed between genotypes (Table 2). For both
297 genotypes, and both harvests, the root:shoot ratio was significantly higher in plants supplied
298 with low P (Table 1). For cv. A, whether at low or high P, there was a decrease in relative root
299 biomass with AM colonisation ($F_{3,23}$=14.7, P=0.002; Table 1). Interestingly, when comparing
300 low P grown plants over time, root:shoot did not change with plant age, but was significantly
301 lower in mycorrhizal plants of both genotypes (cv. A: $F_{3,23}$=6.07, P=0.0033 and cv. B:
302 $F_{3,23}$=2.89, P=0.0146). For leaf area, there was a significant interaction between AM and P
303 treatments for cv. A harvested at Time 1, with a slight increase in leaf area with AMF under
304 low P, whereas at high P, consistent with the total biomass data, there was a 19% decrease in
305 total leaf area of plants grown with AMF (Table 1). In cv. B, there was a trend towards
306 increased leaf area with AM colonisation; however, the increase was only significant in the
307 same size comparison (Table 2). At Time 1, no differences in leaf area ratio (LAR) were
308 detected for cv. A or cv. B, whereas specific leaf area (SLA) was significantly higher in low P
309 than in high P plants (cv. A: $F_{3,23}$=9.64, P<0.0001 and cv. B: $F_{3,23}$=5.63, P<0.001) (data not
310 shown). By contrast, when plants of the same size were compared both SLA and LAR were
311 significantly greater in high P grown plants. A significant interaction between AM and P
312 treatments was detected for SLA in cv. A (P=0.0295), and LAR in cv. B (P=0.0350), with, in
313 each case, the magnitude of the difference between low and high P treatments being reduced
314 in the mycorrhizal treatment.
315

316 **Plant nutrition and cyanogenic glucosides**

317 ***P concentration***. The concentration of P in both the shoots and roots of both genotypes was
318 significantly higher in the plants supplied with high P compared with those supplied with low

319 P, whether plants of the same age or same size were compared, and irrespective of AM
320 colonisation (Fig. 2a, b). Few significant mycorrhizal effects on tissue P concentration were
321 detected. Interestingly, for both genotypes, it was primarily only in the high P treatment in
322 plants harvested at Time 1, where AM colonisation of roots was very low, that significant
323 differences in tissue P concentrations were detected between mycorrhizal and non-
324 mycorrhizal plants, where plants grown with AMF inoculum had lower root and shoot P
325 concentrations (Fig. 2a, b). By contrast, at low P, despite some trends towards increased shoot
326 or root P with AM colonisation, only root P concentrations of mycorrhizal cv. B plants were
327 found to be significantly greater than in non-mycorrhizal plants of the same size (Fig. 2b).
328

329 ***Cyanogenic glucosides***. The HCNp varied significantly with genotype, treatment, harvest
330 time and between shoots and roots (Fig. 3). Overall, across all treatments there were seven
331 main observations. First, shoot HCNp was 42% higher in cv. A compared with cv. B, and root
332 HCNp was 27% higher, consistent with expected differences in cyanogenic capacity of the
333 two cultivars (Fig. 3a, b). Second, HCNp was more than 5-fold higher in shoots than in roots
334 of both cultivars (Fig. 3a, b). Third, low P-grown plants had a higher shoot HCNp than high
335 P-grown plants when compared at the same age (Time 1). Averaged across AM treatments,
336 the shoot HCNp of low P grown plants was 2.9 and 3.6 fold greater than high P grown plants
337 in cv. A and cv. B, respectively (Fig. 3a, b). Fourth, when compared at the same ontogenetic
338 stage (Time 2 for low P-grown plants and Time 1 for high P-grown plants), the shoot HCNp
339 of the low P plants was still higher than high P plants, although the magnitude of the
340 difference was much smaller than when compared at the same time point (Fig. 3a, b). In both
341 genotypes, shoot HCNp was 1.8-1.9 times greater at low P than high P, pooling AM
342 treatments. Fifth, there were few differences in shoot HCNp between plants from the different
343 mycorrhizal treatments and those that were found were broadly consistent with changes in N
344 status and ontogeny (see below). Sixth, shoot and root cyanogen concentrations responded in

345 the opposite directions to changes in P supply (Fig 3a, b). Most notably, shoots of smaller low
346 P plants had significantly higher HCNp than larger high P plants, while roots of low P plants
347 had significantly lower (1.3-2.4 times) HCNp than high P plants. Finally, the direction of
348 ontogenetic changes in shoot HCNp and root HCNp differed, and in roots was significantly
349 affected by AMF (based on the comparison between Time 1 and Time 2 for low P grown
350 plants). In contrast to the significant reduction in shoot HCNp with plant age in both
351 genotypes (47% and 39% reductions for cv. A and cv. B, respectively), root HCNp increased
352 significantly, by 90% and 40% for cv. A and cv. B, respectively, pooling AM treatments. This
353 increase in root HCNp with age was significantly greater in mycorrhizal plants than non-
354 mycorrhizal plants for both genotypes (2-way GLM, cv. A P=0.0065 and cv. B P=0.0047,
355 AM treatment main effect). For example, in cv. A, root HCNp of plants with AMF increased
356 107% between harvests, compared with a 68% increase in non-mycorrhizal plants (Fig. 3a, b).
357
358 ***Total N concentration.*** When plants of the same age were compared, the concentration of N
359 in the shoots and roots of plants of both genotypes was greater in the low P treatment,
360 irrespective of mycorrhizal treatment (Fig. 2c, d). In cv. A, there was a trend towards greater
361 shoot N in non-mycorrhizal plants, while in cv. B, the effect of AM treatment was significant
362 in both P treatments, with mycorrhizal plants having 12% and 25% lower mean shoot N than
363 non-mycorrhizal plants in low and high P treatments, respectively (Fig. 2d). Similarly, when
364 plants of different ages but the same size were compared, the concentration of N was again
365 generally higher in the low P addition treatment than in the high P treatment, for both
366 genotypes (Fig. 2c, d). In both cultivars, both shoot and root N concentrations decreased with
367 development from Time 1 to Time 2, in low P grown plants. Consistent with observations of
368 plants at the same chronological age, any significant AM effects again showed non-
369 mycorrhizal plants to have higher shoot N concentration than mycorrhizal plants, for example,
370 in the low P plants of cv. A at Time 2 shoot N was 19% higher in non-mycorrhizal plants

371 (Fig. 2c). In the case of root N concentrations, no significant differences between mycorrhizal
372 treatments were detected for either cultivar; however, root N was higher in plants grown with
373 low P plants than with high P, in both same age and same size comparisons, for both
374 genotypes (Fig. 2c, d).
375
376 Significant differences in shoot N:P were detected with AM colonisation (data not shown).
377 For both same age and same size comparisons, and for both genotypes, AM colonisation was
378 associated with a greater relative P content, and a significant reduction in shoot N:P. Similarly
379 in roots of cv. B, AM colonised plants had significantly lower N:P in both comparisons. A
380 significant interaction between AM and P was detected such that this difference was only
381 significant in low P treatments, where AM colonisation was greater.
382
383 ***Comparison of N and HCNp.*** Irrespective of AM treatment, genotype or whether comparing
384 same aged or same sized plants, the proportion of N allocated to cyanogenic glucosides (CN-
385 N/N%) was significantly higher in shoots, but lower in roots, of plants supplied with low P
386 (Fig. 3c, d). The magnitude of this P effect on CN-N/N (%) was greater in the same age
387 comparison than the same size comparison, as shoot CN-N/N (%) of low P plants decreased
388 with development (Time 1 versus Time 2). For example, in non-mycorrhizal cv. A, in the
389 same age comparison, shoot N allocation to CN in low P grown plants was 2.5-fold greater
390 than in high P grown plants, but was only 1.4-fold greater when plants of the same size were
391 compared (Fig. 3c). Conversely, for roots of the same plants, N allocation to CN in high P
392 grown plants was 4.6-fold greater than in low P grown plants in the same age comparison, but
393 was 2.5-fold greater in the same size comparison, as root CN-N/N (%) of low P plants
394 increased over time. When compared at the same age, CN-N/N (%) was higher in both shoots
395 and roots of mycorrhizal cv. B plants (Fig. 3d). When cv. B plants of the same size were
396 compared, this same effect was seen in the roots but no AM effect on shoot CN-N/N (%) was

397 detected (Fig. 3d). The same trend was seen in the same size comparison of cv. A but the
398 differences were not significant. Despite similar percent decreases in shoot and root N%
399 between harvests for low P grown plants, shoot CN-N/N (%) decreased and root CN-N/N (%)
400 increased significantly with ontogeny (Fig. 3c, d). For example, in cv. A, percent N allocation
401 to CN in shoots decreased 35% between Time 1 and Time 2, but increased 117% in roots,
402 averaged across AM treatments (Fig 3; $F_{3,23}$=18.1, *P*<0.0001 harvest time main effect).
403 Whereas there was no difference between AM treatments for shoots, in roots, the relative
404 increase in CN-N/N (%) was significantly greater in mycorrhizal plants (143%) than non-
405 mycorrhizal plants (85%; cv. A *P*=0.0067 AM treatment main effect).
406
407 To examine N allocation in different tissues in more detail, relationships between N and
408 cyanogenic glucoside concentrations were further explored using regression analysis (Fig. 4).
409 For shoots, irrespective of whether plants were being analysed from the same age comparison
410 or same size comparison, there were significant positive correlations between foliar N and
411 HCNp (Fig. 4a, c). When plants of the same age were compared, that is, where P treatments
412 resulted in plants of significantly different size (i.e. and developmental stage), highly
413 significant positive correlations between foliar N content and HCNp were found in both
414 genotypes, with 87% of variation in HCN explained by variation in N in cv. A (Fig. 4a). In
415 cv. B this value was 48% (Fig. 4a). When plants of the same size were analysed, similarly
416 strong positive correlations between foliar N and HCNp were still evident, with 71% and 56%
417 of the variation in HCNp explained by variation in N, in cv. A and cv. B, respectively (Fig.
418 4c).
419
420 In contrast to shoots, when examining roots from plants of the same age, a strong negative
421 correlation between root N concentration and HCNp was found for both genotypes (Fig. 4b).
422 At Time 1, variation in root N was driven by P treatments and consequential differences in

423 plant size, with high P grown, larger plants having lower root N concentrations. In this
424 analysis, 44% and 45% of the variation in HCNp could be explained by variation in root N in
425 cv. A and cv. B, respectively. By contrast, when examining plants of the same size, no
426 significant relationship between N and HCNp in roots was detected.
427
428

429 **DISCUSSION**

430 Even though many crop plants are thought to be cyanogenic (Jones 1998), little is known
431 about how P affects the synthesis and turnover of cyanogenic glucosides. We hypothesised
432 that improved P and N nutrition, either by higher P supply or AM association, would result in
433 a higher HCNp in sorghum tissues, but that the change would only be appropriately quantified
434 when ontogenetically controlled comparisons are made. We found that P-limited plants had
435 higher shoot HCNp when plants were compared at the same age, a change not entirely
436 consistent with those plants being smaller and developmentally younger (Busk & Møller,
437 2002) as ontogenetically controlled comparisons, using plants with the same leaf number and
438 biomass, also showed that there was an increase, of lesser magnitude, in the allocation of N to
439 dhurrin in shoots when P was limiting. By contrast, in the roots there was a decrease in HCNp
440 in the low P-grown plants, but no obvious trend when ontogenetic differences were taken into
441 account. Colonisation of sorghum roots by AM did not consistently affect shoot HCNp, but
442 there was a significant interaction with P, such that the effects of low P on growth and shoot
443 HCNp were ameliorated by AM. By contrast, in low P grown plants, AM effected a
444 significant increase in root HCNp over time. Interestingly, there was no appreciable growth
445 sacrifice associated with the formation of AM. Results were broadly similar across the two
446 genotypes despite differences in HCNp and rates of AM colonisation.
447

448 **Stage versus age**

449 Assessing phenotypic plasticity in the expression of sorghum cyanogenesis in response to
450 variation in soil P supply is confounded by significant developmental and age related changes
451 in tissue CN (and likely N) concentrations (Busk & Møller, 2002), to such an extent that
452 strikingly different conclusions about the significance of P supply for HCNp would be drawn
453 depending on whether plants are compared at the same age or same stage. Results presented
454 here support our hypothesis that differences in HCNp associated with P supply are primarily,
455 but not entirely, mediated via changes in plant growth rate and developmental stage.
456 Previously published research on sorghum has been confounded by differences in plant size
457 and developmental stage, making it hard to assess the extent to which the reported variation in
458 HCNp with P was 'apparent' plasticity, and a consequence of changes in growth rate in
459 response to varied P supply. Wheeler *et al.* (1980), for example, found higher P more than
460 doubled forage yield and reduced HCNp by 34%; Kriedeman (1964) also found that increased
461 P enhanced dry matter production more rapidly than CN production. Consistent with these
462 earlier studies, we also found that HCNp was higher in P-deficient plants when they were
463 harvested at the same time, with a 3.2-fold difference in HCNp and a 5.5-fold difference in
464 total plant biomass due to P supply (pooling data for both genotypes). When compared at the
465 same size, the magnitude of P effect on shoot HCNp was reduced, from a 3.2-fold difference
466 to a 1.8-fold difference. The P effect on HCNp was still significant, despite an average 43%
467 decrease in shoot HCNp between harvests in low P plants. In order to understand how dhurrin
468 is regulated it is essential, therefore, that ontogenetically controlled comparisons be made
469 rather than simply comparing plants sampled at the same time when the treatments
470 differentially stimulate plant growth. The potential for ontogenetic change to obscure
471 phenotypic variation in defence allocation is also evident in other systems, such as *Plantago*
472 *lanceolata* L., where reported developmental variation in iridoid glycoside concentrations is
473 of similar or greater magnitude than variation due to environment or genotype (Bowers *et al.*,

474 1992, Bowers & Stamp, 1993, Quintero & Bowers, 2011, Quintero & Bowers, 2012).
475 Together, the results here and in other systems in which ontogenetic changes in chemical
476 defence are well known (e.g. cyanogenic *Eucalyptus* spp.; (Goodger *et al.*, 2006, Neilson *et*
477 *al.*, 2011)), indicate the potential for a comparison of any two time points on a complex non-
478 linear trajectory to yield different conclusions, and emphasise the importance of appropriate
479 (e.g. size dependent) comparisons for studies examining the extent of true phenotypic
480 plasticity in response to the environment. Just as size dependent comparisons are considered
481 most relevant to testing models of optimal biomass allocation, into which ontogeny is better
482 integrated (Coleman *et al.*, 1994), they may be more appropriate to studies probing the trade-
483 offs associated with resource allocation to plant chemical defence. This is an area that is
484 clearly open to further investigation, especially where a wider range of plant ages and more
485 detailed developmental trajectories are considered.

486 Few studies have examined the effect of P supply on either C-based or N-based chemical
487 defences, with no consistent pattern. Sorghum has being the species subject to most research
488 given the economic impact of forage toxicity (Wheeler & Mulcahy, 1989). Under P-limited
489 conditions, increased concentrations of constitutive C-based defences (condensed tannins and
490 phenolics) have been reported (Keski-Saari & Julkunen-Tiito, 2003, Sampedro *et al.*, 2011),
491 whereas no effect of P limitation on was found on the concentration of N-based alkaloids in
492 tobacco leaves (Andrade *et al.*, 2013), or on pyrrolizidine alkaloids in shoots or roots of
493 ragwort (Vrieling & van Wijk, 1994), although plants were not the same size in the latter
494 study. In coffee seedlings, P deficiency led to a 20% reduction in foliar caffeine (Mazzafera,
495 1999), while Andrade et al. (2013) found the response of alkaloids to P supply varied with
496 species, tissue and the alkaloid compound. In the present study, in the same size (and same
497 age) comparison, high P plants were N deficient, and had low shoot HCNp, whereas low P
498 grown plants were P deficient, with a higher N:P and higher shoot HCNp, suggesting changes
499 in HCNp with P treatment may reflect changes in plant N status. This finding is consistent

500 with the many studies of woody and herbaceous species showing a strong positive correlation
501 between foliar N and HCNp (e.g. Busk & Møller, 2002, Gleadow & Woodrow, 2000, but see
502 Miller, Simon & Woodrow, 2006, Miller & Tuck, 2013). In the roots, however, we found the
503 opposite, with a strong negative correlation between N and HCNp in plants harvested at the
504 same time. Moreover, when plants were harvested at the same ontogenetic stage there was no
505 correlation between N and root HCNp. Thus, not only are conclusions about the magnitude of
506 the effect of P on HCNp confounded by developmental factors, but conclusions about the
507 importance of N in regulating HCNp in sorghum roots are different depending on whether
508 comparisons are made on a same age versus same size basis. There are few reports of
509 variation in root cyanogenesis (Kaplan *et al.*, 2008, van Dam, 2009). Interestingly, one study
510 of the highly cyanogenic *Prunus turneriana*, similarly reported no correlation between root
511 HCNp and N; however foliar HCNp and N were also not correlated in that species (Miller *et*
512 *al.*, 2004).

513 **Roots versus shoots**

514 Based on the divergent responses of shoot and root HCNp to soil P supply and tissue N
515 concentration, and with ontogeny found here, it seems probable that dhurrin concentrations in
516 sorghum are regulated independently in the roots and the shoots. This is not completely
517 unexpected given the number of cyanogenic species that are reported to have non-cyanogenic
518 roots (e.g. *Trifolium repens* L., (Hughes, 1991); *Eucalyptus cladocalyx* F. Muell. (Gleadow &
519 Woodrow, 2000)). Further, Blomstedt *et al.* (2012) identified several mutants of sorghum
520 that, as mature plants, lack dhurrin completely in the leaves, have substantially reduced levels
521 in the sheath, but have completely normal root HCNp. These have been termed adult cyanide
522 deficient class (*acdc1, acdc2, acdc3*) and are possibly regulatory mutants. Cassava (*Manihot*
523 *esculenta* Crantz.) also has the capacity for cyanogenic glucoside synthesis in both shoots and
524 roots (Du *et al.*, 1995, McMahon, White & Sayre, 1995), although significant transport from
525 shoots to roots also occurs in that species (Jørgensen *et al.*, 2005). The types of herbivores

526 feeding on roots and shoots are also quite different. Given the importance of herbivores in
527 driving defence strategies (Agrawal *et al.*, 2012, Barton & Koricheva, 2010, Kaplan *et al.*,
528 2008), it is not surprising that the environmental and molecular regulation of dhurrin in roots
529 and shoots may have evolved in different directions. Similarly divergent developmental
530 changes in 4β-hydroxiwithanolide concentrations between seedlings and mature plants of
531 cape gooseberry (*Physalis peruviana* L.) have been reported, with concentrations increasing
532 in roots but decreasing in shoots (Calderon, Ruiz & Castellanos, 2012). Dissimilar responses
533 of root and shoot defences to experimental treatments (e.g. nitrogen; Jamieson, Seastedt &
534 Bowers, 2012), and dissimilar ontogenetic changes in root and shoot defence chemistry have
535 been described for other defences, although changes typically appear to be in the same
536 direction (e.g. Lohman & McConnaughay, 1998, Quintero & Bowers, 2011). The ontogenetic
537 increase in sorghum root HCNp reported here between two time points merits more detailed
538 investigation given the non-linear changes in sorghum root HCNp (Loyd & Gray, 1970) and
539 other root defences reported in studies sampling at multiple time points (Beninger, Cloutier &
540 Grodzinski, 2009, Williams & Ellis, 1989). Nevertheless, it is clear that shoots cannot be
541 assumed to be representative of the whole plant, and this complexity may challenge the
542 interpretation/application of existing defence theories to whole plants, the emphasis and tests
543 of which to date, tend to focus on above ground tissues (Parker, Salminen & Agrawal, 2012,
544 Rasmann & Agrawal, 2008).

545

546 **Resource allocation as a result of changes in P and AM**

547 A role for AM in the uptake of P and N in other species is well known (e.g. Berntson &
548 Bazzazz, 1996). No studies to our knowledge, however, have previously been published on
549 the interaction of AM and P on cyanogenesis. We found AM root colonisation levels (8-64%
550 in low P plants) consistent with those reported for sorghum in other studies (Albert &

551 Sathianesan, 2009, Ortas, Harris & Rowell, 1996, Raju *et al.*, 1990), but despite some AM
552 effects on biomass (increased leaf area, decreased root:shoot in some comparisons), we did
553 not find consistent significant biomass enhancement with AM colonisation. Moreover, while
554 we detected some AM-associated differences in plant chemistry (lower N:P, lower N, and
555 increased CN-N/N (%) in some comparisons), we found no consistent significant AM
556 enhancement of P or N uptake. This does not, however, necessarily imply that uptake of
557 nutrients via the AM pathway was not important (Li *et al.*, 2006, Smith, Smith & Jakobsen,
558 2003). This differs some from other sorghum studies that reported an increase shoot biomass
559 and P content with AM colonisation (e.g. Ortas *et al.*, 1996, Raju *et al.*, 1990), and an
560 increase in the uptake of N not available to non-mycorrhizal plants (Ames *et al.*, 1984). The
561 efficacy of different AMF in enhancing plant nutrient status and growth varies with AMF
562 species and plant genotype (Raju *et al.*, 1990). Further, other factors such as temperature and
563 the form of N (NH_4^+ or NO_3^-) can also affect AM function, including AM-mediated uptake of
564 P in sorghum (Ortas *et al.*, 1996).

565 The absence of consistent significant AM effects on growth and nutrient uptake here limits
566 our ability to fully probe AM effects on resource allocation to defence as hypothesised;
567 nevertheless, some important insights were gained. For example, whereas no main effect of
568 AM on shoot HCNp was detected, there was a significant interaction with P. This could
569 indicate some amelioration of P stress by AM, although despite a relative increase in P
570 compared to N, there was no significant difference in leaf P between AM and non-AM plants
571 from the low P treatment. We observed a small decrease in allocation of shoot N to HCNp,
572 when comparing plants colonised by AM with those that did not form AM at the same time
573 point. The reduced allocation of N to CN in AM-low P compared with low P-alone plants
574 when compared at the same age (Time 1) is consistent with a demand for photosynthate to
575 support the AM. There was a relatively small, but significant, growth depression in the
576 mycorrhizal plants, despite low levels of AM colonisation in high P plants, indicating that

577 there was a significant demand for C by the AM in the first growth interval. It is perhaps
578 unsurprising that we found no AM effect on shoot HCNp in the absence of enhanced shoot N
579 with AM; however, AM had significant effects on below-ground cyanogenic capacity,
580 effecting an increase in root HCNp and the allocation of N to defence over time. The impact
581 of AM on other root defences has previously been reported (e.g. Bennett, Alers-Garcia &
582 Bever, 2006, Vierheilig *et al.*, 2000). The effects of AM on shoot and root alkaloids was
583 found to vary with species, organ and compound in a study by Andrade et al. (2013), AM
584 colonisation stimulating increased production of some root alkaloids in *Catharanthus roseus*
585 (L.) G. Don. In *P. lanceolata*, genotype specific increases in root iridoid glycoside
586 concentrations with AM colonisation were found (De Deyn *et al.*, 2009) . This however, is to
587 our knowledge the first study to show an effect of AM on HCNp.

588

589 The effects of AM on P and N nutrition and the fate of those nutrients *in planta* merits further
590 study, particularly in the light of increasing interest in above- and below-ground connections,
591 the effects of AM on relationships between plants and herbivores (e.g. Bennett *et al.*, 2006,
592 Cavagnaro *et al.*, 2011, Vannette & Hunter, 2011, Vannette & Rasmann, 2012), and the
593 potential for AM to modify the ontogenetic trajectory of growth via changes to plant resource
594 acquisition and allocation. Indeed studies of medicinally important species have shown AM
595 effects on foliar secondary metabolites including phenolics (Ceccarelli *et al.*, 2010, Toussaint,
596 Smith & Smith, 2007), terpenoids (Kapoor, Chaudhary & Bhatnagar, 2007), and alkaloids
597 (Abu-Zeyad, Khan & Khoo, 1999), potentially mediated via changes to plant nutrition.

598 **CONCLUSION**

599 In comparing the effects of nutrients and other environmental variables on plant defence it is
600 important to consider ontogenetic variation in assessing true phenotypic plasticity. The
601 increase in HCNp in shoots of plants grown at low P compared at the same point is not

602 completely consistent with a simple increase in concentration in the smaller and
603 developmentally younger low P-limited plants as ontogenetically consistent comparisons of
604 plants harvested at the same leaf stage also showed that there was an increase in the allocation
605 of N to dhurrin in P-limited plants. We propose that changes in the availability of P relative to
606 N within the plant allow N to be reallocated from the primary metabolism to dhurrin but
607 critically, that the magnitude of this response can be obscured when comparing plants at
608 different stages of development. N supply in shoots is the most significant driver of
609 differences in shoot HCNp, but conclusions about N regulation of HCNp in roots differ
610 depending whether same age or same sized plants are compared. Further, root HCNp was also
611 enhanced by AM colonisation. Results presented here show that P supply, AM and ontogeny
612 affect HCNp in quite different ways in roots and shoots. This result further highlights the need
613 to consider above- and below-ground linkages. Such tissue-specific differences could be the
614 result of independent biosynthetic pathways or other regulatory mechanisms that evolved in
615 response to different suites of herbivores or selective forces above- and below-ground. As
616 recognised elsewhere (Barton & Koricheva, 2010, Cavagnaro *et al.*, 2011, Parker *et al.*, 2012,
617 Vannette & Hunter, 2011, Vannette & Rasmann, 2012), it is clear that above- and below-
618 ground tissues, ontogeny and biotic interactions are all factors which require greater
619 integration into current theories and investigations of plant defence.

620

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629

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TABLES

Table 1. Summary growth and biomass allocation of Sorghum cv. A grown at high and low phosphorus (HP, LP), with and without AM colonisation (+/-AM), harvested to enable comparisons of plants at the same age (T1, 24days after transplantation), and at the same size (T1 for HP plants, and T2, 63-64 days after transplantation, for LP plants). Data are means ± SE of *n*=6 plants. Results of 2-way GLMs for same age and same size comparisons are listed separately showing the main effects and interactions. Superscript letters indicate significant differences between treatments at *P*<0.05 where a significant P*AM interaction was detected. Lower case letters (abc) indicate differences in the same age comparison; upper case letters (XYZ) indicate differences in the same size comparison.

Treatment/harvest	Root colonisation (%)	Total biomass (g dwt)	RGR ($g\ g^{-1}\ day^{-1}$)	Leaf area (cm^2)	Root:shoot
LP –AM T1	0.0 ± 0.0^{a}	0.64 ± 0.10	0.12 ± 0.006	45.51 ± 4.43^{a}	2.62 ± 0.28^{a}
LP +AM T1	8.7 ± 2.0^{b}	0.54 ± 0.05	0.12 ± 0.003	48.50 ± 2.72^{a}	1.67 ± 0.09^{b}
HP –AM T1	0.0 ± 0.0^{aX}	3.64 ± 0.20	0.19 ± 0.002^{X}	322.42 ± 12.87^{bX}	1.45 ± 0.06^{b}
HP +AM T1	2.2 ± 1.0^{aX}	2.98 ± 0.17	0.18 ± 0.002^{X}	260.89 ± 6.78^{cY}	1.35 ± 0.06^{b}
LP –AM T2	0.0 ± 0.0^{X}	3.49 ± 0.38	0.082 ± 0.002^{Y}	196.01 ± 20.68^{Z}	2.02 ± 0.11
LP +AM T2	51.2 ± 6.2^{Y}	3.58 ± 0.30	0.082 ± 0.001^{Y}	234.75 ± 15.05^{YZ}	1.86 ± 0.12

2-way ANOVA	Same age	Same size	Same age	Same size	Same age	Same size	Same age	Same size	Same age	Same size
P	*P*=0.0088	*P*<0.0001	*P*<0.0001	ns	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001
AM	*P*<0.0001	*P*<0.0001	*P*=0.0146	ns	ns	ns	*P*=0.0012	ns	*P*=0.0024	ns
P*AM	*P*=0.0088	*P*<0.0001	ns	ns	ns	*P*=0.0491	*P*=0.0005	*P*=0.0028	*P*=0.0110	ns
F ratio	$F_{3,23}=13.4$	$F_{3,23}=87.6$	$F_{3,23}=124.9$	$F_{3,23}=1.2$	$F_{3,23}=90.4$	$F_{3,23}=950.5$	$F_{3,23}=345.0$	$F_{3,23}=13.0$	$F_{3,23}=14.7$	$F_{3,23}=12.4$

Table 2. Summary of growth and biomass allocation of Sorghum cv. B grown at high and low phosphorus (HP, LP), with and without AM colonisation (+/-AM), harvested to enable comparisons of plants at the same age (T1, 24 days after transplantation), and at the same size (T1 for HP plants, and T2, 63-64 days after transplantation, for LP plants). Data are means ± SE of *n*=6 plants. Results of 2-way GLMs for same age and same size comparisons are listed separately showing the main effects and interactions. Superscript letters indicate significant differences between treatments at *P*<0.05 where a significant P*AM interaction was detected. Lower case letters (abc) indicate differences in the same age comparison; upper case letters (XYZ) indicate differences in the same size comparison.

Treatment/harvest	Root colonisation (%)	Total biomass (g dwt)	RGR ($g\ g^{-1}\ day^{-1}$)	Leaf area (cm^2)	Root:shoot
LP –AM T1	0.8 ± 0.5^{a}	0.56 ± 0.04	0.11 ± 0.004	45.58 ± 3.53	2.24 ± 0.12
LP +AM T1	12.8 ± 2.5^{b}	0.72 ± 0.08	0.12 ± 0.002	56.86 ± 3.33	2.04 ± 0.08
HP –AM T1	1.7 ± 0.7^{aX}	3.64 ± 0.14	0.18 ± 0.001	315.44 ± 8.42	1.42 ± 0.09^{X}
HP +AM T1	3.7 ± 0.6^{aX}	4.01 ± 0.17	0.18 ± 0.002	326.50 ± 10.03	1.49 ± 0.04^{X}
LP –AM T2	2.0 ± 0.4^{X}	3.59 ± 0.69	0.080 ± 0.003	180.97 ± 22.95	2.45 ± 0.18^{Y}
LP +AM T2	64.0 ± 3.6^{Y}	3.76 ± 0.32	0.081 ± 0.001	232.56 ± 13.13	1.97 ± 0.18^{Z}

2-way ANOVA	Same age	Same size	Same age	Same size	Same age	Same size	Same age	Same size	Same age	Same size
P	*P*=0.0064	*P*<0.0001	*P*<0.0001	ns	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001
AM	*P*<0.0001	*P* <0.0001	*P*=0.0359	ns	*P*=0.0255	Ns	ns	*P*=0.0465	ns	ns
P*AM	*P*=0.0016	*P* <0.0001	ns	ns	ns	Ns	ns	ns	ns	*P*<0.0266
F ratio	$F_{3,23}$=16.3	$F_{3,23}$=267.9	$F_{3,23}$=242.6	$F_{3,23}$=0.225	$F_{3,23}$=242.9	$F_{3,23}$=898.5	$F_{3,23}$=498.0	$F_{3,23}$=22.1	$F_{3,23}$=22.1	$F_{3,23}$=17.6

903

904 ## FIGURE LEGENDS

905

906 **Fig. 1.** (a) Biomass (dry weight) and (b) HCNp of roots and shoots of 27 day old *Sorghum* cv. A
907 seedlings grown at five different soil P concentrations. Different letters on the bars indicate
908 significant differences (1-way ANOVA) between treatments. Data area means ± SE of n=3
909 plants. Superscript letters indicate significant differences between treatments at P<0.05.

910

911 **Fig. 2**. Mean (a, b) phosphorus concentration, and (c, d) nitrogen concentration in *Sorghum* cv.
912 A (a, c) and cv. B (b, d) shoots (above line) and roots (below line) grown at high (HP) and low
913 (LP) phosphorus treatments, with (hatched bars) and without (white bars) arbuscular
914 mycorrhizas. Plants were harvested at two times; when HP and LP plants were the same age (T1,
915 24 days after transplantation), and (T2, 63-64 days after transplantation) when low P plants
916 reached the same size as HP at T1. Data are means ± SE of n=6. Different letters indicate
917 significant differences between treatments at P<0.05 (2-way GLM and Tukey's HSD) when
918 plants were compared at the same age (lower case abc) and the same size (upper case XYZ). For
919 full statistical information see the Electronic Supplemental Material.

920

921 **Fig. 3.** Mean (a, b) HCNp and (c, d) mean total N allocated to cyanogenic glucosides (CN-N/N
922 %) in *Sorghum* cv. A (a, c) and cv. B (b, d) shoots (above line) and roots (below line) grown at
923 high (HP) and low (LP) phosphorus treatments, with (hatched bars) and without (white bars)
924 arbuscular mycorrhizas. Plants were harvested at two times (T); when HP and LP plants were the
925 same age (T1, 24 days after transplantation), and (T2, 63-64 days after transplantation) when low
926 P plants reached the same size as HP at T1. Data are means ± SE of n=6. Different letters
927 indicate significant differences between treatments at P<0.05 (2-way GLM and Tukey's HSD)
928 when plants were compared at the same age (lower case abc) and the same size (upper case
929 XYZ). For full statistical information see the Electronic Supplemental Material.

930

931 **Fig. 4.** Relationship between HCNp and total nitrogen concentration in shoots (a, c) and roots (b,
932 d) of *Sorghum* cv. A (open circles) and cv. B (closed circles) plants grown at high and low P,
933 with and without arbuscular mycorrhizas (AM). Relationships are shown for when high P and
934 low P plants were the same age (a, b), and for when low P and high P plants were the same size
935 (c, d). Significant linear relationships are shown for both genotypes. Equations: (a) same age cv.
936 A: shoot CN=0.239*shoot N-3.98, cv. B: shoot CN=0.129*shoot N-1.51 (b) same age cv. A:

937 root CN=-0.031*root N+0.862, cv. B: root CN=-0.020*root N+0.603 (c) same size cv. A: shoot
938 CN=0.129*shoot N-1.50, cv. B: shoot CN=0.074*shoot CN-0.504.

Figure 1

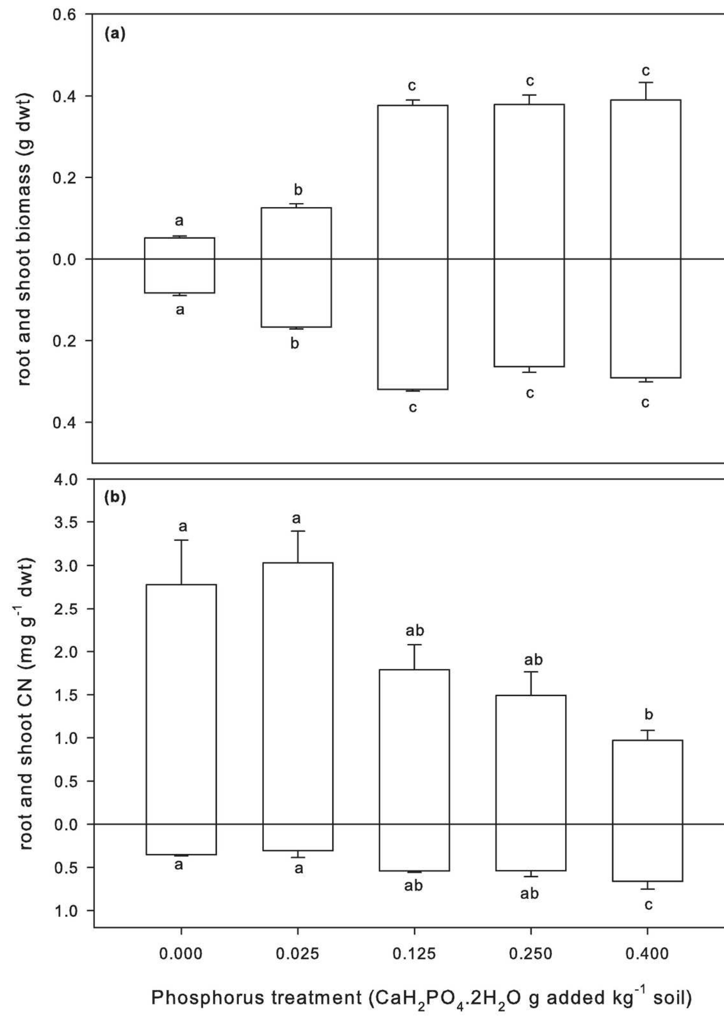

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Figure 2

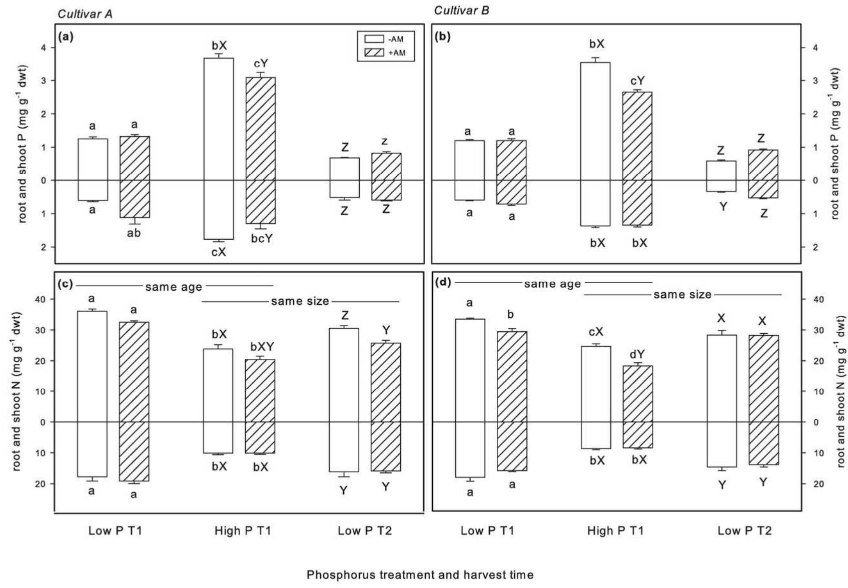

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Figure 3

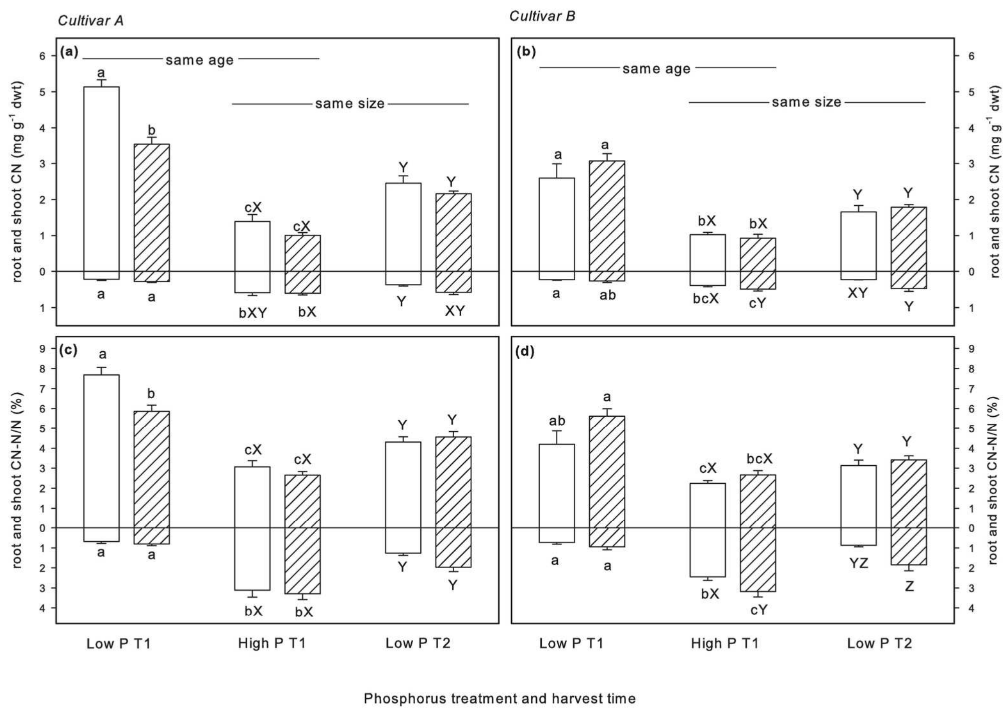

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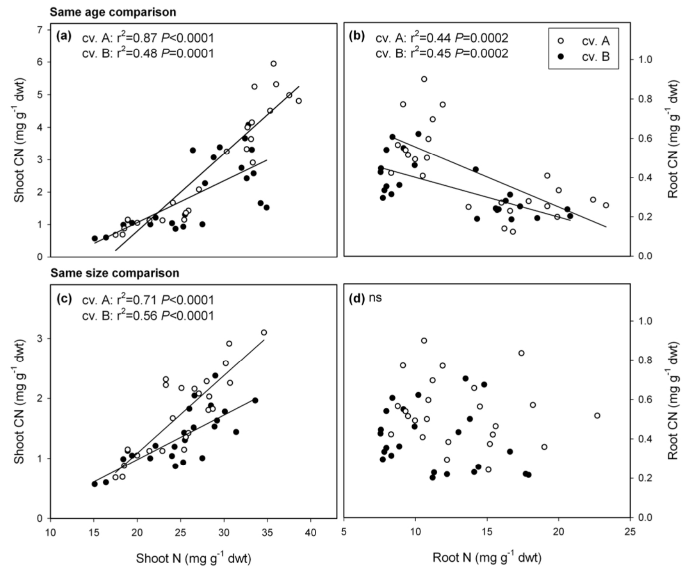
Figure 4.



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