Plant, Cell & Environment

Plant, Cell and Environment (2014) **37**, 929–942 doi: 10.1111/pce.12209

Original Article

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

Rebecca E. Miller[1]*, Roslyn M. Gleadow[1] & Timothy R. Cavagnaro[1†]

[1]*School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia*

ABSTRACT

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

Key-words: cyanogenesis; dhurrin; nitrogen; phenotypic plasticity; plant defence; roots; resource allocation.

INTRODUCTION

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

Among environmental variables, the effect of soil P on chemical defences has been little explored (e.g. Bryant *et al*. 1993). This is surprising because P is important for photosynthesis and growth and consequently has the potential to affect the trade-off in resources between primary and secondary metabolism implicit in most defence theories (e.g. Sampedro *et al*. 2010, 2011). The potential impact on plant defence of below-ground associations such as arbuscular mycorrhizal (AM) associations, an important pathway for the uptake of P and other nutrients in plants (Smith & Read 2008), has similarly been neglected (see also Bezemer & van Dam 2005; Erb *et al*. 2009; Schloter & Matyssek 2009; Cavagnaro *et al*. 2011). There are few studies of the effect of P and/or AM on chemical defence and none, to our knowledge, on a possible link with

Correspondence: R. E. Miller. Tel: +61 3 90356800; e-mail: millerr2@unimelb.edu.au

Present address: *Melbourne School of Land and Environment, University of Melbourne Burnley Campus, Richmond, Victoria 3121, Australia; †School of Agriculture, Food and Wine, The University of Adelaide, PMB1, Glen Osmond, South Australia 5064, Australia.

cyanogenic glycosides, an important class of constitutive N-containing allelochemicals found in over 5% of all species, including many crops (Jones 1998). Cyanogenic glycosides are safe when stored *in planta*, but release toxic hydrogen cyanide (HCN) when plant tissues are crushed or chewed, and high concentrations can have significant impacts on human and animal health (Cliff *et al*. 1985; Wheeler *et al*. 1990).

Here, we use the cyanogenic species *Sorghum bicolor* (L.) Moench – one of the top five crops in terms of area, with over 300 million ha planted worldwide in 2011 (FAOSTAT 2011) – as a model to address several questions about phenotypic plasticity in chemical defence in response to P and AM. Sorghum is an appropriate model for this study as it is capable of forming AM (Rillig *et al*. 2001), and owing to the agronomic significance of understanding factors affecting forage toxicity, has been the subject of the only previous work on the effects of P on cyanogenesis. Understanding P effects on plants (in particular crops) is of growing importance given peak P, the point where the maximum global phosphorus production rate is reached, is predicted to occur in about 2030 (Cordell *et al*. 2009). Data in the literature report a significant negative association between P supply and cyanogenic potential (HCNp) in sorghum shoots (e.g. Patel & Wright 1958; Harms & Tucker 1973; Wheeler *et al*. 1990) and significant stimulation of HCNp by N supply (e.g. Patel & Wright 1958; McBee & Miller 1980; Busk & Møller 2002). HCNp is the amount of cyanide released from all cyanogenic compounds present in plant tissue. The majority of these studies failed to effectively take account of plant size and variations in growth rate and developmental stage – several not reporting biomass data, and none comparing plants at an equivalent size or developmental stage. This is important because the HCNp of sorghum is known to change significantly with ontogeny: HCNp reaches a maximum 4 days after germination where dhurrin content in the young seedling tips may account for 6% of the dry weight, and decreases more than fivefold by about 40 days old (Akazawa *et al*. 1960; Loyd & Gray 1970; Halkier & Møller 1989; Busk & Møller 2002). Against such significant ontogenetic drift in cyanogenic capacity, any interpretation of the effects of nutrients on plant defence phenotype is likely confounded by differences in plant size and development. This is illustrated by the study of Patel & Wright (1958) in which sorghum shoot HCNp of same aged plants differed by a maximum of ~450 ppm across nutrient treatments, while within treatments, shoot HCNp varied by ~400 ppm with ontogeny. Despite significant developmental changes in HCNp, biomass data was not presented in this study so no comparisons of same-sized plants could be made (Patel & Wright 1958). While comparing plants at the same time point may be appropriate for some studies of defensive traits, such as simultaneous comparisons of chemical composition in relation to herbivore abundance (Coleman *et al*. 1994), size-dependent comparisons may be more appropriate for studies focused on assessing phenotypic plasticity and the costs of resource allocation to chemical defence in tests of plant defence theories (Goodger *et al*. 2006; Barton & Koricheva 2010; Simon *et al*. 2010).

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

MATERIALS AND METHODS

Soils, plant material and nutrient treatments

A soil:sand mix (18:82 w/w on a dry weight basis) was used in all experiments. This mixture was comprised of coarse washed sand and collected from the 0 to 15 cm soil layer at the Wallenjoe Swamp State Game Reserve, northern Victoria, Australia. Soil from this site has low plant available (Colwell) P (10.2 mg g^{-1} dry soil) and a high AM inoculum potential (see Cavagnaro & Martin 2011; Watts-Williams & Cavagnaro 2012); this soil:sand mix is referred to as 'soil' hereafter. Non-mycorrhizal treatments were established following Smith & Smith (1981) and others (Lovelock & Miller 2002; Asghari & Cavagnaro 2011).

The forage sorghum cultivars used here are hybrids between grain sorghum, *S. bicolor* (L.) Moench ssp. *bicolor* and sudan grass [*S. bicolor* (L.) Moench ssp. *drummondii* (Nees ex Steud.) de Wet & Harlan; syn. *S. sudanense* (Piper) Stapf]. Experiments were performed using two genetically related hybrids (cv. A and cv. B) known to differ in HCNp, and therefore potentially also in the allocation of N to cyanogenesis. Seeds were surface sterilized by immersion for 10 min in an aerated 3% sodium hypochlorite solution, rinsed with reverse osmosis (RO) water to remove any trace of NaOCl, and them imbibed in aerated RO water for 20 min. Seeds were then directly sown into seedling trays, containing sterilized (twice autoclaved) seed raising mix (Debco, Tyabb, Victoria, Australia). After 7 days, seedlings were carefully washed from the seed raising mix with RO water and seedlings of a uniform size were transplanted into 1 L pots

containing the soil:sand mix. Ten additional seedlings of each genotype (equivalent to those used in the experiments) were oven dried to determine an initial biomass for relative growth rate (RGR) determination.

Plants were grown in a glasshouse with supplemental lighting (MK-1 Just-a-shade, Ablite, Allambie Heights, NSW, Australia) on the Monash University Clayton campus in April 2009, with a 16/8 h day/night photoperiod. Air temperature and relative humidity were logged as 5 min averages throughout the experiment using a Veriteq Spectrum SP-2000-20R logger (Veriteq, Richmond, BC, Canada) while photosynthetic photon flux density (PPFD) was logged for a 2-week period as 15 min averages using two visible light sensors (LI-190SA, Li-Cor Inc., Lincoln, NE, USA). Conditions in the glasshouse were as follows: mean day time temperature 22.1 °C (minimum 18.3 °C/ maximum 25.9 °C); mean night time temperature 20.1 °C (minimum 17.3 °C/maximum 22.6 °C); mean light intensity 144.2 ± 30.3 μmol quanta m^{-2} s^{-1}; mean daily photon load 495.1 ± 108.6 mol quanta m^{-2} and mean relative humidity 44 ± 9 %. Plants were watered three times weekly with 20% modified Long Ashton solution minus P (Cavagnaro *et al*. 2001; Cavagnaro & Martin 2011) with a total of 8 mM nitrogen supplied as 4 mM $NaNO_3$ and 2 mM $(NH_4)_2SO_4$. Plants were also flushed with RO water once a week to prevent salt accumulation over the course of the experiment. Plants within treatments and treatments were randomized spatially in the glasshouse weekly.

Experimental design

Experiment 1

In order identify the optimal amount of P to be added to the soil in the main experiment, five P-addition treatments were established by mixing $CaHPO_4.2H_2O$, with the 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 used, and only mycorrhizal treatments were included in this preliminary experiment to confirm the high inoculum potential of the soil. Plants were harvested when they were at the *ca.* five-leaf stage, 23–24 days after transplanting (or 27 days after sowing).

Experiment 2

Based on the results of the first experiment described above, P was added to the soil at 0.025 and 0.125 g $CaHPO_4.2H_2O$ kg^{-1} dry soil (as above) to establish low- and high-P addition treatments. To investigate chronological and ontogenetic patterns in plant responses, there were two harvests. The first harvest (time 1) was 24 days after transplanting (31 days after sowing), when plants are approximately at the five- to six-leaf stage. Because of the known ontogenetic variation in sorghum cyanogenesis (Patel & Wright 1958; Loyd & Gray 1970; McBee & Miller 1980), a second harvest (time 2) was included to enable comparison of plants at the same size (developmental stage); that is, 63–64 days after transplanting, when the low-P plants had reached the same size (on the basis of leaf number, and biomass, see Results) as the high-P plants harvested at time 1. The first harvest time was chosen as it is well after the maximum seedling HCNp, which occurs within a week of germination (Loyd & Gray 1970; Busk & Møller 2002).

Plant biomass sampling and analysis

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

Leaf nitrogen and carbon

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

Cyanogenic glucoside concentration

The concentration of cyanogenic glucosides was measured by trapping the cyanide (CN), liberated following hydrolysis of the glucoside, in a 1 M NaOH well (Brinker & Seigler 1989) as modified by Miller *et al*. (2004). Freeze-dried, ground plant tissue (10–15 mg dwt) was incubated for 24 h at 37 °C with 1 mL of 0.1 M citrate–HCl (pH 5.5). β-glucosidase emulsin from *Prunus amygdalus* Batsch (EC 3.2.1.21, Sigma G-0395, Sigma-Aldrich Pty. Ltd., Sydney, NSW, Australia) was added to the buffer at the rate of 1.12 units mL^{-1}. Cyanide in the NaOH well was determined using a photometric microplate reader (Labsystems Multiskan Ascent, with incubator, Labsystems, Helsinki, Finland). The absorbance was measured at 595 nm with NaCN as the standard.

Statistical Analyses

The effects of P supply on biomass and tissue chemistry in Experiment 1 were analysed using one-way analysis of variance (ANOVA). Data from Experiment 2 were analysed in two separate two-factor general linear models (GLMs). The first assessed the impacts of P and AM treatments on plants of the same age (i.e. harvested at time 1). The second assessed

the impacts of P and AM treatments on plants of the same size (i.e low P-grown plants harvested at time 2 and high P-grown plants harvested at time 1). In order to satisfy the assumptions of ANOVA, several variables were transformed. Significant differences between treatments at the $P = 0.05$ level were assessed using Tukey's HSD *post hoc* analysis. Some additional two-factor GLM analyses were conducted to investigate changes in shoot and root N concentrations over time in low-P treated plants. All analyses were conducted using JMP v.9 (SAS Institute Inc., Lane Cover, NSW, Australia). In order to compare the extent of mycorrhizal colonization across genotypes and treatments, a three-way GLM was conducted. This analysis also included harvest 2, high-P plants to compare the effect of P on the extent of colonization at this longer time period and to compare the colonization of genotypes. GLM results for data relating to Figs 2 and 3 are presented in the Supporting Information Table S1.

RESULTS

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

In the initial experiment, sorghum was grown at five different levels of P and harvested 27 days after sowing. Shoot and root biomass of seedlings increased with increasing soil P addition, up to an addition of 0.125 g $CaHPO_4.2H_2O$ kg^{-1} dry soil, after which further P addition had no detectable effect on growth (Fig. 1a). Leaf area increased, while root:shoot decreased with increasing P supply (see Supporting Information Table S1). Whereas the concentrations of cyanogenic glucosides in the shoots of plants decreased significantly (by 65%) with P addition, root cyanogenic glucoside concentrations increased by 88% (Fig. 1b). These divergent responses of shoot and root cyanogenic glucoside concentration are despite similar declines in root and shoot N with increasing soil P supply (data not shown). On the basis of these results, P addition treatments selected for inclusion in the main experiments were 0.025 and 0.125 g $CaHPO_4.2H_2O$ kg^{-1} dry soil. In addition, colonization of roots by AMF was detected (but not quantified) in plants at the time of harvest, indicating that the soil used contained appropriate inoculum.

Experiment 2: Mycorrhizal colonization and plant growth

In the main experiment, plants were grown supplied with two concentrations of P (low P, high P), in either sterilized soil or soil containing AM inoculum. Plants were harvested 27 days after planting, when high-P plants had five to six leaves (time 1). A second set of low-P plants was harvested after 63–64 days when they attained the same developmental stage as the high-P plants at the first harvest (time 2). This combination of harvests provided the opportunity to directly compare plants of the same size (ontogeny), as well as at the same age (chronology). Overall, total biomass of low-P plants harvested at time 2 was not significantly different from the biomass of high-P plants harvested at time 1, validating the use of leaf number as a determinant of plant size (Tables 1 & 2).

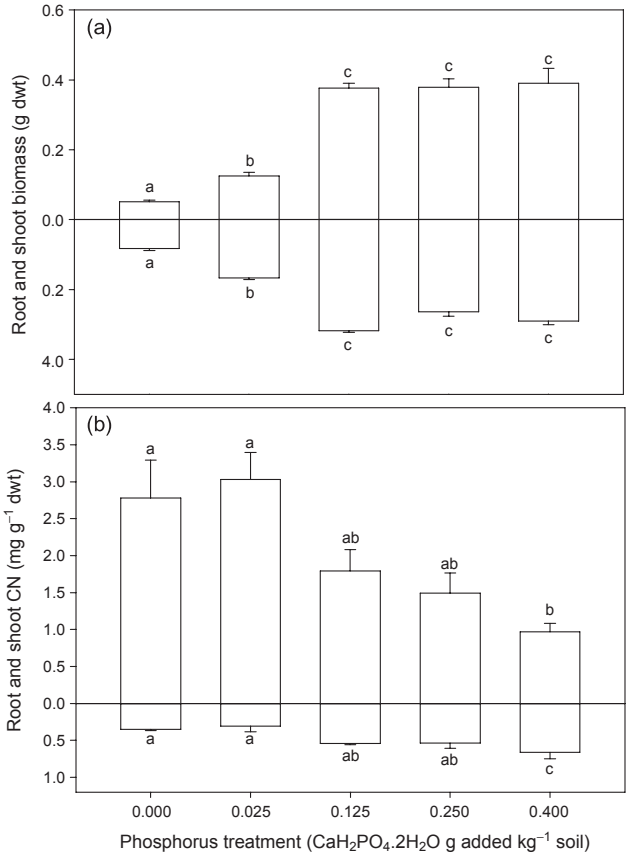

Figure 1. (a) Biomass (dry weight) and (b) HCNp of roots and shoots of 27-day-old *Sorghum* cv. A seedlings grown at five different soil P concentrations. Different letters on the bars indicate significant differences (one-way ANOVA) between treatments. Data area means ± SE of $n = 3$ plants. Letters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

Colonization rates

Significant differences in percent root colonization were detected with genotype ($F_{7,47} = 60.4$; $P = 0.0045$), harvest time ($P < 0.0001$) and P treatment ($P < 0.0001$). Plants in the non-mycorrhizal treatment were either not colonized by AMF (cv. A) or showed only very low levels of colonization (<2%; cv. B) (Table 2). Rates of colonization increased significantly with time from 2.2–12.8% at time 1 to 7.8–64.0% at time 2, depending on the cultivar and the treatment (Tables 1 & 2). Root colonization was approximately fivefold greater in low-P plants compared to high-P plants and roots of plants of cv. B were significantly ($P = 0.004$) more colonized than those of cv. A.

Biomass and biomass partitioning

When plants of the same age, grown under different P regimes, were compared, total biomass of both genotypes was significantly higher in the high-P treatment, irrespective of AM treatment (Tables 1 & 2). For both genotypes, significant effects of AM colonization on total biomass were detected at

Table 1. Summary growth and biomass allocation of *Sorghum* cv. A grown at high and low phosphorus (HP, LP), with and without AM colonization (±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)

Treatment/harvest	Root colonization (%)	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

Two-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	$F_{3,23} = 13.4$	$F_{3,23} = 87.6$	$F_{3,23} = 124.9$	$F_{3,23} = 1.2$	$F_{3,23} = 90.4$	$F_{3,23} = 950.5$	$F_{3,23} = 345.0$	$F_{3,23} = 13.0$	$F_{3,23} = 14.7$	$F_{3,23} = 12.4$

Data are means ± SE of $n = 6$ plants. Results of two-way general linear models (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.
AM, arbuscular mycorrhizal; ANOVA, analysis of variance; RGR, relative growth rate.

Table 2. Summary of growth and biomass allocation of *Sorghum* cv. B grown at high and low phosphorus (HP, LP), with and without AM colonization (±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)

Treatment/harvest	Root colonization (%)	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}

Two-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	$F_{3,23} = 16.3$	$F_{3,23} = 267.9$	$F_{3,23} = 242.6$	$F_{3,23} = 0.225$	$F_{3,23} = 242.9$	$F_{3,23} = 898.5$	$F_{3,23} = 498.0$	$F_{3,23} = 22.1$	$F_{3,23} = 22.1$	$F_{3,23} = 17.6$

Data are means ± SE of $n = 6$ plants. Results of two-way general linear models (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.
AM, arbuscular mycorrhizal; ANOVA, analysis of variance; RGR, relative growth rate.

time 1, when plants were the same age, but in opposite directions. Specifically, for cv. B, which had higher levels of AMF colonization, the AM treatment was associated with increased total biomass, an effect most evident at low P, where there was a 28% increase in total biomass (Table 2). Conversely, for cv. A, there was a significant, albeit relatively small, growth depression in the mycorrhizal plants at time 1, despite low levels of AM colonization in the high-P plants. When both genotypes were harvested at the same ontogenetic stage, there was no detectable effect of AM colonization on total biomass, unlike in the same age comparison. Consequently, high-P plants had a higher RGR than low-P plants in both genotypes, with 1.5–1.6-fold increase when compared at the same age (time 1), and 2.2–2.3-fold increase when compared at the same stage. AM colonization was only associated with enhanced RGR in cv. B in the same age comparison (by 2.2 and 7.0% in high-P and low-P treatments, respectively) but not same size analysis (Tables 1 & 2).

Treatment effects on biomass partitioning differed between genotypes (Table 2). For both genotypes, and both harvests, the root:shoot ratio was significantly higher in plants supplied with low P (Table 1). For cv. A, whether at low or high P, there was a decrease in relative root biomass with AM colonization ($F_{3,23}$=14.7, P = 0.002; Table 1). Interestingly, when comparing low P-grown plants over time, root:shoot did not change with plant age, but was significantly lower in mycorrhizal plants of both genotypes (cv. A: $F_{3,23}$=6.07, P = 0.0033 and cv. B: $F_{3,23}$=2.89, P = 0.0146). For leaf area, there was a significant interaction between AM and P treatments for cv. A harvested at time 1, with a slight increase in leaf area with AMF under low P, whereas at high P, consistent with the total biomass data, there was a 19% decrease in total leaf area of plants grown with AMF (Table 1). In cv. B, there was a trend towards increased leaf area with AM colonization; however, the increase was only significant in the same size comparison (Table 2). At time 1, no differences in leaf area ratio (LAR) were detected for cv. A or cv. B, whereas specific leaf area (SLA) was significantly higher in low-P 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 shown). By contrast, when plants of the same size were compared both SLA and LAR were significantly greater in high P-grown plants. A significant interaction between AM and P treatments was detected for SLA in cv. A (P = 0.0295) and LAR in cv. B (P = 0.0350), with, in each case, the magnitude of the difference between low- and high-P treatments being reduced in the mycorrhizal treatment.

Plant nutrition and cyanogenic glucosides

P concentration

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

Cyanogenic glucosides

The HCNp varied significantly with genotype, treatment, harvest time and between shoots and roots (Fig. 3). Overall, across all treatments there were seven main observations. First, shoot HCNp was 42% higher in cv. A compared with cv. B, and root HCNp was 27% higher, consistent with expected differences in cyanogenic capacity of the two cultivars (Fig. 3a,b). Second, HCNp was more than fivefold higher in shoots than in roots of both cultivars (Fig. 3a,b). Third, low P-grown plants had a higher shoot HCNp than high P-grown plants when compared at the same age (time 1). Averaged across AM treatments, the shoot HCNp of low P-grown plants was 2.9- and 3.6-fold greater than high P-grown plants in cv. A and cv. B, respectively (Fig. 3a,b). Fourth, when compared at the same ontogenetic stage (time 2 for low P-grown plants and time 1 for high P-grown plants), the shoot HCNp of the low-P plants was still higher than high-P plants, although the magnitude of the difference was much smaller than when compared at the same time point (Fig. 3a,b). In both genotypes, shoot HCNp was 1.8–1.9 times greater at low P than high P, pooling AM treatments. Fifth, there were few differences in shoot HCNp between plants from the different mycorrhizal treatments and those that were found were broadly consistent with changes in N status and ontogeny (see below under Total N concentration). Sixth, shoot and root cyanogen concentrations responded in the opposite direction to changes in P supply (Fig. 3a,b). Most notably, shoots of smaller low-P plants had significantly higher HCNp than larger high-P plants, while roots of low-P plants had significantly lower (1.3–2.4 times) HCNp than high-P plants. Finally, the direction of ontogenetic changes in shoot HCNp and root HCNp differed, and in roots was significantly affected by AMF (based on the comparison between time 1 and time 2 for low P-grown plants). In contrast to the significant reduction in shoot HCNp with plant age in both genotypes (47 and 39% reductions for cv. A and cv. B, respectively), root HCNp increased significantly, by 90 and 40% for cv. A and cv. B, respectively, pooling AM treatments. This increase in root HCNp with age was significantly greater in mycorrhizal plants than non-mycorrhizal plants for both genotypes (two-way GLM, cv. A P = 0.0065 and cv. B P = 0.0047, AM treatment main effect). For example, in cv. A, root HCNp of plants with AMF increased 107% between harvests compared with a 68% increase in non-mycorrhizal plants (Fig. 3a,b).

Total N concentration

When plants of the same age were compared, the concentration of N in the shoots and roots of plants of both genotypes

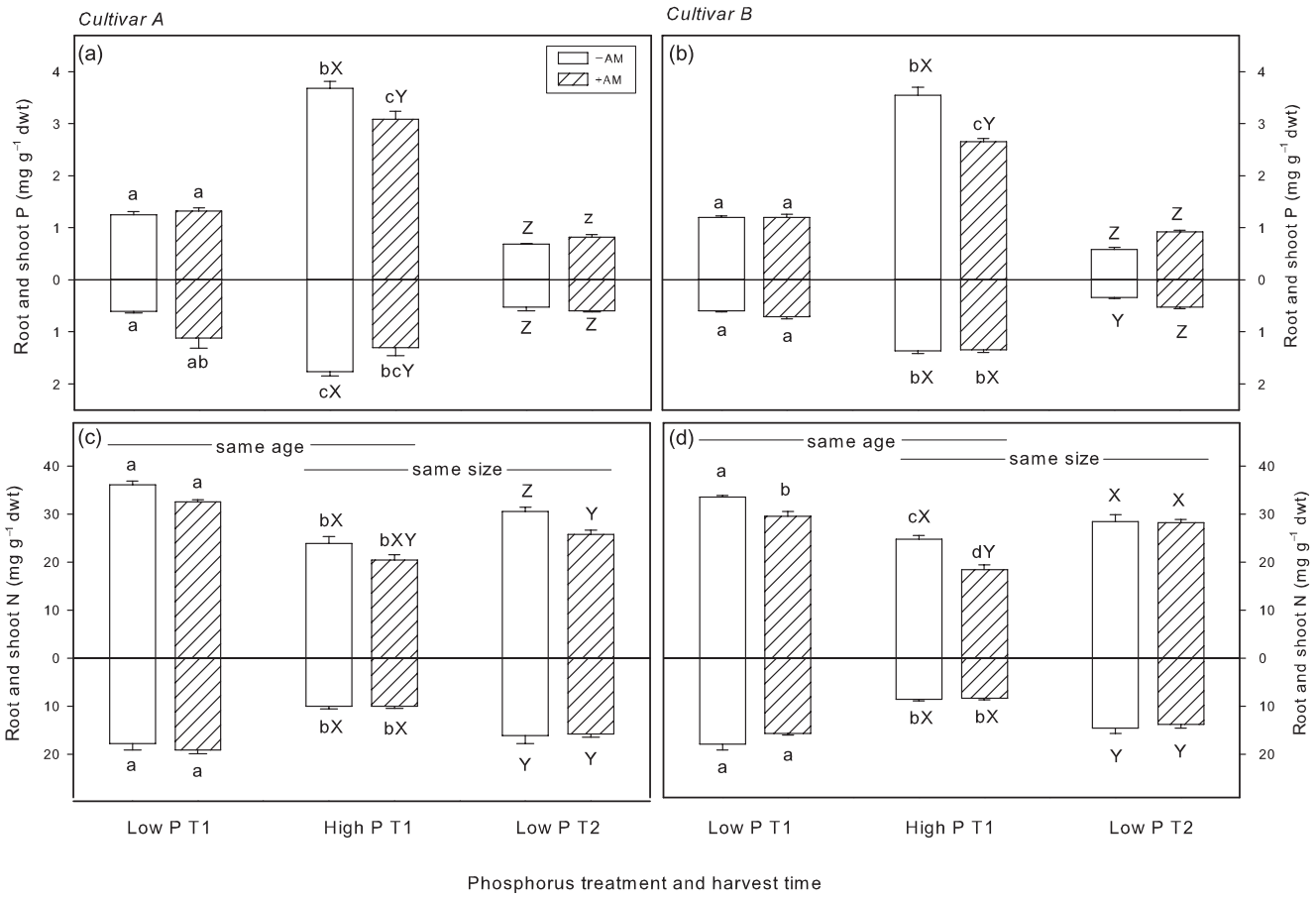

Figure 2. Mean (a, b) phosphorus concentration and (c, d) nitrogen concentration in *Sorghum* cv. A (a, c) and cv. B (b, d) shoots (above line) and roots (below line) grown at high (HP) and low (LP) phosphorus treatments, with (hatched bars) and without (white bars) arbuscular mycorrhizas. Plants were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

was greater in the low-P treatment, irrespective of mycorrhizal treatment (Fig. 2c,d). In cv. A, there was a trend towards greater shoot N in non-mycorrhizal plants, while in cv. B, the effect of AM treatment was significant in both P treatments, with mycorrhizal plants having 12 and 25% lower mean shoot N than non-mycorrhizal plants in low- and high-P treatments, respectively (Fig. 2d). Similarly, when plants of different ages but the same size were compared, the concentration of N was again generally higher in the low-P addition treatment than in the high-P treatment, for both genotypes (Fig. 2c,d). In both cultivars, both shoot and root N concentrations decreased with development from time 1 to time 2, in low P-grown plants. Consistent with observations of plants at the same chronological age, any significant AM effects again showed non-mycorrhizal plants to have higher shoot N concentration than mycorrhizal plants, for example, in the low-P plants of cv. A at time 2 shoot N was 19% higher in non-mycorrhizal plants (Fig. 2c). In the case of root N concentrations, no significant differences between mycorrhizal treatments were detected for either cultivar; however, root N was higher in plants grown with low P than with high P, in both same age and same size comparisons, for both genotypes (Fig. 2c,d).

Significant differences in shoot N:P were detected with AM colonization (data not shown). For both same age and same size comparisons, and for both genotypes, AM colonization was associated with a greater relative P content, and a significant reduction in shoot N:P. Similarly, in roots of cv. B, AM colonized plants had significantly lower N:P in both comparisons. A significant interaction between AM and P was detected such that this difference was only significant in low-P treatments, where AM colonization was greater.

Comparison of N and HCNp

Irrespective of AM treatment, genotype or whether comparing same aged or same sized plants, the proportion of N allocated to cyanogenic glucosides (CN-N/N%) was significantly higher in shoots, but lower in roots, of plants supplied with low P (Fig. 3c,d). The magnitude of this P effect on

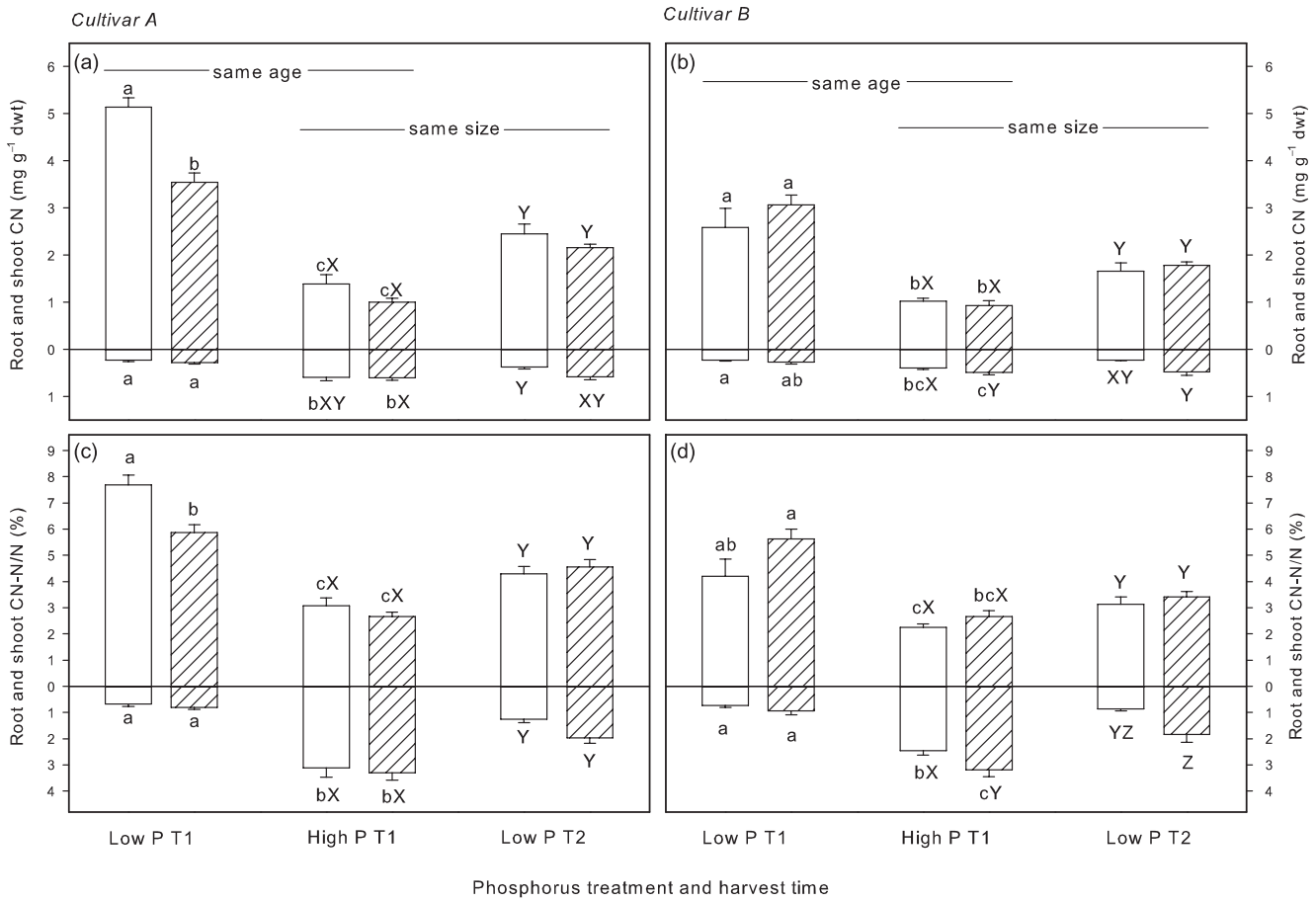

Figure 3. Mean (a, b) HCNp and (c, d) mean total N allocated to cyanogenic glucosides (CN-N/N%) in *Sorghum* cv. A (a, c) and cv. B (b, d) shoots (above line) and roots (below line) grown at high (HP) and low (LP) phosphorus treatments, with (hatched bars) and without (white bars) arbuscular mycorrhizas.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

CN-N/N (%) was greater in the same age comparison than the same size comparison, as shoot CN-N/N (%) of low-P plants decreased with development (time 1 versus time 2). For example, in non-mycorrhizal cv. A, in the same age comparison, shoot N allocation to CN in low P-grown plants was 2.5-fold greater than in high P-grown plants, but was only 1.4-fold greater when plants of the same size were compared (Fig. 3c). Conversely, for roots of the same plants, N allocation to CN in high P-grown plants was 4.6-fold greater than in low P-grown plants in the same age comparison, but was 2.5-fold greater in the same size comparison, as root CN-N/N (%) of low-P plants increased over time. When compared at the same age, CN-N/N (%) was higher in both shoots and roots of mycorrhizal cv. B plants (Fig. 3d). When cv. B plants of the same size were compared, this same effect was seen in the roots but no AM effect on shoot CN-N/N (%) was detected (Fig. 3d). The same trend was seen in the same size comparison of cv. A but the differences were not significant. Despite similar percent decreases in shoot and root N% between harvests for low P-grown plants, shoot CN-N/N (%) decreased and root CN-N/N (%) increased significantly with ontogeny (Fig. 3c,d). For example, in cv. A, percent N allocation to CN in shoots decreased 35% between time 1 and time 2, but increased 117% in roots, averaged across AM treatments (Fig. 3; $F_{3,23} = 18.1$, $P < 0.0001$ harvest time main effect). Whereas there was no difference between AM treatments for shoots, in roots, the relative increase in CN-N/N (%) was significantly greater in mycorrhizal plants (143%) than non-mycorrhizal plants (85%; cv. A $P = 0.0067$ AM treatment main effect).

To examine N allocation in different tissues in more detail, relationships between N and cyanogenic glucoside concentrations were further explored using regression analysis (Fig. 4). For shoots, irrespective of whether plants were being analysed from the same age comparison or same size comparison, there were significant positive correlations between foliar N and HCNp (Fig. 4a,c). When plants of the same age were compared, that is, where P treatments resulted in plants of significantly different size (i.e. and developmental stage), highly significant positive correlations between foliar N

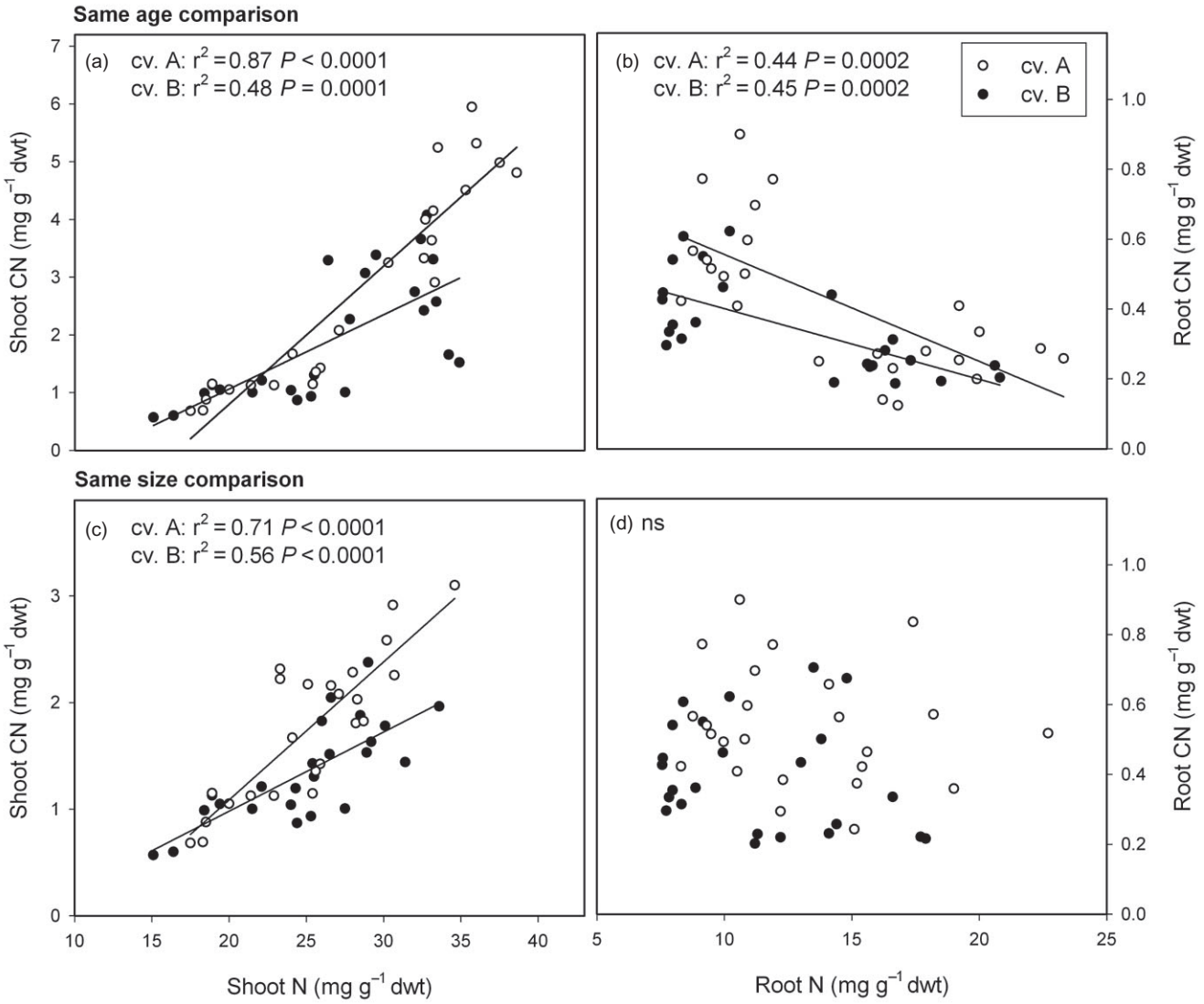

Figure 4. Relationship between HCNp and total nitrogen concentration in shoots (a, c) and roots (b, d) of *Sorghum* cv. A (open circles) and cv. B (closed circles) plants grown at high and low P, with and without arbuscular mycorrhizas (AM). Relationships are shown for when high-P and low-P plants were the same age (a, b), and for when low-P and high-P plants were the same size (c, d). Significant linear relationships are shown for both genotypes. Equations: (a) same age cv. A: shoot CN = 0.239*shoot N-3.98, cv. B: shoot CN = 0.129*shoot N–1.51 (b) same age cv. A: root CN = –0.031*root N+0.862, cv. B: root CN = –0.020*root N+0.603 (c) same size cv. A: shoot CN = 0.129*shoot N–1.50, cv. B: shoot CN = 0.074*shoot CN–0.504.

content and HCNp were found in both genotypes, with 87% of variation in HCN explained by variation in N in cv. A (Fig. 4a). In cv. B, this value was 48% (Fig. 4a). When plants of the same size were analysed, similarly strong positive correlations between foliar N and HCNp were still evident, with 71 and 56% of the variation in HCNp explained by variation in N, in cv. A and cv. B, respectively (Fig. 4c).

In contrast to shoots, when examining roots from plants of the same age, a strong negative correlation between root N concentration and HCNp was found for both genotypes (Fig. 4b). At time 1, variation in root N was driven by P treatments and consequential differences in plant size, with high P-grown, larger plants having lower root N concentrations. In this analysis, 44 and 45% of the variation in HCNp could be explained by variation in root N in cv. A and cv. B, respectively. By contrast, when examining plants of the same size, no significant relationship between N and HCNp in roots was detected.

DISCUSSION

Even though many crop plants are thought to be cyanogenic (Jones 1998), little is known about how P affects the synthesis and turnover of cyanogenic glucosides. We hypothesized that improved P and N nutrition, either by higher P supply or AM association, would result in a higher HCNp in sorghum tissues, but that the change could only be appropriately quantified when ontogenetically controlled comparisons were

made. We found that P-limited plants had higher shoot HCNp when plants were compared at the same age, a change not entirely consistent with those plants being smaller and developmentally younger (Busk & Møller 2002) as ontogenetically controlled comparisons, using plants with the same leaf number and biomass, also showed that there was an increase, of lesser magnitude, in the allocation of N to dhurrin in shoots when P was limiting. By contrast, in the roots there was a decrease in HCNp in the low P-grown plants, but no obvious trend when ontogenetic differences were taken into account. Colonization of sorghum roots by AM did not consistently affect shoot HCNp, but there was a significant interaction with P, such that the effects of low P on growth and shoot HCNp were ameliorated by AM. By contrast, in low P-grown plants, AM effected a significant increase in root HCNp over time. Interestingly, there was no appreciable growth sacrifice associated with the formation of AM. Results were broadly similar across the two genotypes despite differences in HCNp and rates of AM colonization.

Stage versus age

Assessing phenotypic plasticity in the expression of sorghum cyanogenesis in response to variation in soil P supply is confounded by significant developmental and age-related changes in tissue CN (and likely N) concentrations (Busk & Møller 2002), to such an extent that strikingly different conclusions about the significance of P supply for HCNp would be drawn depending on whether plants are compared at the same age or same stage. Results presented here support our hypothesis that differences in HCNp associated with P supply are primarily, but not entirely, mediated via changes in plant growth rate and developmental stage. Previously published research on sorghum has been confounded by differences in plant size and developmental stage, making it hard to assess the extent to which the reported variation in HCNp with P was 'apparent' plasticity, and a consequence of changes in growth rate in response to varied P supply. Wheeler *et al*. (1980), for example, found higher P more than doubled forage yield and reduced HCNp by 34%; Kriedeman (1964) also found that increased P enhanced dry matter production more rapidly than CN production. Consistent with these earlier studies, we also found that HCNp was higher in P-deficient plants when they were harvested at the same time, with a 3.2-fold difference in HCNp and a 5.5-fold difference in total plant biomass due to P supply (pooling data for both genotypes). When compared at the same size, the magnitude of P effect on shoot HCNp was reduced, from a 3.2-fold difference to a 1.8-fold difference. The P effect on HCNp was still significant, despite an average 43% decrease in shoot HCNp between harvests in low-P plants. In order to understand how dhurrin is regulated, it is essential, therefore, that ontogenetically controlled comparisons be made rather than simply comparing plants sampled at the same time when the treatments differentially stimulate plant growth. The potential for ontogenetic change to obscure phenotypic variation in defence allocation is also evident in other systems, such as *Plantago lanceolata* L., where reported developmental variation in iridoid glycoside concentrations is of similar or greater magnitude than variation due to environment or genotype (Bowers *et al*. 1992; Bowers & Stamp 1993; Quintero & Bowers 2011, 2012). Together, the results here and in other systems in which ontogenetic changes in chemical defence are well known [e.g. cyanogenic *Eucalyptus* spp.; (Goodger *et al*. 2006; Neilson *et al*. 2011)], indicate the potential for a comparison of any two time points on a complex non-linear trajectory to yield different conclusions, and emphasize the importance of appropriate (e.g. size dependent) comparisons for studies examining the extent of true phenotypic plasticity in response to the environment. Just as size-dependent comparisons are considered most relevant to testing models of optimal biomass allocation, into which ontogeny is better integrated (Coleman *et al*. 1994), they may be more appropriate to studies probing the trade-offs associated with resource allocation to plant chemical defence. This is an area that is clearly open to further investigation, especially where a wider range of plant ages and more detailed developmental trajectories are considered.

Few studies have examined the effect of P supply on either C-based or N-based chemical defences, with no consistent pattern. Sorghum has been the species subject to most research given the economic impact of forage toxicity (Wheeler & Mulcahy 1989). Under P-limited conditions, increased concentrations of constitutive C-based defences (condensed tannins and phenolics) have been reported (Keski-Saari & Julkunen-Tiito 2003; Sampedro *et al*. 2011), whereas no effect of P limitation was found on the concentration of N-based alkaloids in tobacco leaves (Andrade *et al*. 2013), or on pyrrolizidine alkaloids in shoots or roots of ragwort (Vrieling & van Wijk 1994), although plants were not the same size in the latter study. In coffee seedlings, P deficiency led to a 20% reduction in foliar caffeine (Mazzafera 1999), while Andrade *et al*. (2013) found the response of alkaloids to P supply varied with species, tissue and the alkaloid compound. In the present study, in the same size (and same age) comparison, high-P plants were N deficient, and had low shoot HCNp, whereas low P-grown plants were P deficient, with a higher N:P and higher shoot HCNp, suggesting changes in HCNp with P treatment may reflect changes in plant N status. This finding is consistent with the many studies of woody and herbaceous species showing a strong positive correlation between foliar N and HCNp (e.g. Busk & Møller 2002; Gleadow & Woodrow 2000, but see Miller *et al*. 2006; Miller & Tuck 2013). In the roots, however, we found the opposite, with a strong negative correlation between N and HCNp in plants harvested at the same time. Moreover, when plants were harvested at the same ontogenetic stage, there was no correlation between N and root HCNp. Thus, not only are conclusions about the magnitude of the effect of P on HCNp confounded by developmental factors, but conclusions about the importance of N in regulating HCNp in sorghum roots are different depending on whether comparisons are made on a same age versus same size basis. There are few reports of variation in root cyanogenesis (Kaplan *et al*. 2008; van Dam 2009). Interestingly, one study of the highly cyanogenic *Prunus turneriana*, similarly reported no

correlation between root HCNp and N; however, foliar HCNp and N were also not correlated in that species (Miller *et al*. 2004).

Roots versus shoots

Based on the divergent responses of shoot and root HCNp to soil P supply and tissue N concentration, and with ontogeny found here, it seems probable that dhurrin concentrations in sorghum are regulated independently in the roots and the shoots. This is not completely unexpected given the number of cyanogenic species that are reported to have non-cyanogenic roots [e.g. *Trifolium repens* L., (Hughes 1991); *Eucalyptus cladocalyx* F. Muell. (Gleadow & Woodrow 2000)]. Further, Blomstedt *et al*. (2012) identified several mutants of sorghum that, as mature plants, lack dhurrin completely in the leaves, have substantially reduced levels in the sheath, but have completely normal root HCNp. These have been termed adult cyanide deficient class (*acdc1, acdc2, acdc3*) and are possibly regulatory mutants. Cassava (*Manihot esculenta* Crantz.) also has the capacity for cyanogenic glucoside synthesis in both shoots and roots (Du *et al*. 1995; McMahon *et al*. 1995), although significant transport from shoots to roots also occurs in that species (Jørgensen *et al*. 2005). The types of herbivores feeding on roots and shoots are also quite different. Given the importance of herbivores in driving defence strategies (Kaplan *et al*. 2008; Barton & Koricheva 2010; Agrawal *et al*. 2012), it is not surprising that the environmental and molecular regulation of dhurrin in roots and shoots may have evolved in different directions. Similarly, divergent developmental changes in 4β-hydroxiwithanolide concentrations between seedlings and mature plants of cape gooseberry (*Physalis peruviana* L.) have been reported, with concentrations increasing in roots but decreasing in shoots (Calderon *et al*. 2012). Dissimilar responses of root and shoot defences to experimental treatments (e.g. nitrogen; Jamieson *et al*. 2012), and dissimilar ontogenetic changes in root and shoot defence chemistry have been described for other defences, although changes typically appear to be in the same direction (e.g. Lohman & McConnaughay 1998; Quintero & Bowers 2011). The ontogenetic increase in sorghum root HCNp reported here between two time points merits more detailed investigation given the non-linear changes in sorghum root HCNp (Loyd & Gray 1970) and other root defences reported in studies sampling at multiple time points (Williams & Ellis 1989; Beninger *et al*. 2009). Nevertheless, it is clear that shoots cannot be assumed to be representative of the whole plant, and this complexity may challenge the interpretation/application of existing defence theories to whole plants, the emphasis and tests of which to date, tend to focus on above ground tissues (Rasmann & Agrawal 2008; Parker *et al*. 2012).

Resource allocation as a result of changes in P and AM

A role for AM in the uptake of P and N in other species is well known (e.g. Berntson & Bazzazz 1996). No studies to our knowledge, however, have previously been published on the interaction of AM and P on cyanogenesis. We found AM root colonization levels (8–64% in low-P plants) consistent with those reported for sorghum in other studies (Raju *et al*. 1990; Ortas *et al*. 1996; Albert & Sathianesan 2009), but despite some AM effects on biomass (increased leaf area, decreased root:shoot in some comparisons), we did not find consistent significant biomass enhancement with AM colonization. Moreover, while we detected some AM-associated differences in plant chemistry [lower N:P, lower N and increased CN-N/N (%) in some comparisons], we found no consistent significant AM enhancement of P or N uptake. This does not, however, necessarily imply that uptake of nutrients via the AM pathway was not important (Smith *et al*. 2003; Li *et al*. 2006). These findings differ from some other sorghum studies that reported an increase shoot biomass and P content with AM colonization (e.g. Raju *et al*. 1990; Ortas *et al*. 1996), and an increase in the uptake of N not available to non-mycorrhizal plants (Ames *et al*. 1984). The efficacy of different AMF in enhancing plant nutrient status and growth varies with AMF species and plant genotype (Raju *et al*. 1990). Further, other factors such as temperature and the form of N (NH_4^+ or NO_3^-) can also affect AM function, including AM-mediated uptake of P in sorghum (Ortas *et al*. 1996).

The absence of consistent significant AM effects on growth and nutrient uptake here limits our ability to fully probe AM effects on resource allocation to defence as hypothesized; nevertheless, some important insights were gained. For example, whereas no main effect of AM on shoot HCNp was detected, there was a significant interaction with P. This could indicate some amelioration of P stress by AM, although despite a relative increase in P compared to N, there was no significant difference in leaf P between AM and non-AM plants from the low-P treatment. We observed a small decrease in allocation of shoot N to HCNp, when comparing plants colonized by AM with those that did not form AM at the same time point. The reduced allocation of N to CN in AM low P compared with low P-alone plants when compared at the same age (time 1) is consistent with a demand for photosynthate to support the AM. There was a relatively small, but significant, growth depression in the mycorrhizal plants, despite low levels of AM colonization in high-P plants, indicating that there was a significant demand for C by the AM in the first growth interval. It is perhaps unsurprising that we found no AM effect on shoot HCNp in the absence of enhanced shoot N with AM; however, AM had significant effects on below-ground cyanogenic capacity, effecting an increase in root HCNp and the allocation of N to defence over time. The impact of AM on other root defences has previously been reported (e.g. Vierheilig *et al*. 2000; Bennett *et al*. 2006). The effects of AM on shoot and root alkaloids was found to vary with species, organ and compound in a study by Andrade *et al*. (2013), AM colonization stimulating increased production of some root alkaloids in *Catharanthus roseus* (L.) G. Don. In *Plantago lanceolata*, genotype specific increases in root iridoid glycoside concentrations with AM colonization were found (De Deyn *et al*. 2009). This however, is to our knowledge the first study to show an effect of AM on HCNp.

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

CONCLUSION

In comparing the effects of nutrients and other environmental variables on plant defence, it is important to consider ontogenetic variation in assessing true phenotypic plasticity. The increase in HCNp in shoots of plants grown at low P compared with the same point is not completely consistent with a simple increase in concentration in the smaller and developmentally younger P-limited plants as ontogenetically consistent comparisons of plants harvested at the same leaf stage also showed that there was an increase in the allocation of N to dhurrin in P-limited plants. We propose that changes in the availability of P relative to N within the plant allow N to be reallocated from the primary metabolism to dhurrin but critically, that the magnitude of this response can be obscured when comparing plants at different stages of development. N supply in shoots is the most significant driver of differences in shoot HCNp, but conclusions about N regulation of HCNp in roots differ depending whether same age or same sized plants are compared. Further, root HCNp was also enhanced by AM colonization. Results presented here show that P supply, AM and ontogeny affect HCNp in quite different ways in roots and shoots. This result further highlights the need to consider above- and below-ground linkages. Such tissue-specific differences could be the result of independent biosynthetic pathways or other regulatory mechanisms that evolved in response to different suites of herbivores or selective forces above- and below-ground. As recognized elsewhere (Barton & Koricheva 2010; Cavagnaro *et al*. 2011; Vannette & Hunter 2011; Parker *et al*. 2012; Vannette & Rasmann 2012), it is clear that above- and below-ground tissues, ontogeny and biotic interactions are all factors which require greater integration into current theories and investigations of plant defence.

ACKNOWLEDGMENTS

We thank Stewart Crowley, Nicholas Cody and Kiara O'Gorman for technical assistance. This project was initiated through a small grant from the School of Biological Sciences to R.E.M. and funded in part by the Australian Research Council (LP100100434) and Pacific Seeds Pty Ltd. (Advanta Group) to T.R.C. and R.M.G. We thank Pacific Seeds for supplying the seed used in this experiment. R.E.M. was supported by a Monash University Faculty of Science Margaret Clayton Fellowship. T.R.C. gratefully acknowledges the Australian Research Council for supporting his research through the Future Fellowship programme (FT120100463).

REFERENCES

Abu-Zeyad R., Khan A.G. & Khoo C. (1999) Occurrence of arbuscular mycorrhiza in *Castanospermum australe* A. Cunn. & C. Fraser and effects on growth and production of castanospermine. *Mycorrhiza* **9,** 111–117.

Agrawal A.A., Hastings A.P., Johnson M.T.J., Maron J.L. & Salminen J.-P. (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science* **338,** 113–116.

Akazawa T., Miljanich P. & Conn E.E. (1960) Studies on cyanogenic glycoside of *Sorghum vulgare*. *Plant Physiology* **35,** 535–538.

Albert E.S.R. & Sathianesan M.S. (2009) Studies on the status of arbuscular mycorrhizal fungi on the fodder crop *Sorghum bicolor* (L.) Moench. *Tropical Life Sciences Research* **20,** 99–109.

Ames R.N., Reid C.P.P., Porter L.K. & Cambardella C. (1983) Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by *Glomus mosseae*, a vesicular arbuscular mycorrhizal fungus. *New Phytologist* **95,** 381–396.

Ames R.N., Porter L.K., St John T.V. & Reid C.P.P. (1984) Nitrogen sources and 'A' values for vesicular-arbuscular and non-mycorrhizal sorghum grown at three rates of ^{15}N-ammonium sulphate. *New Phytologist* **97,** 269–276.

Andrade S.A.L., Malik S., Sawaya A., Bottcher A. & Mazzafera P. (2013) Association with arbuscular mycorrhizal fungi influences alkaloid synthesis and accumulation in *Catharanthus roseus* and *Nicotiana tabacum* plants. *Acta Physiologiae Plantarum* **35,** 867–880.

Asghari H.R. & Cavagnaro T.R. (2011) Arbuscular mycorrhizas enhance plant interception of leached nutrients. *Functional Plant Biology* **38,** 219–226.

Barton K.E. & Koricheva J. (2010) The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. *The American Naturalist* **175,** 481–493.

Beninger C.W., Cloutier R.R. & Grodzinski B. (2009) A comparison of antirrhinoside distribution in the organs of two related Plantaginaceae species with different reproductive strategies. *Journal of Chemical Ecology* **35,** 1363–1372.

Bennett A.E., Alers-Garcia J. & Bever J.D. (2006) Three-way interactions among mutualistic mycorrhizal fungi, plants, and plant enemies: hypotheses and synthesis. *The American Naturalist* **167,** 141–152.

Berntson G.M. & Bazzazz F.A. (1996) Belowground positive and negative feedbacks on CO_2 growth enhancement. *Plant and Soil* **187,** 119–131.

Bezemer T.M. & van Dam N.M. (2005) Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology and Evolution* **20,** 617–624.

Blomstedt C.K., Gleadow R.M., O'Donnell N.H., Naur P., Jensen K., Laursen T., . . . Neale A.D. (2012) A combined biochemical screen and TILLING approach identifies mutations in *Sorghum bicolor* L. Moench resulting in acyanogenic forage production. *Plant Biotechnology Journal* **10,** 54–66.

Boege K. & Marquis R.J. (2005) Facing herbivory as you grow up: the ontogeny of resistance in plants. *Trends in Ecology and Evolution* **20,** 441–448.

Bowers M.D. & Stamp N.E. (1993) Effects of plant age, genotype, and herbivory on *Plantago* performance and chemistry. *Ecology* **74,** 1778–1791.

Bowers M.D., Collinge S.K., Gamble S.E. & Schmitt J. (1992) Effects of genotype, habitat, and seasonal variation on iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and the implications for insect herbivores. *Oecologia* **91,** 201–207.

Brinker A.M. & Seigler D.S. (1989) Methods for the detection and quantitative determination of cyanide in plant materials. *Phytochemical Bulletin* **21,** 24–31.

Bryant J.P., Reichardt P.B., Clausen T.P. & Werner R.A. (1993) Effects of mineral nutrition on delayed inducible resistance in Alaska paper birch. *Ecology* **74,** 2072–2084.

Busk P.K. & Møller B.L. (2002) Dhurrin synthesis in sorghum is regulated at the transcriptional level and induced by nitrogen fertilization in older plants. *Plant Physiology* **129,** 1222–1231.

Calderon J.M., Ruiz N. & Castellanos L. (2012) Within and between plant variation of 4 beta-hydroxiwithanolide E in cape gooseberry (*Physalis peruviana*; Solanaceae). *Biochemical Systematics and Ecology* **41,** 21–25.

Cavagnaro T.R. & Martin A.W. (2011) Arbuscular mycorrhizas in south-eastern Australian processing tomato farm soils. *Plant and Soil* **340,** 327–336.

Cavagnaro T.R., Smith F.A., Lorimer M.F., Haskard K.A., Ayling S.M. & Smith S.E. (2001) Quantitative development of Paris-type arbuscular mycorrhizas formed between *Asphodelus fistulosus* and *Glomus coronatum*. *New Phytologist* **149,** 105–113.

Cavagnaro T.R., Jackson L.E., Six J., Ferris H., Goyal S., Asami D. & Scow K.M. (2006) Arbuscular mycorrhizas, microbial communities, nutrient availability, and soil aggregates in organic tomato production. *Plant and Soil* **282,** 209–225.

Cavagnaro T.R., Gleadow R.M. & Miller R.E. (2011) Plant nutrient acquisition and utilisation in a high carbon dioxide world. *Functional Plant Biology* **38,** 87–96.

Ceccarelli N., Curadi M., Martelloni L., Sbrana C., Picciarelli P. & Giovannetti M. (2010) Mycorrhizal colonization impacts on phenolic content and antioxidant properties of artichoke leaves and flower heads two years after field transplant. *Plant and Soil* **335,** 311–323.

Cliff J., Martensson J., Lundqvist P., Rosling H. & Sorbo B. (1985) Association of high cyanide and low sulfur intake in cassava-induced spastic paraparesis. *Lancet* **2,** 1211–1213.

Coleman J.S., McConnaughay K.D.M. & Ackerly D.D. (1994) Interpreting phenotypic variation in plants. *Trends in Ecology and Evolution* **9,** 187–191.

Coley P.D., Bryant J.P. & Chapin F.S. (1985) Resource availability and plant antiherbivore defense. *Science* **230,** 895–899.

Cordell D., Drangert J.-O. & White S. (2009) The story of phosphorus: global food security and food for thought. *Global Environmental Change* **19,** 292–305.

van Dam N.M. (2009) Belowground herbivory and plant defenses. *Annual Review of Ecology Evolution and Systematics* **40,** 373–391.

De Deyn G.B., Biere A., van der Putten W.H., Wagenaar R. & Klironomos J.N. (2009) Chemical defense, mycorrhizal colonization and growth responses in *Plantago lanceolata* L. *Oecologia* **160,** 433–442.

Du L., Bokanga M., Møller B.L. & Halkier B.A. (1995) The biosynthesis of cyanogenic glucosides in roots of cassava. *Phytochemistry* **39,** 323–326.

Elger A., Lemoine D.G., Fenner M. & Hanley M.E. (2009) Plant ontogeny and chemical defence: older seedlings are better defended. *Oikos* **118,** 767–773.

Erb M., Lenk C., Degenhardt J. & Turlings T.C.J. (2009) The underestimated role of roots in defense against leaf attackers. *Trends in Plant Science* **14,** 653–659.

FAOSTAT (2011) Food and agricultural commodities production. Food and Agriculture Organization of The United Nations Statistics Database. Available at: http://faostat3.fao.org/faostat-gateway/go/to/home/E

Gleadow R.M. & Woodrow I.E. (2000) Temporal and spatial variation in cyanogenic glycosides in *Eucalyptus cladocalyx*. *Tree Physiology* **20,** 591–598.

Goodger J.Q.D., Gleadow R.M. & Woodrow I.E. (2006) Growth cost and ontogenetic expression pattern of defence in cyanogenic *Eucalyptus* spp. *Trees* **20,** 757–765.

Halkier B.A. & Møller B.L. (1989) Biosynthesis of the cyanogenic glucoside dhurrin in seedlings of *Sorghum bicolor* (L) Moench and partial purification of the enzyme system involved. *Plant Physiology* **90,** 1552–1559.

Harms C.L. & Tucker B.B. (1973) Influence of nitrogen fertilization and other factors on yield, prussic acid, nitrate, and total nitrogen concentrations of Sudangrass cultivars. *Agronomy Journal* **65,** 21–26.

Herms D.A. & Mattson W.J. (1992) The dilemma of plants: to grow or defend. *The Quarterly Review of Biology* **67,** 283–335.

Hughes M.A. (1991) The cyanogenic polymorphism in *Trifolium repens* L (white clover). *Heredity* **66,** 105–115.

Jamieson M.A., Seastedt T.R. & Bowers M.D. (2012) Nitrogen enrichment differentially affects above- and belowground plant defense. *American Journal of Botany* **99,** 1630–1637.

Jones D.A. (1998) Why are so many food plants cyanogenic? *Phytochemistry* **47,** 155–162.

Jørgensen K., Bak S., Busk P.K., Sørensen C., Olsen C.E., Puonti-Kaerlas J. & Møller B.L. (2005) Cassava plants with a depleted cyanogenic glucoside content in leaves and tubers. Distribution of cyanogenic glucosides, their site of synthesis and transport, and blockage of the biosynthesis by RNA interference technology. *Plant Physiology* **139,** 363–374.

Kaplan I., Halitschke R., Kessler A., Sardanelli S. & Denno R.F. (2008) Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology* **89,** 392–406.

Kapoor R., Chaudhary V. & Bhatnagar A.K. (2007) Effects of arbuscular mycorrhiza and phosphorus application on artemisinin concentration in *Artemisia annua* L. *Mycorrhiza* **17,** 571–587.

Keski-Saari S. & Julkunen-Tiito R. (2003) Early developmental responses of mountain birch (*Betula pubescens* subsp c*zerepanovii*) seedlings to different concentrations of phosphorus. *Tree Physiology* **23,** 1201–1208.

Kriedeman P.E. (1964) Cyanide formation in *Sorghum almum* in relation to nitrogen and phosphorus nutrition. *Australian Journal of Experimental Agriculture and Animal Husbandry* **4,** 15–16.

Li H., Smith S.E., Holloway R.E., Zhu Y. & Smith F.A. (2006) Arbuscular mycorrhizal fungi contribute to phosphorus uptake by wheat grown in a phosphorus-fixing soil even in the absence of positive growth responses. *New Phytologist* **172,** 536–543.

Lohman D.J. & McConnaughay K.D. (1998) Patterns of defensive chemical production in wild parsnip seedlings (Apiaceae: *Pastinaca sativa* L.). *Chemoecology* **8,** 195–200.

Lovelock C.E. & Miller R. (2002) Heterogeneity in inoculum potential and effectiveness of arbuscular mycorrhizal fungi. *Ecology* **83,** 823–832.

Loyd R.C. & Gray E. (1970) Amount and distribution of hydrocyanic acid potential during the life cycle of plants of three sorghum cultivars. *Agronomy Journal* **62,** 394–397.

McBee G.G. & Miller F.R. (1980) Hydrocyanic acid potential in several sorghum breeding lines as affected by nitrogen fertilization and variable harvests. *Crop Science* **20,** 232–234.

McCarthy M.C. & Enquist B.J. (2007) Consistency between an allometric approach and optimal partitioning theory in global patterns of plant biomass allocation. *Functional Ecology* **21,** 713–720.

McConnaughay K.D.M. & Coleman J.S. (1999) Biomass allocation in plants: ontogeny or optimality? A test along three resource gradients. *Ecology* **80,** 2581–2593.

McMahon J.M., White W.L.B. & Sayre R.T. (1995) Cyanogenesis in cassava (*Manihot esculenta* Crantz). *Journal of Experimental Botany* **46,** 731–741.

Mazzafera P. (1999) Mineral nutrition and caffeine content in coffee leaves. *Bragantia* **58,** 387–391.

Miller R.E. & Tuck K.L. (2013) Reports on the distribution of aromatic cyanogenic glycosides in Australian tropical rainforest tree species of the Lauraceae and Sapindaceae. *Phytochemistry* **92,** 146–152.

Miller R.E., Gleadow R.M. & Woodrow I.E. (2004) Cyanogenesis in tropical *Prunus turneriana*: characterisation, variation and response to low light. *Functional Plant Biology* **31,** 491–503.

Miller R.E., Simon J. & Woodrow I.E. (2006) Cyanogenesis in the Australian tropical rainforest endemic *Bromyba platynema* (Rutaceae): chemical characterisation and polymorphism. *Functional Plant Biology* **33,** 477–486.

Moles A.T., Peco B., Wallis I.R., Foley W.J., Poore A.G., Seabloom E.W., . . . Hui F.K. (2013) Correlations between physical and chemical defences in plants: tradeoffs, syndromes, or just many different ways to skin a herbivorous cat? *New Phytologist* **198,** 252–263.

Moriuchi K.S. & Winn A.A. (2005) Relationships among growth, development and plastic response to environment quality in a perennial plant. *New Phytologist* **166,** 149–158.

Neilson E.H., Goodger J.Q.D., Motawia M.S., Bjarnholt N., Frisch T., Olsen C.E., . . . Woodrow I.E. (2011) Phenylalanine derived cyanogenic diglucosides from *Eucalyptus camphora* and their abundances in relation to ontogeny and tissue type. *Phytochemistry* **72,** 2325–2334.

Neilson E.H., Goodger J.Q.D., Woodrow I.E. & Møller B.L. (2013) Plant chemical defense: at what cost? *Trends in Plant Science* **18,** 250–258.

Ortas I., Harris P.J. & Rowell D.L. (1996) Enhanced uptake of phosphorus by mycorrhizal sorghum plants as influenced by forms of nitrogen. *Plant and Soil* **184,** 255–264.

Parker J.D., Salminen J. & Agrawal A.A. (2012) Evolutionary potential of root chemical defense: genetic correlations with shoot chemistry and plant growth. *Journal of Chemical Ecology* **38,** 992–995.

Patel C.J. & Wright M.J. (1958) The effect of certain nutrients upon the hydrocyanic acid content of sudangrass grown in nutrient solution. *Agronomy Journal* **50,** 645–647.

Quintero C. & Bowers M.D. (2011) Plant induced defenses depend more on plant age than previous history of damage: implications for plant-herbivore interactions. *Journal of Chemical Ecology* **37,** 992–1001.

Quintero C. & Bowers M.D. (2012) Changes in plant chemical defenses and nutritional quality as a function of ontogeny in *Plantago lanceolata* (Plantaginaceae). *Oecologia* **168,** 471–481.

Raju P.S., Clark R.B., Ellis J.R. & Maranville J.W. (1990) Effects of species of VA-mycorrhizal fungi on growth and mineral uptake of sorghum at different temperatures. *Plant and Soil* **121,** 165–170.

Rasmann S. & Agrawal A.A. (2008) In defense of roots: a research agenda for studying plant resistance to belowground herbivory. *Plant Physiology* **146,** 875–880.

Rillig M.C., Wright S.F., Kimball B.A., Pinter P.J., Wall G.W., Ottman M.J. & Leavitt S.W. (2001) Elevated carbon dioxide and irrigation effects on water stable aggregates in a *Sorghum* field: a possible role for arbuscular mycorrhizal fungi. *Global Change Biology* **7,** 333–337.

Sampedro L., Moreira X., Llusia J., Peñuelas J. & Zas R. (2010) Genetics, phosphorus availability, and herbivore-derived induction as sources of phenotypic variation of leaf volatile terpenes in a pine species. *Journal of Experimental Botany* **61,** 4437–4447.

Sampedro L., Moreira X. & Zas R. (2011) Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. *Journal of Ecology* **99,** 818–827.

Schloter M. & Matyssek R. (2009) Tuning growth versus defence–belowground interactions and plant resource allocation. *Plant and Soil* **323,** 1–5.

Simon J., Gleadow R.M. & Woodrow I.E. (2010) Allocation of nitrogen to chemical defence and plant functional traits is constrained by soil N. *Tree Physiology* **30,** 1111–1117.

Smith F.A. & Smith S.E. (1981) Mycorrhizal infection and growth of *Trifolium subterraneum*: use of sterilized soil as a control treatment. *New Phytologist* **88,** 299–309.

Smith S.E. & Read D.J. (2008) *Mycorrhizal Symbiosis* 3rd edn, Academic Press Ltd., Cambridge, UK.

Smith S.E., Smith F.A. & Jakobsen I. (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiology* **133,** 16–20.

Stamp N. (2003) Out of the quagmire of plant defense hypotheses. *The Quarterly Review of Biology* **78,** 23–55.

Toussaint J.P., Smith F.A. & Smith S.E. (2007) Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. *Mycorrhiza* **17,** 291–297.

Vannette R.L. & Hunter M.D. (2011) Plant defence theory re-examined:nonlinear expectations based on the costs and benefits of resource mutualisms. *Journal of Ecology* **99,** 66–76.

Vannette R.L. & Rasmann S. (2012) Arbuscular mycorrhizal fungi mediate below-ground plant-herbivore interactions: a phylogenetic study. *Functional Ecology* **26,** 1033–1042.

Vierheilig H., Bennett R., Kiddle G., Kaldorf M. & Ludwig-Müller J. (2000) Differences in glucosinolate patterns and arbuscular mycorrhizal status of glucosinolate-containing plant species. *New Phytologist* **146,** 343–352.

Vrieling K. & van Wijk C.A.M. (1994) Cost assessment of the production of pyrrolizidine alkaloids in ragwort (*Senecio jacobaea* L.). *Oecologia* **97,** 541–546.

Watts-Williams S. & Cavagnaro T.R. (2012) Arbuscular mycorrhizas modify tomato responses to soil zinc and phosphorus addition. *Biology And Fertility Of Soils* **48,** 285–294.

Wheeler J.L. & Mulcahy C. (1989) Consequences for animal production of cyanogenesis in sorghum forage and hay – a review. *Tropical Grasslands* **23,** 193–202.

Wheeler J.L., Hedges D.A., Archer K.A. & Hamilton B.A. (1980) Effect of nitrogen, sulphur and phophorus fertilizer on the production, mineral content and cyanide potential of forage sorghum. *Australian Journal of Experimental Agriculture and Animal Husbandry* **20,** 330–338.

Wheeler J.L., Mulcahy C., Walcott J.J. & Rapp G.G. (1990) Factors affecting the hydrogen cyanide potential of forage sorghum. *Australian Journal of Agricultural Research* **41,** 1093–1100.

Williams R.D. & Ellis B.E. (1989) Age and tissue distribution of alkaloids in *Papaver somniferum*. *Phytochemistry* **28,** 2085–2088.

Received 16 May 2013; received in revised form 18 September 2013; accepted for publication 21 September 2013

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Summary of two-factor general linear model analysis of variance results for plant chemical composition of the roots and shoots of two sorghum cultivars (cv. A and cv. B) compared when harvested at the same age (i.e. harvested after 28–29 days at Time 1) or at the same size, that is, a comparison between high P-grown plants harvested at Time 1 and low P-grown plants harvested at Time 2 (64–65 days) when the latter had reached the same size as the younger high-P. In addition to the two P treatments, plants were grown with and without arbuscular mycorrhizas. N is total inorganic N measured using a CHN analyser. HCNp is the cyanide potential, measured as the total amount of CN released from endogenous cyanogenic glycosides. CN-N/N% is the proportion of N allocated to CN in the relevant tissues.