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RESEARCH PAPER

Plant growth responses to elevated atmospheric CO_2 are increased by phosphorus sufficiency but not by arbuscular mycorrhizas

Iver Jakobsen[1,2,*], Sally E. Smith[1,3], F. Andrew Smith[1,3], Stephanie J. Watts-Williams[1,4], Signe S. Clausen[1,2] and Mette Grønlund[1,2]

[1] Department of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark
[2] Present address: Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, DK-1871, Thorvaldsensvej 40, Frederiksberg C, Denmark.
[3] Present address: Soils Group, School of Agriculture, Food and Wine, Waite Campus, The University of Adelaide, SA 5005, Australia.
[4] Present address: Boyce Thompson Institute, Tower Rd, Ithaca, NY 14853, USA.

* Correspondence: ivja@plen.ku.dk

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Abstract

Capturing the full growth potential in crops under future elevated CO_2 (eCO_2) concentrations would be facilitated by improved understanding of eCO_2 effects on uptake and use of mineral nutrients. This study investigates interactions of eCO_2, soil phosphorus (P), and arbuscular mycorrhizal (AM) symbiosis in *Medicago truncatula* and *Brachypodium distachyon* grown under the same conditions. The focus was on eCO_2 effects on vegetative growth, efficiency in acquisition and use of P, and expression of phosphate transporter (PT) genes. Growth responses to eCO_2 were positive at P sufficiency, but under low-P conditions they ranged from non-significant in *M. truncatula* to highly significant in *B. distachyon*. Growth of *M. truncatula* was increased by AM at low P conditions at both CO_2 levels and eCO_2×AM interactions were sparse. Elevated CO_2 had small effects on P acquisition, but enhanced conversion of tissue P into biomass. Expression of PT genes was influenced by eCO_2, but effects were inconsistent across genes and species. The ability of eCO_2 to partly mitigate P limitation-induced growth reductions in *B. distachyon* was associated with enhanced P use efficiency, and requirements for P fertilizers may not increase in such species in future CO_2-rich climates.

Key words: Arbuscular mycorrhizal symbiosis, *Brachypodium distachyon*, elevated atmospheric CO_2, gene expression, *Medicago truncatula*, phosphate transporters, plant growth, plant phosphorus uptake, soil phosphorus.

Introduction

Dramatic increases in atmospheric concentrations of carbon dioxide (CO_2) since pre-industrial times are predicted to produce CO_2 levels of ~500 to ~900 ppm by the end of this century, according to different climate scenarios (IPCC, 2013). Elevated CO_2 concentrations (eCO_2) are expected to increase growth of C_3 plants primarily because the current CO_2 concentration is suboptimal for the Rubisco enzyme that catalyzes carbon fixation; in particular, eCO_2 will competitively inhibit the oxygenation reaction and so reduce CO_2 loss and energy costs associated with photorespiration.

Other factors in the growth environment such as soil phosphorus (P) levels will influence the magnitude of the 'carbon fertilizer' effects on future crop productivity (Cavagnaro *et al.*, 2011; Pandey *et al.*, 2015*b*) and many soils are already characterized by decreasing P availability (Obersteiner *et al.*, 2013). Global abundance of such soils may further increase as rock P is non-renewable on a human time scale (Scholz and Wellmer, 2013), or because P fertilizer becomes prohibitively expensive for farmers, especially in developing countries. It is therefore important to understand if or how the expected eCO_2 effects on crop yields can be realized under P-limiting conditions (Pandey *et al.*, 2015*a*). Possible requirements for higher inputs of P fertilizers under eCO_2 conditions could even accelerate the depletion of rock P reserves.

In general, the efficiency of plant acquisition and utilization of soil P should be maximized to sustain food production in low-P soils (López-Arredondo *et al.*, 2014) and attempts to improve production need to consider interactions with eCO_2. Plant P acquisition is enhanced by extensive development of roots and is therefore determined by the C status of plants; on the other hand, the P status influences plant photosynthesis and growth rate, leading to multiple C–P interactions. The physiological background for the C–P trade balance as influenced by eCO_2 and low P conditions are appropriately investigated in experiments under controlled conditions to minimize possible masking of C–P trading by non-nutritional influences that are common under field conditions. Previous studies on eCO_2×P interactions have shown that eCO_2 can increase growth of C_3 grasses even in low-P soil (Newbery *et al.*, 1995; Imai and Adachi, 1996; Newbery and Wolfenden, 1996; Pandey *et al.*, 2015*a*), whereas the response in legumes (also C3) is usually limited under low-P conditions (Stocklin and Körner, 1999; Edwards *et al.*, 2005; Jin *et al.*, 2012; Lam *et al.*, 2012; Singh *et al.*, 2014). Such differences between functional plant groups are influenced by patterns of C partitioning and by efficiencies in P acquisition by their root systems (Jin *et al.*, 2015). Plant responses to eCO_2 are likely to be modulated by their mutualistic root symbionts, and there is a body of evidence showing that eCO_2 increases nitrogen fixation in legumes (Rogers *et al.*, 2009). It is also highly relevant to ask whether eCO_2 will amplify the development and function of the arbuscular mycorrhizal (AM) symbiosis that delivers soil P to most land plants in return for photosynthate, resulting in increased growth responses to eCO_2 via positive feedback. A possible C limitation of AM development both in the soil and within the roots might be mitigated at eCO_2 and lead to increased mycorrhiza formation. This may, in turn, result in an increased mycorrhizal P uptake and facilitate an eCO_2 growth response in (e.g.) legumes grown at low P.

Two meta-analyses have found that the abundance of AM colonization increases under eCO_2 (Treseder, 2004; Alberton *et al.*, 2005), but data are variable and can be positive or neutral (Gavito *et al.*, 2002, 2003; Hartwig *et al.*, 2002; Lukac *et al.*, 2003; Gamper *et al.*, 2004; Cavagnaro *et al.*, 2007). The eCO_2 effect on growth of AM plants will depend on the AM C–P trade balance. Growth responses to eCO_2 were reported to be similar for AM-colonized and non-colonized *Pisum sativum* and *Trifolium repens* (Jongen *et al.*, 1996; Gavito *et al.*, 2000, 2002, 2003; i.e. there were no CO_2×AM interactions), but AM-amplified growth responses to eCO_2 have also been reported (Rouhier and Read, 1998; Hartwig *et al.*, 2002). Even when eCO_2 has no net effect on growth of AM plants, there may still be a concealed physiological effect. That is, P uptake in AM plants is the combined contribution of the direct root pathway and of the AM pathway, and the latter can be highly functional even when overall growth and P uptake are similar in AM and non-mycorrhizal (NM) plants, indicating reductions in the activity of the direct pathway (Smith *et al.*, 2004; Grace *et al.*, 2009; Nagy *et al.*, 2009). The function of the AM pathway can only be quantitatively assessed by using radioactive phosphate (^{33}P or ^{32}P; Pearson and Jakobsen, 1993), but the potential function can be evaluated in studies of expression of phosphate (Pi) transporter (PT) genes and combined approaches are becoming increasingly common (see Smith *et al.*, 2011). The two P uptake pathways involve a number of PT proteins, some of which are specific to or induced by AM fungi (Javot *et al.*, 2007*b*; Smith *et al.*, 2011). Importantly for this investigation, the PT genes expressed in roots have been identified in both *Brachypodium distachyon* (Hong *et al.*, 2012) and *Medicago truncatula* (Liu *et al.*, 2008). In *M. truncatula*, roles in direct and AM pathways have been identified, but roles of individual genes are less clear in *B. distachyon*. Few studies have analyzed the effects of eCO_2 on PT gene expression, and these have been in the non-AM plant *Arabidopsis thaliana* (Niu *et al.*, 2013; Pandey *et al.*, 2015*b*).

As soil P availability is a major determinant for growth responses to AM symbiosis, it is striking that this factor has been considered in only a few studies concerning eCO_2×AM interactions, which have included only two experimentally imposed P levels (Syvertsen and Graham, 1999; Johnson *et al.*, 2005). In the first case, the eCO_2 response in Citrus was amplified by AM symbiosis under P limitation, while such an effect was not observed in the other study that included a range of plant species. There is an obvious need for more studies on eCO_2×AM interactions, including not only a wider range of soil P availability but also different plant species (Syvertsen and Graham, 1999; Johnson *et al.*, 2005; Cavagnaro *et al.*, 2011).

The aim of this work was to study how soil P level and AM symbiosis influence eCO_2 effects on growth of two plant species differing in responses to soil P limitation and to AM colonization: *Medicago truncatula* Gaertn. (barrel medic) and *Brachypodium distachyon* (L.) P. Beauv. *Medicago truncatula* has been well studied; it generally shows positive growth and P responses to AM colonization and the contribution of the AM P uptake pathway has been tracked with $^{32/33}P$ (see for example Smith *et al.*, 2004; Facelli *et al.*, 2014; Watts-Williams *et al.*, 2015). It has rather poor P uptake efficiency when non-mycorrhizal (NM). *Brachypodium distachyon* has (to our knowledge) been subject to only one investigation involving AM symbioses (Hong *et al.*, 2012). Responses in low-P soil based on fresh shoot weight or P content varied with the symbiotic AM fungus and were in some cases neutral or negative. It was expected that this species would show quite high P uptake efficiency, regardless of mycorrhizal status.

Thus, the following hypotheses were tested: (1) growth responses to eCO_2 under low-P conditions depend on the efficiency of P acquisition and use in the plant species when AM or NM; (2) growth responses to eCO_2 will increase with increasing soil P levels; and (3) AM functioning is little affected by eCO_2.

Materials and methods

A pot experiment was carried out at both ambient (aCO_2 = 400 ppm) and elevated (eCO_2 = 900 ppm) atmospheric CO_2 concentrations, with *M. truncatula* cv. Jemalong A17 and *B. distachyon* line 21–3 growing in soil supplied with 0, 10, 20, 40, or 80 mg KH_2PO_4-P kg^{-1}. Plants were inoculated with an AM fungus or not.

Experimental set-up

Seeds of *M. truncatula* were scarified in concentrated H_2SO_4 for 8 min, rinsed in sterile water, surface-sterilized with 2% $NaHClO_3$ for 5 min, rinsed in sterile water and pre-germinated on water-agar (0.8%) plates in the dark at 4 °C (5 d) and at 22 °C (2 d). Seeds of *B. distachyon* were surface-sterilized and pre-germinated in the same way.

The experimental soil was a semi-sterile (15 kGy, 10 MeV electron beam) 1:1 (w:w) mixture of a sandy loam (10% clay, 12% silt, 46% fine sand, and 30% coarse sand) and quartz sand, which was supplemented and thoroughly mixed with basal nutrients (Merrild *et al.*, 2013). The five P treatments are referred to as 0P, 10P, 20P, 40P, and 80P and resulted in the following levels of 0.5 M $NaHCO_3$-extractable P (Olsen *et al.*, 1954): 4.3, 7.8, 11.2, 19.8, and 39.6 mg P kg^{-1} soil.

The pots held 1430 g soil, of which 50 g was mixed with 262 kBq of carrier-free $H_3{}^{33}PO_4$. This labelled soil was contained in a 40-mm diameter plastic cylinder capped with 25-µm nylon mesh at both ends, and this compartment for ingrowth of AM fungal hyphae (hyphal compartment: HC) was placed at 10 cm depth in all pots (see Smith *et al.*, 2003, which shows a diagram of the compartmented pot). Mycorrhizal pots had a mixture of 1000 g soil and 100 g inoculum of *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C.Walker & A.Schüßler 2010 (previously named *Glomus intraradices*) culture BEG87 sandwiched between the bottom (200 g) and top (80 g) layers of non-inoculated soil. The AM fungal inoculum was a mixture of dry soil, spores, and root fragments of *Trifolium subterraneum* L. pot cultures. All NM and AM pots received 15 ml inoculum leachate that was prepared by wet-sieving 1 l aqueous suspension of 100 g inoculum through two layers of 25-µm nylon mesh. Each of the 15 treatments for each species had three replicates.

The soil in the pots was watered to 60% of the water-holding capacity and pots were placed in two separate walk-in growth rooms set at 400 and 900 ppm CO_2. Two or three pre-germinated seeds were sown in each pot and emerged seedlings were thinned to one per pot. Plants were maintained at a 16/8 h light/dark cycle at 20/15 °C, respectively. Fluorescent daylight lamps (Osram GmbH, Munich, Germany) provided 500 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR; 400–700 nm). As plants grew bigger, pots were watered to 70% of water-holding capacity and fertilized twice with NH_4NO_3, resulting in a total supply of 112 mg N per pot. To avoid chamber-specific bias in the experiment, pots and their corresponding CO_2 treatment were relocated between the two climate chambers every week.

Harvesting and physiological analysis

Plants were harvested 35 d after sowing (about half the life-cycles of the two species); shoots were dried at 70 °C for 48 h and dry weights recorded. Harvest time was chosen to take into account the half-life of ^{33}P (25.4 d) and the influence of P supply on the specific activity, and hence detectability, of ^{33}P transferred to the plants. Roots were washed, blotted, and weighed, and a weighed subsample of 500–700 mg was stored in 50% ethanol for determination of AM colonization. Another ~500 mg subsample of root material was flash-frozen in liquid N_2, crushed, and kept at −80 °C for RNA isolation. The remaining root tissue was dried at 70 °C for 48 h and dry weights were determined. Growth responses to eCO_2 (% eCO_2 response) and to AM inoculation (% AM response) were calculated from shoot dry weights as follows: % eCO_2 = 100×(eCO_2 – mean aCO_2)/mean aCO_2, and % AM = 100×(AM – mean NM)/mean NM. Dried shoot and root samples were digested in a 4:1 mixture (v:v) of 65% nitric and 70% perchloric acids, and total P was determined by the molybdate blue method using AutoAnalyzer 3 (Bran + Luebbe, Norderstedt, Germany). The ^{33}P in shoot and root tissue was determined in the same digests in a Packard TR 1900 liquid scintillation counter (PerkinElmer, Waltham, MA).

Root samples were cleared in 10% KOH and stained with trypan blue (Kormanik and McGraw, 1982), and were then assessed for AM-colonized root length (Newman, 1966). Quantification of hyphae in the root-free HC soil was investigated for *M. truncatula* by measuring the length of hyphae collected on membrane filters (Jakobsen *et al.*, 1992). After correction for isotopic decay, uptake of ^{33}P from the small HCs (in which the soil specific activity was measured and varied between P treatments) was extrapolated to uptake from the whole pot as described previously (Smith *et al.*, 2003) to give % of total plant P uptake by AM fungal hyphae: 100 × ($SA^{33}P$ plant/$SA^{33}P$ HC) × (P in pot/P in HC), where SA is specific activity and P is bicarbonate-extractable P. The calculations did not take into account the possibility of different hyphal length densities (HLDs) in the main pot and HC (Smith *et al.*, 2004) as HLDs were only measured in the HCs.

RNA isolation and real-time qPCR analysis

Total RNA was extracted from ~70 mg of root samples of both species, using miRNeasy Mini Kit (Qiagen Hilden, Germany) with on-column DNase treatment following the manufacturer's protocol. RNA concentration was measured on a Nanodrop ND-1000 Spectrophotometer (Saveen 1 Werner, Malmö, Sweden). cDNA was synthesized from 200 ng of total RNA using dNTP Mix (Qiagen) and Expand Reverse Transcriptase (Roche) including Protector RNase inhibitor (Roche).

The real-time primers (Eurofins MWG operon, Germany) were: *MtEF1α*, *MtPT1*, *MtPT3*, and *MtPT5* (Liu *et al.*, 2008); *MtPT4* (Javot *et al.*, 2007*a*), and *BdUBC18* (Hong *et al.*, 2008). Primers for *BdPT4*, *BdPT8*, and *BdPT7* are given in Supplementary Table SI at *JXB* online. Gene expression analysis was carried out on three replicate plants from each treatment, with technical duplicates. Real-time PCR analysis was performed using the Rotor Gene 2000 Real Time Cycler (Qiagen). Each 20 µl of PCR reaction contained 8 µl of a 1/8 dilution of RT reaction (see above), and 12 µl of SYBR Green Master Mix (Fermentas, Thermo Scientific), which included 500 nM of each primer. Samples were heated to 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. After each PCR reaction, the specificity of the amplification was verified by running a melt-curve analysis. The Rotor Gene 2000 software calculated relative amounts of RNA based on PCR cycle threshold values obtained from a dilution series from 1/4 to 1/160 (each step was a 1:3 dilution in H_2O) of a standard RT sample from an AM or NM plant (depending on the primer of interest). Data were normalized to *MtEF1α* mRNA levels.

Statistics

All data were assessed for normality using the Shapiro–Wilk test and by viewing QQ-plots. Any data that appeared non-normal were square-root or log transformed so that they conformed to the assumption of normality before further statistical analysis. All response variable data (except for gene expression, % eCO_2 response

and % AM response) were analyzed by multi-factor analyses of variance (ANOVA). Factors in the three-way analyses were: CO_2 level, soil P level, and arbuscular mycorrhiza. Root colonization was analyzed by two-way ANOVA (CO_2 level and soil P level) after removing the nil-values for NM plants. Gene expression data were split between the AM and non-mycorrhizal (NM) treatments and also analyzed by two-way ANOVA (CO_2 level and soil P level). For % eCO_2 response, the factors in the two-way ANOVA were arbuscular mycorrhiza and soil P level, while for % AM response the factors were CO_2 level and soil P level. Where significant ($P<0.05$) interactions or main effects were found, comparisons were made using Tukey's honestly significant difference (HSD). Linear or polynomial regression analyses were performed in Microsoft Excel (version 14.5.1) to determine the relationship between shoot dry weights and shoot P contents (the P use efficiency, PUE) at each CO_2 level, respectively. All other statistical analyses were performed with JMP Pro 12.0.1 (SAS Institute Inc.).

Results

Table 1 shows probabilities of significance for the main treatment effects and treatment interactions derived from ANOVA.

Effects of P fertilization and elevated CO_2 on plant growth and root colonization by AM fungi

Growth of both plant species increased significantly with increasing P fertilization, but the effect was much stronger for *M. truncatula* than for *B. distachyon*, with shoot dry weight in the legume failing to reach a plateau (Fig. 1). Furthermore, interactions between soil P supply and inoculation by AM fungi differed between species (Table 1), as discussed below.

Table 1. *Probabilities of significance for main treatment effects and treatment interactions of the variables measured in M. truncatula and B. distachyon as derived from three-way ANOVA. Gene expression data were analyzed separately for AM and NM plants by two-way ANOVA. Mycorrhizal and CO_2 growth responses (% AM response, % eCO_2 response) were also analyzed by two-way ANOVA.*

Variable	AM	CO_2	P level	AM×CO_2	AM×P	CO_2×P	AM×CO_2×P
M. truncatula							
Shoot DW	<0.0001	<0.0001	<.0001	ns*	<0.0001	0.001	ns
Shoot P conc	<0.0001	<0.0001	<0.0001	0.042	<0.0001	ns	ns
Shoot P cont	<0.0001	0.0025	<0.0001	0.0044	<0.0001	ns	ns
Root colonization		ns	0.0002			ns	
Root DW	<0.0001	<0.0001	<0.0001	ns	<0.0001	ns	ns
Root length	ns	0.041	<0.0001	ns	0.007	ns	ns
RL-spec P uptake	<0.0001	ns	<0.0001	ns	0.001	ns	ns
AM P uptake	<0.0001	ns	0.0123	ns	<0.0001	ns	ns
% AM response		0.001	<0.0001			0.0273	
% eCO_2 response	0.0129		ns		ns		
Expression of PT genes**							
MtPT1 NM		ns (0.056)	0.002			ns	
MtPT3 NM		0.038	ns			ns (0.096)	
MtPT5 NM		<0.0001	ns			ns	
MtPT4 AM***		0.001	0.004			ns	
MtPT1 AM		0.001	0.001			ns	
MtPT3 AM		<0.0001	0.003			ns	
MtPT5 AM		0.004	ns			ns	
B. distachyon							
Shoot DW	ns	<0.0001	<0.0001	ns	ns	0.007	ns (0.087)
Shoot P conc	ns	ns	<0.0001	ns	ns (0.057)	ns	ns
Shoot P cont	ns	<0.0001	<0.0001	ns	0.0193	ns	0.0287
Root colonization		0.0077	<0.0001	0.004	<0.0001	ns	ns
RL-spec P uptake	ns	ns	<0.0001	ns	ns	ns	ns
Root DW	0.0322	<0.0001	<0.0001	ns	ns	0.0009	ns
Root length	ns	<0.0001	0.004	ns	ns	ns	ns
AM P uptake	<0.0001	ns	0.0093	ns	<0.0001	ns	ns
% AM response		ns	0.042			ns (0.052)	
% eCO_2 response	ns		0.002		0.011		
Expression of PT genes**							
BdPT4 NM		ns	0.0003			ns	
BdPT8 NM		0.009	<0.0001			ns	
BdPT7 AM***		ns (0.066)	0.0003			ns	
BdPT4 AM		0.021	ns			ns	
BdPT8 AM		ns	0.0002			ns	

* ns, not significant
** no AM component in ANOVA since NM and AM plants were analysed separately
*** *MtPT4* and *BdPT7* are not expressed in NM plants

Growth responses to eCO_2 differed markedly between the legume and the grass. In *M. truncatula*, growth was severely P-limited in the 0P–20P range and was only increased by eCO_2 (% eCO_2 response) at the two highest P levels, as reflected by the significant CO_2×P interaction (Fig. 1, Tables 1 and 2). The eCO_2 response was similar in AM and NM plants although the P-limited growth was partly mitigated by AM colonization (Tables 1 and 2, Fig. 1). In contrast, shoot DW of *B. distachyon* was significantly higher at eCO_2 than at aCO_2 at all P levels except the highest (80P). Further, at eCO_2, dry weight accumulation reached a plateau at 40P in *B. distachyon*, while no such plateau was observed in the plants grown at aCO_2.

Mycorrhizal growth response (% AM response, Table 2) was strongest for *M. truncatula*, due to the suppression of growth at 0P when non-mycorrhizal. Growth of *M. truncatula* responded significantly to AM development in the 0P to 20P range and responses were lowest at eCO_2 (Fig. 1, Tables 1 and 2). With both CO_2 treatments, % AM response declined with increasing soil P level in accordance with the significant AM×P interaction (Table 1). Colonization by AM fungi had no significant effects on growth of *B. distachyon* and responses to the addition of P were matched between the AM and NM plants at eCO_2 (Tables 1 and 2, Fig. 1). At aCO_2 the data trended towards a positive, but then negative % AM response at 0P and 80P, respectively (Tables 1 and 2). The effect of CO_2, AM colonization, and soil P treatments on root dry weights closely reflected the shoot growth (Supplementary Fig. S1).

Elevated CO_2 also resulted in increased root length in both *M. truncatula* and *B. distachyon* (Fig. 2). Colonization by AM fungi did not affect root length in *B. distachyon*, but in *M. truncatula* root length was increased and decreased by AM symbiosis at low-P and high-P conditions, respectively. This modification by AM colonization was associated with a lower specific root length (m g^{-1} DW) in AM plants, in particular in the 0P to the 20P range (data not shown). Roots of all inoculated plants were colonized by AM fungi and non-inoculated plants remained non-colonized. Percentage of root length colonized at 0P was higher than 55% in both species and decreased significantly with increasing P application. This decrease was largest in *B. distachyon* (from ~60% at 0P to 20% at 80P) (Fig. 2). The percentage of root length colonized was not strongly affected by eCO_2 except for moderate but significant increases in *B. distachyon* (Fig. 2). However, because eCO_2 increased total root length, the absolute length

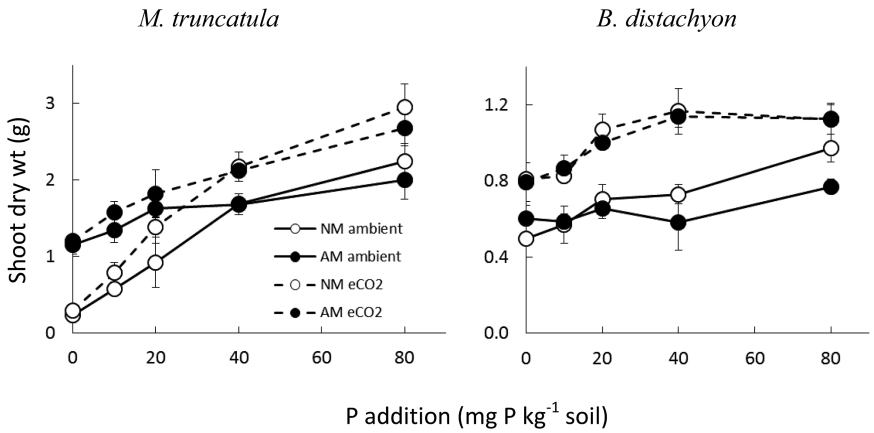


Fig. 1. Shoot dry weights of *M. truncatula* and *B. distachyon* grown at aCO_2 (solid lines) and eCO_2 (dashed lines), in the presence or absence of AM colonization (AM or NM: filled or open symbols) and at different soil P levels. Data points are means ±SEM with *n*=3.

Table 2. *Relative shoot growth responses to elevated CO_2 [= 100×(eCO_2 – mean aCO_2)/mean aCO_2] and to AM inoculation [= 100×(AM – mean NM)/mean NM] in M. truncatula and B. distachyon grown at different soil P supplies.*

P supply (mg kg^{-1})	% CO_2 response				% AM response			
	NM		AM		aCO_2		eCO_2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
			M. truncatula					
0	25.0	13.4	4.3	2.5	381.9	32.0	302.2	9.7
10	36.2	12.1	17.4	4.8	131.0	15.6	99.2	8.1
20	49.6	14.7	12.1	8.5	75.5	6.8	31.5	9.9
40	28.9	4.9	27.0	1.5	–0.4	2.8	–1.8	1.2
80	31.1	8.5	33.4	7.0	–11.0	5.4	–9.4	4.8
			B. distachyon					
0	63.3	5.1	31.8	13.8	21.1	11.8	–2.2	10.3
10	45.1	3.3	47.5	9.7	3.1	5.5	4.8	6.9
20	52.0	9.1	52.2	4.7	–6.7	6.3	–6.6	2.9
40	59.7	13.5	95.3	8.3	–20.1	11.8	–2.3	4.2
80	15.6	4.6	46.5	8.5	–20.8	3.2	0.3	5.8

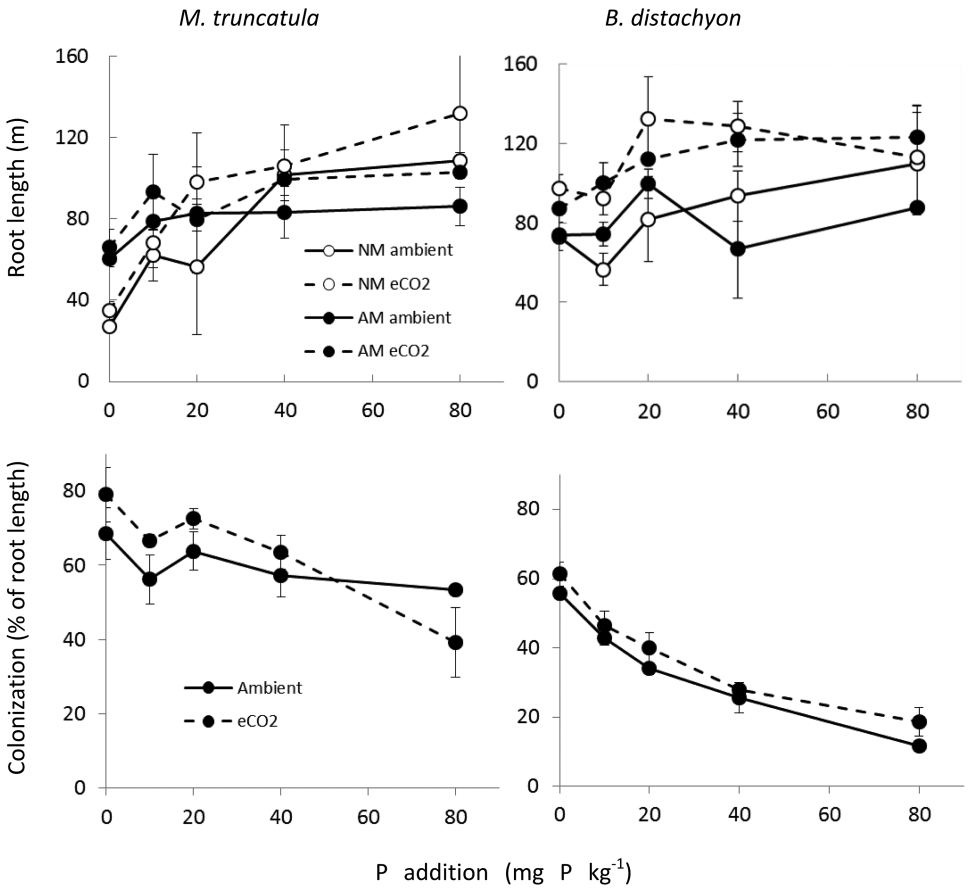


Fig. 2. Total root length of *M. truncatula* and *B. distachyon* grown at aCO_2 (solid lines) and eCO_2 (dashed lines), in the presence or absence of AM colonization (AM or NM: filled or open symbols) and at different soil P levels. Percentage of root length colonized is shown in the lower panel for AM plants of the two species grown at aCO_2 and eCO_2 and different soil P levels. Data points are means ±SEM with n=3.

of colonized roots was increased by approx. 10% in *M. truncatula* and by as much as 50% in *B. distachyon* grown at P40 (data not shown).

Effects of elevated CO_2 on shoot P concentrations and P use efficiency

Shoot tissue P content in both species increased significantly over the range of P application, as did shoot P concentrations (Table 1, Supplementary Fig. S2, Fig. 3a). Exposure to eCO_2 resulted in decreased shoot P concentrations in *M. truncatula* at higher soil P levels. In *B. distachyon* eCO_2 did not alter tissue P concentrations significantly. Root colonization by AM fungi increased P concentrations in *M. truncatula* at 0P and 10P (Fig. 3a). This reflected a general shift from a positive AM effect at the low P levels towards a neutral or negative effect at the highest P levels. Shoot P concentrations in *M. truncatula* were similar in AM-colonized plants grown at 0P and in NM plants grown at 20P (Fig. 3a).

The P use efficiency (PUE), being the reciprocal of shoot P concentration, is derived from the *y*/*x*-axis ratios in Fig. 3b, which shows the relationship between shoot dry weight and shoot P content. This relationship, which facilitates the analysis of AM or CO_2 treatment effects on PUE in plants having similar P contents, was curvilinear in *M. truncatula*, where PUE was increased by eCO_2 when P uptake was beyond a certain threshold (about 3 mg P). In *B. distachyon*, the relationship was linear and PUE was increased by eCO_2 over the full range of shoot P contents studied (Fig. 3b). In contrast, PUE seemed to be unaffected by AM colonization when comparing plants of similar shoot P content in each species.

No effects of elevated CO_2 on root length-specific P uptake and AM contribution to P uptake

Uptake of P per unit root length (root length-specific: RL-spec) was not significantly affected by the CO_2 level, but increased in response to P addition in both species and in response to AM root colonization in *M. truncatula* (up to 20P only), but not in *B. distachyon* (Table 1, Fig. 4). The effect of P and AM colonization interacted significantly in *M. truncatula*, such that RL-spec P uptake increased more steeply with increasing P addition in NM than in AM plants.

The contribution of the AM pathway to uptake was estimated from ^{33}P uptake via hyphae accessing the small hyphal compartments (HCs) containing ^{33}P-labelled soil. Irrespective of the different AM growth response observed in the two species, the calculation showed that they both received 65–75% of their shoot P uptake via the AM pathway at 20P, and that this was not affected by CO_2 level (Fig. 4). The activity of the AM pathway decreased markedly between 20P and 40P and appeared to be non-operational at 80P in both species. In AM

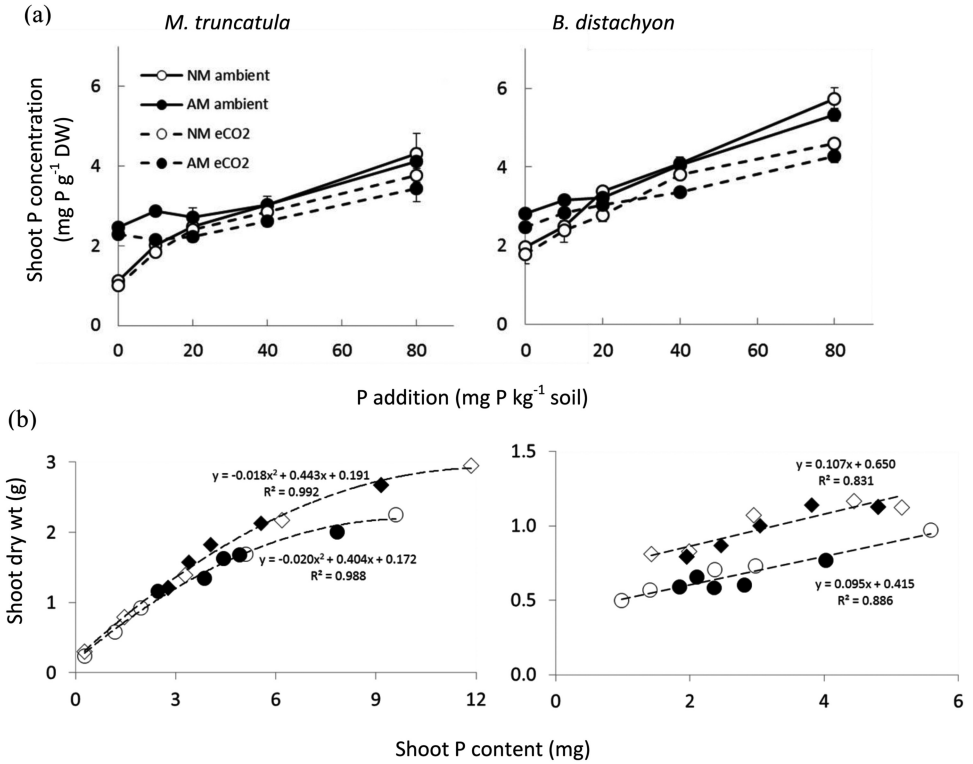


Fig. 3. Phosphorus concentrations (a) and dry weight vs. P content relationships (b) for shoots of *M. truncatula* and *B. distachyon* grown at aCO_2 (solid lines) and eCO_2 (dashed lines) (a), in the presence or absence of AM colonization (AM or NM: filled or open symbols) and at different soil P levels. In (b), aCO_2 and eCO_2 treatments are denoted by circles and diamonds, respectively, and P use efficiency is derived from the *y*/*x*-axis ratios. Data points are means (*n*=3) and bars in (a) are ±SEM.

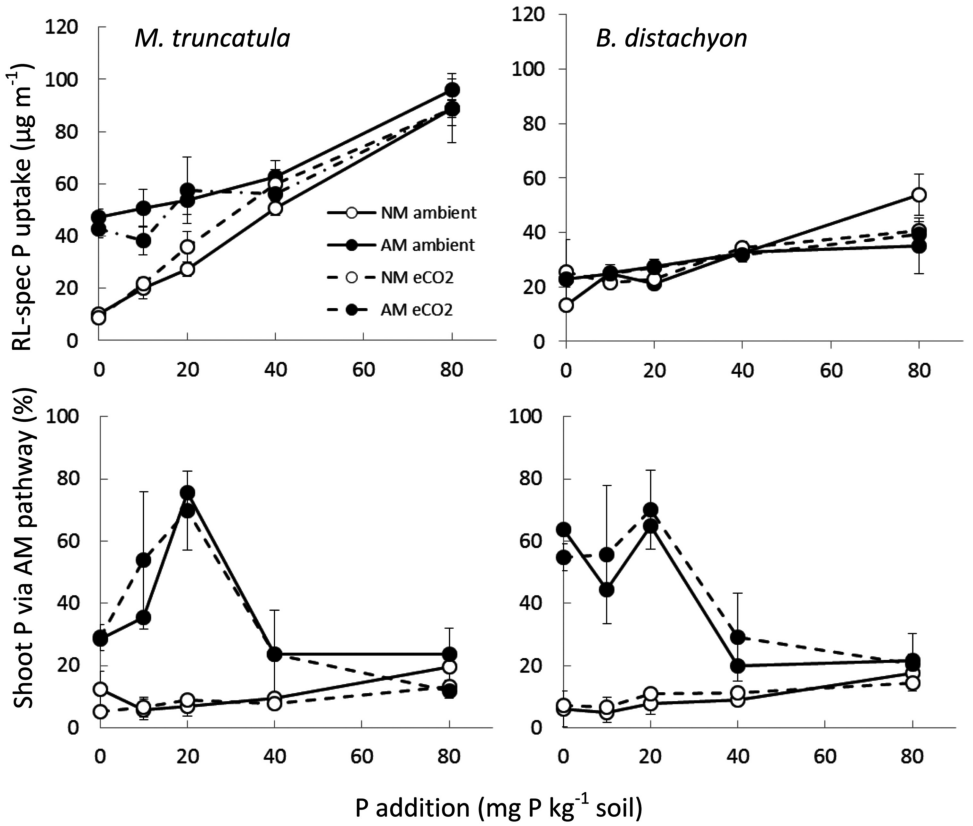
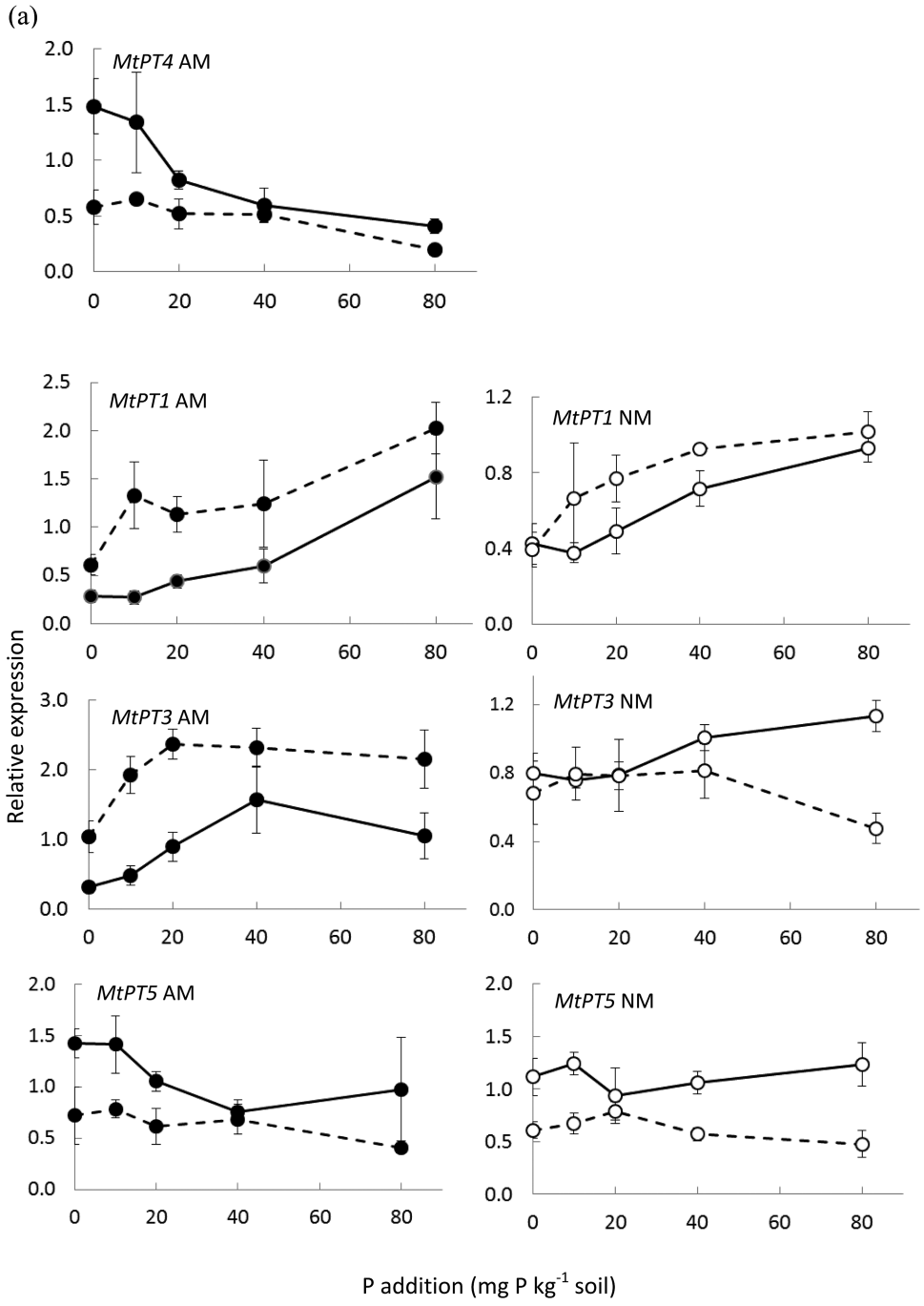


Fig. 4. Root length (RL)-specific P uptake in *M. truncatula* and *B. distachyon* grown at aCO_2 (solid lines) and eCO_2 (dashed lines), in the presence or absence of AM colonization (AM or NM: filled or open symbols) and at different soil P levels. Shoot P uptake via the AM pathway is shown in the lower panel. Data points are means ±SEM with *n*=3.

M. truncatula, the calculated uptake of ^{33}P was clearly lower at 0P and 10P than at 20P. However, there was a linear correlation between hyphal length densities in the HC soil and ^{33}P uptake from the same soil (Supplementary Fig. S3). The uptake of ^{33}P by NM plants was very low with low soil P and increased with increasing soil P level (Fig. 4). This uptake was probably caused by the combined effect of root hairs penetrating the mesh and diffusion of ^{33}P in the opposite direction.

Effects of elevated CO_2 on expression levels of phosphate transporter (PT) genes

Expression analyses of PT genes were performed by RT-qPCR to evaluate the measured contribution of the two P uptake pathways (direct and mycorrhizal) through roots, using ^{33}P transfer against expression patterns of genes involved in these pathways. The effects of eCO_2 on gene expression were analyzed in roots of AM plants and of NM plants separately. Elevated CO_2 and increasing soil P concentration both decreased the expression of the AM-induced PT gene *MtPT4* in *M. truncatula* (Fig. 5, Table 1). For the direct-pathway PT genes (*MtPT1*, *MtPT3*, and *MtPT5*), the effect of eCO_2 varied: the expression of *MtPT1* increased and *MtPT5* decreased at eCO_2 in both AM and NM plants while *MtPT3* expression increased and decreased in AM and NM plants, respectively. The addition of P to the soil decreased the expression of *MtPT1* in both AM and NM plants and of *MtPT3* in AM plants only. Expression of *MtPT5* was not affected by soil P level.



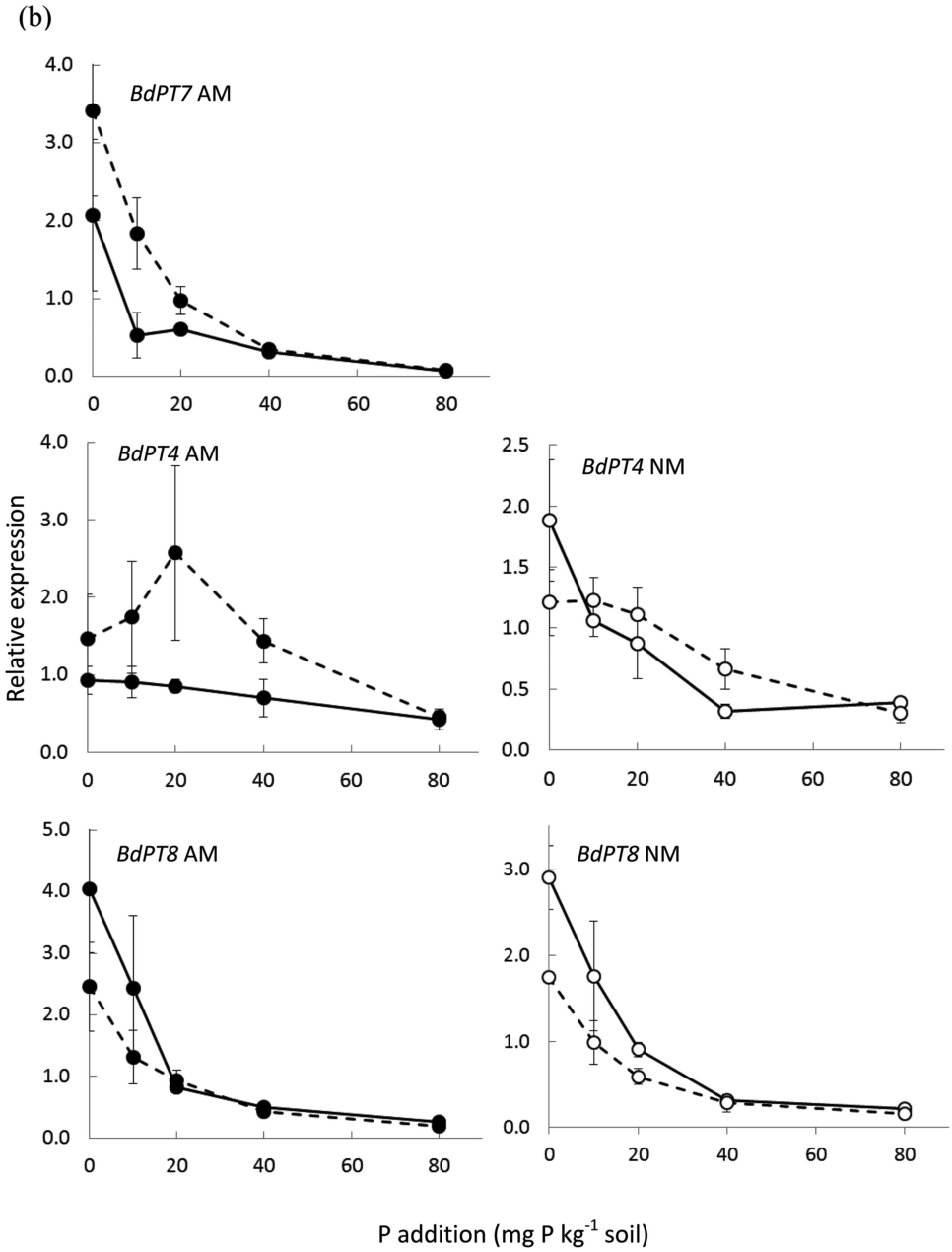


Fig. 5. Effects of eCO_2 and soil P additions on expression of phosphate transporter (PT) genes *MtPT1*, *MtPT3*, *MtPT4*, and *MtPT5* in roots of *M. truncatula* (a) and *BdPT4*, *BdPT7*, and *BdPT8* in roots of *B. distachyon* (b). *MtPT4* and *BdPT7* are induced by AM colonization. Expression levels at aCO_2 (solid lines) and eCO_2 (dashed lines) and at different P additions can be compared only within each of the 12 panels. AM and NM plants are represented by filled and open symbols, respectively. Data points are means ±SEM with n=3.

In *B. distachyon*, eCO_2 had no significant effect on the expression of the AM-induced PT gene *BdPT7*, but its expression decreased with increasing soil P level, as in *M. truncatula* (Fig. 5, Table 1). Direct-pathway PT genes have not yet been characterized in *B. distachyon*. However, phylogenetic studies (Signe S. Clausen, unpublished data; Hong *et al.*, 2012) and expression studies at high and low soil P (Signe S. Clausen, unpublished data) suggest that *BdPT4* and *BdPT8* are active in the direct P uptake pathway. The expression of both genes was slightly increased by eCO_2 (significant for *BdPT4* in AM plants only and for *BdPT8* in NM plants only). Furthermore, their expression declined with increasing P addition (Fig. 5, Table 1). Trends of declining expression in response to AM colonization were observed for the same two genes in plants grown at the three lowest P levels, but the reductions were not statistically significant (data not shown).

Discussion

The present work is considerably more comprehensive than previous studies and hence is novel as it exposes plant species of two functional types (a pasture legume and a grass) to factorial combinations of eCO_2, AM fungal inoculation, and a range of P fertilizer additions under controlled conditions. Our comprehensive physiological measurements (including AM P uptake using ^{33}P) and gene expression data have

not previously been combined to address our hypotheses. If short-term studies such as this one could be extrapolated to longer-term growth, such a multifactor approach would facilitate the ability to predict effects of future climates on crop nutrition and growth (Cavagnaro *et al.*, 2011). In accordance with our first hypothesis, responses to eCO_2 under soil P limitation were high in the grass, *B. distachyon*, with a root system that allowed efficient P uptake, but low in the legume, *M. truncatula*, with a less efficient root system. The expected positive relationship between eCO_2 stimulation of plant growth and increasing P supply (second hypothesis) was confirmed for both species. Furthermore, AM symbiotic functioning was not influenced by eCO_2 (confirming our third hypothesis), at least in terms of % root length colonized and symbiosis-mediated plant P uptake. In general terms, at the time of experimental harvest, under eCO_2 less P fertilizer was required to produce the same amount of shoot dry matter as obtained in plants grown at aCO_2 at non-limiting P fertilizer supplies.

CO_2 fertilizer effects on plant growth: interaction with soil P level and mycorrhiza

Our finding that the CO_2 effect was enhanced at increasing soil P availability in *B. distachyon* and *M. truncatula* supports previous observations that plant growth responses to eCO_2 are lower under nutrient-limited conditions (Poorter and Perez-Soba, 2001; Nord *et al.*, 2015; Pandey *et al.*, 2015*b*), particularly in relation to N supply, which has been more extensively researched than P. The larger growth response to eCO_2 in *B. distachyon* than in *M. truncatula* accords with free-air CO_2 enrichment (FACE) studies showing a greater stimulation of photosynthetic C uptake in grasses than in legumes (summarized in Leakey *et al.*, 2009). This would be expected from the long-established, but sometimes overlooked, 'law of the minimum' (Liebig, 1843) and more general principles of plant growth-limiting factors (Blackman, 1905). Put simply, where growth is strongly limited by soil P supply, it would not be increased by eCO_2 unless there is a physiological interaction whereby higher C supply directly increases P uptake or PUE, or both. There was no such interaction in *M. truncatula* at the lowest P levels (Fig. 1, Supplementary Fig. S1), while at higher, but still growth-limiting soil P levels, eCO_2 supply increased growth. Possible reasons for such C/P co-limitation are discussed by Pandey *et al.* (2015*b*).

Whereas many legumes are potentially strongly P limited under eCO_2 (e.g. Edwards *et al.*, 2005; Rogers *et al.*, 2009), P fertilization does not always increase the eCO_2 response in grasses (Grunzweig and Körner, 2003) and this variation appears to relate to differences in root production between the two plant groups (*B. distachyon* had higher root length at low P). The limited growth response to eCO_2 in the highly P-responsive *M. truncatula* exposed to low soil P accords with previous findings in chickpea, field pea, barrel medic, and soybean (Sa and Israel, 1998; Jin *et al.*, 2012; Lam *et al.*, 2012; Singh *et al.*, 2014). In contrast, the ~50% growth response to eCO_2 over the full soil P range in *B. distachyon* is very much in line with a predicted 46% increase in C gain in C3 plants at the atmospheric concentrations of CO_2 expected for the middle of this century (Leakey *et al.*, 2009). In *B. distachyon*, the lower eCO_2 response at 80P reflected the observation that plants became C saturated at a lower P level under eCO_2 than under aCO_2 conditions.

Medicago truncatula displayed the expected AM growth response under P-limiting growth conditions (see for example Smith *et al.*, 2004; Li *et al.*, 2005; Konvalinkova *et al.*, 2015) while *B. distachyon* exhibited neutral or negative AM growth responses. Such response patterns are typical for the vegetative growth phase of grasses such as barley (Grace *et al.*, 2009) and wheat (Li *et al.*, 2005; Stonor *et al.*, 2014), suggesting that *B. distachyon* provides a suitable model for (temperate) grasses with regards to AM research (Brkljacic *et al.*, 2011; Hong *et al.*, 2012). The borderline significant AM×CO_2×P interaction for shoot DW in *B distachyon* (Table 1) reflected a P level-dependent AM response at aCO_2 but not at eCO_2. This AM response was negative at aCO_2 above 20P and its absence at eCO_2 suggests that any C drain by the AM fungi that might decrease growth at high-P conditions was fully compensated by increased C assimilation at eCO_2.

The slight enhancement of % AM colonization caused by eCO_2 in *B. distachyon* and the lack of effect in *M. truncatula* agree with many previous reports (e.g. Rillig *et al.*, 1999; Gavito *et al.*, 2002, 2003; Cavagnaro *et al.*, 2007), although a meta-study has reported an average increase of 21% (Alberton *et al.*, 2005). However, the eCO_2-elicited increase in root length means that the absolute colonized root length was markedly increased (up to 50% in *B. distachyon*) and thus the absolute growth of the AM fungi must have responded to eCO_2 at the same rate as root growth, as also suggested by previous studies (Staddon *et al.*, 1998; Alberton *et al.*, 2005).

Plants at eCO_2 have unchanged P uptake efficiency but P use efficiency is increased

The observed lack of CO_2 fertilizer effects on the P uptake capacity per unit root length in both plant species confirms previous reports (Newbery *et al.*, 1995; Jin *et al.*, 2012) and the observed increased P uptake at eCO_2 may be explained by the increased root growth (Fig. 2), which contributes to shorten the diffusion pathway in the soil for Pi. External hyphae of AM fungi also contribute to shorten the diffusion pathway for Pi and the calculated AM-mediated Pi uptake generally correlates with the length density of AM hyphae in the soil (Supplementary Fig. S3; Jakobsen *et al.*, 1992; Munkvold *et al.*, 2004). The lack of an eCO_2 effect on the contribution of the AM pathway to total P uptake in *M. truncatula* and *B. distachyon* adds to previous studies with pea (Gavito *et al.*, 2002, 2003), but is novel by finding this lack of interaction to be independent of P level. This suggests that AM development and function was not C-limited at aCO_2. In this context, it remains unclear why the growth of AM hyphae into the HC and hence their uptake of ^{33}P were greatly reduced at the two lower soil P levels in *M. truncatula* plants, but it has been recognized that AM development can be impaired under extreme P limitation (Bolan *et al.*, 1984). The calculated % contribution of the AM P uptake pathway at 0 and 10P was

much lower than that expected from the higher biomass and P contents of AM versus NM plants (Figs 1 and 3b). A possible explanation is that hyphal length density in the main pots (not measured) was higher than in the HCs, perhaps due to slow growth into HCs at low P, that would lead to underestimation of the size of AM P uptake as derived from the equation used. However, the % contributions of AM and direct uptake calculated for *B. distachyon* did not show low % contribution of AM P uptake at low soil P (Fig. 4).

Our observation that eCO_2 reduced shoot P concentrations in *M. truncatula* (Fig. 3a) agrees with reports that tissue P and N concentrations are often lower at elevated than at aCO_2 due to greater plant biomass and carbohydrate accumulation (Treseder and Allen, 2000; Jifon *et al.*, 2002). In general, it has been observed that shoot P concentrations are lower or unchanged under eCO_2, regardless of AM status (Newbery *et al.*, 1995; Syvertsen and Graham, 1999; Gavito *et al.*, 2000, 2003; Jifon *et al.*, 2002; Jin *et al.*, 2012). Phosphorus concentration (hence also phosphorus utilization efficiency) was not affected by AM in *B. distachyon*. This is as expected because this species is P efficient when NM. In both species, the absence of an AM effect on PUE was also revealed by the similar shoot DWs of AM and NM plants at each CO_2 treatment when comparing plants of identical shoot P content and hence P physiology (Fig. 3b). In contrast, shoot DW was higher at eCO_2 than at aCO_2 at identical shoot P contents in *M. truncatula* above 3–4 mg P per plant and in *B. distachyon* over the full range of shoot P contents (Fig. 3b). This suggests that the P fertilizer requirement to produce maximum growth in a P-efficient grass (at least in the short-term as studied here) is smaller at eCO_2 than at aCO_2. In the longer term the additional fertilizer needed for full plant development will depend on several factors, including the fact that much of the total plant P is absorbed during early plant growth and the pattern of redistribution of P within the plant, for example to supply developing seed. Effects of eCO_2 on such factors will need to be determined in future research. In any case, the potentially smaller fertilizer requirements in the grass might dampen the expected need for increased exploitation of the non-renewable P rock reserves under eCO_2 (Jin *et al.*, 2015).

Exposure to eCO_2 modulates the expression of phosphate transporter (PT) genes

In contrast to the absence of eCO_2 effects on root length-specific P uptake (see above), eCO_2 influenced the expression of PT genes in roots of *M. truncatula* plants, both in the presence and absence of AM fungi. However, this expression was rather inconsistently induced or suppressed across PT genes and AM treatments. This potentially reflects the high complexity of regulation of PT gene expression, involving P supply, P starvation responses, and AM colonization, and with many shared components interconnected with sugar and phytohormone signalling (see Smith *et al.*, 2011, and references therein). The effects of eCO_2 have yet to be incorporated into this picture. In *B. distachyon*, the magnitude of eCO_2 effects on PT gene expression was much lower than for *M. truncatula*. These results contribute to a field where knowledge is limited: eCO_2 enhanced the expression of transcription factors and PT genes in P-deficient *Arabidopsis thaliana* plants (Niu *et al.*, 2013), but subsequent *in silico* analysis of typical P-responsive genes of *A. thaliana* revealed no significant influence of short-term exposure to eCO_2 on PT gene expression (Pandey *et al.*, 2015*b*). The present work on CO_2×P level interactions in AM species adds to some studies of interactive effects of eCO_2 and abiotic stress, e.g. drought (Allen *et al.*, 2011; Sicher and Barnaby, 2012; Zinta *et al.*, 2014). The lack of correlation between PT gene expression and root length-specific P uptake is in accordance with previous studies (Grace *et al.*, 2009; Grønlund *et al.*, 2013; Facelli *et al.*, 2014; Watts-Williams *et al.*, 2015). This lack of correlation might be caused by the multiple levels of post-translational regulation of PT genes, as reported in *A. thaliana* (Bayle *et al.*, 2011; Chen *et al.*, 2011, 2015), or by the fact that the amount of transporter protein is not the factor that limits P uptake by either the direct or AM pathways. Alternative limiting factors might well be the concentration of P in the soil solution at the uptake sites or the surface area available for uptake.

While eCO_2×P effects were not observed in either of the plant species, the PT genes were in most cases regulated by P level, as expected from earlier work in *M. truncatula* (Chiou *et al.*, 2001; Grunwald *et al.*, 2009; Christophersen *et al.*, 2012) and *B. distachyon* (S.S. Clausen, E. Hammer and M. Grønlund, unpublished data). The slightly increased expression of *MtPT1* (NM and AM roots) and *MtPT3* (AM roots) under high P conditions is in contrast to reports of expression of direct- uptake PT genes in *M. truncatula* being suppressed by high Pi in systems with continuous liquid nutrient supplies (Chiou *et al.*, 2001; Grunwald *et al.*, 2009), but is in accordance with results from experiments with more realistic soil-based growth media (Christophersen *et al.*, 2012; Watts-Williams *et al.*, 2015). The expression of the AM-induced *MtPT4* decreased with increasing soil P, as previously reported for *M. truncatula* (Christophersen *et al.*, 2012) and for homologues in tomato (*Solanum lycopersicum* L.) and petunia (*Petunia hybrida* hort. ex E. Vilm.) (Nagy *et al.*, 2009; Breuillin *et al.*, 2010). This negative effect of high soil P availability on expression of *MtPT4* occurred despite a high level of colonization by AM fungi at 80P. This correlates well with the percentage of P uptake via the AM pathway dropping to background levels above 40P, where *MtPT4* expression was also low, as was observed in tomato (Nagy *et al.*, 2009). The low relative contribution by the AM pathway at the lowest P level was associated with reduced growth of the root-external hyphae and was probably caused by P deficiency in the strongly AM-dependent legume. This P-dependent reduction of AM-derived P uptake was not influenced by CO_2 levels. Expression of PT genes was more strongly suppressed by P in *B. distachyon* than in *M. truncatula*. The clear P-induced repression of the three *B. distachyon* PT genes concurs with reports for other plant species, including barley (Huang *et al.*, 2011) and wheat (Liu *et al.*, 2013). However, the expression *BdPT4* in AM roots was overall low and not significantly affected by P.

Conclusions

It might be argued that the short-time nature of our experiments reduce their relevance for crops. However, the species we chose are both annuals with short life-cycles of between 8 and 12 weeks, with harvest at 5 weeks representing about half this time, during which they would have taken up more than half of their final total P. Furthermore, *M. truncatula* is a pasture legume so short-term vegetative biomass represents the 'crop'. Both species would normally be mycorrhizal in field situations. We therefore consider that these model plants provide a good starting point for analysis of effects of eCO_2 in the contexts of AM colonization and P nutrition.

As already noted, the length of the growth period in this study was limited by the half-life of the ^{33}P used to track P uptake via the AM pathway, and it is premature to extrapolate to later harvests and effects on yields of biomass or seed, particularly for crops such as wheat with much longer life-cycles. Obtaining data for such later growth stages will require modifications of the compartmented pot system to allow addition of ^{33}P to HCs at different times during plant development. Nevertheless, the results presented here show that no consistent effect of eCO_2 in different plant species can be expected over a range of soil P levels, especially where growth is limited in low-P soils, as in nature. With higher soil P (more agricultural conditions), and hence lower P limitation, eCO_2 produces higher growth, but there are differences among species, as there are with responses to AM colonization. Therefore, it is not surprising that meta-analyses have revealed large consistent variations in responses of plant growth to eCO_2 (Treseder, 2004; Parmesan and Hanley, 2015).

The increased P use efficiency (PUE) at eCO_2 indicates that there may be no immediate requirement to increase agricultural P inputs in order to capture the expected CO_2 fertilizer effect. We found little effect of eCO_2 on % root length colonized by AM fungi or on AM function in terms of calculated P uptake in either of the plant species examined, indicating that the plants maintained proportional C supply to the roots and to the AM fungi regardless of CO_2 fertilization. The primary role of AM symbiosis under future growth conditions will remain to ensure an adequate P uptake, but there may be no change in the relative impact of the AM P uptake pathway on total P uptake. Effects on uptake of other plant nutrients, especially soil nitrogen, remain unexplored. However, maintaining a balanced C supply for nutrient uptake directly through the root epidermis and AM fungi seems important and is likely to involve a range of signaling mechanisms. Extrapolation to long-term effects on plant growth and yield is even more challenging, because gradual changes in eCO_2 levels are likely to affect the make-up of AM fungal communities in soil, and AM fungal taxa show differences in C–P trade balance with their host plants (Cotton *et al.*, 2015). However, the role of AM symbiosis in agriculture may find itself gaining more recognition as it becomes one of maintaining general plant fitness, e.g. by improving tolerance to drought and to damaging effects of some pathogens that might be altered under future climates.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Primers for RT-qPCR on *BdPT4*, *BdPT8*, and *BdPT7*.

Figure S1. Root dry weights of *M. truncatula* and *B. distachyon*.

Figure S2. Shoot P content of *M. truncatula* and *B. distachyon*.

Figure S3. ^{33}P uptake vs. hyphal length density in *M. truncatula*.

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References

Alberto O, Kuyper TW, Gorissen A. 2005. Taking mycocentrism seriously: mycorrhizal fungal and plant responses to elevated CO_2. New Phytologist **167,** 859–868.

Allen LH Jr, Kakani VG, Vu JCV, Boote KJ. 2011. Elevated CO_2 increases water use efficiency by sustaining photosynthesis of water-limited maize and sorghum. Journal of Plant Physiology **168,** 1909–1918.

Bayle V, Arrighi JF, Creff A, Nespoulous C, Vialaret J, Rossignol M, Gonzalez E, Paz-Ares J. Nussaume L. 2011. *Arabidopsis thaliana* high-affinity phosphate transporters exhibit multiple levels of posttranslational regulation. Plant Cell **23,** 1523–1535.

Blackman F. 1905. Optima and limiting factors. Annals of Botany **19,** 281–296.

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

Breuillin F, Schramm J, Hajirezaei M, *et al*. 2010. Phosphate systemically inhibits development of arbuscular mycorrhiza in *Petunia hybrida* and represses genes involved in mycorrhizal functioning. Plant Journal **64,** 1002–1017.

Brkljacic J, Grotewold E, Scholl R, *et al*. 2011. *Brachypodium* as a model for the grasses: today and the future. Plant Physiology **157,** 3–13.

Cavagnaro TR, Gleadow RM, Miller RE. 2011. Plant nutrient acquisition and utilisation in a high carbon dioxide world. Functional Plant Biology **38,** 87–96.

Cavagnaro TR, Sokolow SK, Jackson LE. 2007. Mycorrhizal effects on growth and nutrition of tomato under elevated atmospheric carbon dioxide. Functional Plant Biology **34,** 730–736.

Chen AQ, Gu MA, Sun SB, Zhu LL, Hong SA, Xu GH. 2011. Identification of two conserved cis-acting elements, MYCS and P1BS, involved in the regulation of mycorrhiza-activated phosphate transporters in eudicot species. New Phytologist **189,** 1157–1169.

Chen J, Wang Y, Wang F, *et al*. 2015. The rice CK2 kinase regulates trafficking of phosphate transporters in response to phosphate levels. Plant Cell **27,** 711–723.

Chiou TJ, Liu H, Harrison MJ. 2001. The spatial expression patterns of a phosphate transporter (MtPT1) from *Medicago truncatula* indicate a role in phosphate transport at the root/soil interface. Plant Journal **25,** 281–293.

Christophersen HM, Smith FA, Smith SE. 2012. Unraveling the influence of arbuscular mycorrhizal colonization on arsenic tolerance in *Medicago: Glomus mosseae* is more effective than *G. intraradices*, associated with lower expression of root epidermal Pi transporter genes. Frontiers in Physiology **3,** 91.

Cotton TEA, Fitter AH, Miller RM, Dumbrell AJ, Helgason T. 2015. Fungi in the future: interannual variation and effects of climate change on arbuscular mycorrhizal fungal communities. New Phytologist **205,** 1598–1607.

Edwards EJ, McCaffery S, Evans JR. 2005. Phosphorus status determines biomass response to elevated CO_2 in a legume : C_4 grass community. Global Change Biology **11,** 1968–1981.

Facelli E, Duan T, Smith SE, Christophersen H, Facelli J, Smith FA. 2014. Opening the black box: outcomes of interactions between arbuscular mycorrhizal (AM) and non-host genotypes of *Medicago* depend on fungal identity, interplay between P uptake pathways and external P supply. Plant Cell and Environment **37,** 1382–1392.

Gamper H, Peter M, Jansa J, Luscher A, Hartwig UA, Leuchtmann A. 2004. Arbuscular mycorrhizal fungi benefit from 7 years of free air CO_2 enrichment in well-fertilized grass and legume monocultures. Global Change Biology **10,** 189–199.

Gavito ME, Bruhn D, Jakobsen I. 2002. Phosphorus uptake by arbuscular mycorrhizal hyphae does not increase when the host plant grows under atmospheric CO_2 enrichment. New Phytologist **154,** 751–760.

Gavito ME, Curtis PS, Mikkelsen TN, Jakobsen I. 2000. Atmospheric CO_2 and mycorrhiza effects on biomass allocation and nutrient uptake of nodulated pea (*Pisum sativum* L.) plants. Journal of Experimental Botany **51,** 1931–1938.

Gavito ME, Schweiger P, Jakobsen I. 2003. P uptake by arbuscular mycorrhizal hyphae: effect of soil temperature and atmospheric CO_2 enrichment. Global Change Biology **9,** 106–116.

Grace EJ, Cotsaftis O, Tester M, Smith FA, Smith SE. 2009. Arbuscular mycorrhizal inhibition of growth in barley cannot be attributed to extent of colonization, fungal phosphorus uptake or effects on expression of plant phosphate transporter genes. New Phytologist **181,** 938–949.

Grønlund M, Albrechtsen M, Johansen I, Hammer EC, Nielsen TH, Jakobsen I. 2013. The interplay between P uptake pathways in mycorrhizal peas: a combined physiological and gene-silencing approach. Physiologia Plantarum **149,** 234–248.

Grunwald U, Guo WB, Fischer K, Isayenkov S, Ludwig-Muller J, Hause B, Yan XL, Kuster H, Franken P. 2009. Overlapping expression patterns and differential transcript levels of phosphate transporter genes in arbuscular mycorrhizal, Pi-fertilised and phytohormone-treated *Medicago truncatula* roots. Planta **229,** 1023–1034.

Grunzweig JM, Körner C. 2003. Differential phosphorus and nitrogen effects drive species and community responses to elevated CO_2 in semi-arid grassland. Functional Ecology **17,** 766–777.

Hartwig UA, Wittmann P, Raun RB, Hartwig-Raz B, Jansa J, Mozafar A, Luscher A, Leuchtmann A, Frossard E, Nösberger J. 2002. Arbuscular mycorrhiza infection enhances the growth response of *Lolium perenne* to elevated atmospheric pCO_2. Journal of Experimental Botany **53,** 1207–1213.

Hong JJ, Park YS, Bravo A, Bhattarai KK, Daniels DA, Harrison MJ. 2012. Diversity of morphology and function in arbuscular mycorrhizal symbioses in *Brachypodium distachyon*. Planta **236,** 851–865.

Hong SY, Seo PJ, Yang MS, Xiang F, Park CM. 2008. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. BMC Plant Biology **8,** 112.

Huang CY, Shirley N, Genc Y, Shi B, Langridge P. 2011. Phosphate utilization efficiency correlates with expression of low-affinity phosphate transporters and noncoding RNA, IPS1, in barley. Plant Physiology **156,** 1217–1229.

Imai K, Adachi N. 1996. Effects of atmospheric partial pressure of CO_2 and phosphorus nutrition on growth of young rice plants. Environment Control in Biology **34,** 59–66.

IPCC. 2013. *Climate change 2013. The physical science basis. Working Group I contribution to the fifth assessment report of the Intergovernmental Panel on Climate Change*. Cambridge and New York: Cambridge University Press.

Jakobsen I, Abbott LK, Robson AD. 1992. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum*. 1: Spread of hyphae and phosphorus inflow into roots. New Phytologist **120,** 371–380.

Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ. 2007*a*. A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proceedings of the National Academy of Sciences, USA **104,** 1720–1725.

Javot H, Pumplin N, Harrison MJ. 2007*b*. Phosphate in the arbuscular mycorrhizal symbiosis: transport properties and regulatory roles. Plant Cell and Environment **30,** 310–322.

Jifon JL, Graham JH, Drouillard DL, Syvertsen JP. 2002. Growth depression of mycorrhizal *Citrus* seedlings grown at high phosphorus supply is mitigated by elevated CO_2. New Phytologist **153,** 133–142.

Jin J, Tang C, Armstrong R, Sale P. 2012. Phosphorus supply enhances the response of legumes to elevated CO_2 (FACE) in a phosphorus-deficient vertisol. Plant and Soil **358,** 86–99.

Jin J, Tang C, Sale P. 2015. The impact of elevated carbon dioxide on the phosphorus nutrition of plants: a review. Annals of Botany **116,** 987–999.

Johnson NC, Wolf J, Reyes MA, Panter A, Koch GW, Redman A. 2005. Species of plants and associated arbuscular mycorrhizal fungi mediate mycorrhizal responses to CO_2 enrichment. Global Change Biology **11,** 1156–1166.

Jongen M, Fay P, Jones MB. 1996. Effects of elevated carbon dioxide and arbuscular mycorrhizal infection on *Trifolium repens*. New Phytologist **132,** 413–423.

Konvalinkova T, Pueschel D, Janouskova M, Gryndler M, Jansa J. 2015. Duration and intensity of shade differentially affects mycorrhizal growth- and phosphorus uptake responses of *Medicago truncatula*. Frontiers in Plant Science **6,** 65.

Kormanik P, McGraw A. 1982. Quantification of vesicular-arbuscular mycorrhizae in plant roots. In: Schenck NC, ed. *Methods and principles of mycorrhizal research*. St. Paul, MN: The American Phytopathological Society, 37–45.

Lam SK, Chen D, Norton R, Armstrong R. 2012. Does phosphorus stimulate the effect of elevated [CO_2] on growth and symbiotic nitrogen fixation of grain and pasture legumes? Crop & Pasture Science **63,** 53–62.

Leakey AD, Ainsworth EA, Bernacchi CJ, Rogers A, Long SP, Ort DR. 2009. Elevated CO_2 effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. Journal of Experimental Botany **60,** 2859–2876.

Li HY, Zhu YG, Marschner P, Smith FA, Smith SE. 2005. Wheat responses to arbuscular mycorrhizal fungi in a highly calcareous soil differ from those of clover, and change with plant development and P supply. Plant and Soil **277,** 221–232.

Liebig J. 1843. *Chemistry in its applications to agriculture and physiology*. London: Taylor & Watson.

Liu JY, Versaw WK, Pumplin N, Gomez SK, Blaylock LA, Harrison MJ. 2008. Closely related members of the *Medicago truncatula* PHT1 phosphate transporter gene family encode phosphate transporters with distinct biochemical activities. Journal of Biological Chemistry **283,** 24673–24681.

Liu X, Zhao X, Zhang L, Lu W, Li X, Xiao K. 2013. *TaPht1;4*, a high-affinity phosphate transporter gene in wheat (*Triticum aestivum*), plays an important role in plant phosphate acquisition under phosphorus deprivation. Functional Plant Biology **40,** 329–341.

López-Arredondo D, Leyva-Gonzalez M, Gonzalez-Morales S, Lopez-Bucio J, Herrera-Estrella L. 2014. Phosphate nutrition: Improving low-phosphate tolerance in crops. Annual Review of Plant Biology **65,** 95–123.

Lukac M, Calfapietra C, Godbold DL. 2003. Production, turnover and mycorrhizal colonization of root systems of three *Populus* species grown under elevated CO_2 (POPFACE). Global Change Biology **9,** 838–848.

Merrild MP, Ambus P, Rosendahl S, Jakobsen I. 2013. Common arbuscular mycorrhizal networks amplify competition for phosphorus between seedlings and established plants. New Phytologist **200,** 229–240.

Munkvold L, Kjoller R, Vestberg M, Rosendahl S, Jakobsen I. 2004. High functional diversity within species of arbuscular mycorrhizal fungi. New Phytologist **164,** 357–364.

Nagy R, Drissner D, Amrhein N, Jakobsen I, Bucher M. 2009. Mycorrhizal phosphate uptake pathway in tomato is phosphorus-repressible and transcriptionally regulated. New Phytologist **181,** 950–959.

Newbery RM, Wolfenden J. 1996. Effects of elevated CO_2 and nutrient supply on the seasonal growth and morphology of *Agrostis capillaris*. New Phytologist **132,** 403–411.

Newbery RM, Wolfenden J, Mansfield TA, Harrison AF. 1995. Nitrogen, phosphorus and potassium uptake and demand in *Agrostis capillaris* – the influence of elevated CO_2 and nutrient supply. New Phytologist **130,** 565–574.

Newman EI. 1966. A method for estimating the total length of root in a sample. Journal of Applied Ecology **3,** 139–145.

Niu Y, Chai R, Dong H, Wang H, Tang C, Zhang Y. 2013. Effect of elevated CO_2 on phosphorus nutrition of phosphate-deficient *Arabidopsis thaliana* (L.) Heynh under different nitrogen forms. Journal of Experimental Botany **64,** 355–367.

Nord EA, Jaramillo RE, Lynch JP. 2015. Response to elevated CO_2 in the temperate C3 grass *Festuca arundinaceae* across a wide range of soils. Frontiers in Plant Science **6,** 95.

Obersteiner M, Penuelas J, Ciais P, van der Velde M, Janssens IA. 2013. The phosphorus trilemma. Nature Geoscience **6,** 897–898.

Olsen SR, Cole CV, Watanabe FS, Dean LA. 1954. *Estimation of available phosphorus in soils by extraction with sodium bicarbonate*. Washington DC: USDA, Circular No. 939.

Pandey R, Dubey KK, Ahmad A, Nilofar R, Verma R, Jain V, Zinta G, Kumar V. 2015*a*. Elevated CO_2 improves growth and phosphorus utilization efficiency in cereal species under suboptimal phosphorus supply. Journal of Plant Nutrition **38,** 1196–1217.

Pandey R, Zinta G, AbdElgawad H, Ahmad A, Jain V, Janssens IA. 2015*b*. Physiological and molecular alterations in plants exposed to high [CO_2] under phosphorus stress. Biotechnology Advances **33,** 303–316.

Parmesan C, Hanley ME. 2015. Plants and climate change: complexities and surprises. Annals of Botany **116,** 849–864.

Pearson JN, Jakobsen I. 1993. The relative contribution of hyphae and roots to phosphorus uptake by arbuscular mycorrhizal plants measured by dual labelling with ^{32}P and ^{33}P. New Phytologist **124,** 489–494.

Poorter H, Perez-Soba M. 2001. The growth response of plants to elevated CO_2 under non-optimal environmental conditions. Oecologia **129,** 1–20.

Rillig MC, Field CB, Allen MF. 1999. Fungal root colonization responses in natural grasslands after long-term exposure to elevated atmospheric CO_2. Global Change Biology **5,** 577–585.

Rogers A, Ainsworth EA, Leakey AD. 2009. Will elevated carbon dioxide concentration amplify the benefits of nitrogen fixation in legumes? Plant Physiology **151,** 1009–1016.

Rouhier H, Read DJ. 1998. The role of mycorrhiza in determining the response of *Plantago lanceolata* to CO_2 enrichment. New Phytologist **139,** 367–373.

Sa TN, Israel DW. 1998. Phosphorus-deficiency effects on response of symbiotic N_2 fixation and carbohydrate status in soybean to atmospheric CO_2 enrichment. Journal of Plant Nutrition **21,** 2207–2218.

Scholz RW, Wellmer F-W. 2013. Approaching a dynamic view on the availability of mineral resources: What we may learn from the case of phosphorus? Global Environmental Change **23,** 11–27.

Sicher RC, Barnaby JY. 2012. Impact of carbon dioxide enrichment on the responses of maize leaf transcripts and metabolites to water stress. Physiologia Plantarum **144,** 238–253.

Singh SK, Reddy VR, Fleisher DH, Timlin DJ. 2014. Growth, nutrient dynamics, and efficiency responses to carbon dioxide and phosphorus nutrition in soybean. Journal of Plant Interactions **9,** 838–849.

Smith SE, Jakobsen I, Grønlund M, Smith FA. 2011. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant Physiology **156,** 1050–1057.

Smith SE, Smith FA, Jakobsen I. 2003. Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiology **133,** 16–20.

Smith SE, Smith FA, Jakobsen I. 2004. Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytologist **162,** 511–524.

Staddon PL, Graves JD, Fitter AH. 1998. Effect of enhanced atmospheric CO_2 on mycorrhizal colonization by *Glomus mosseae* in *Plantago lanceolata* and *Trifolium repens*. New Phytologist **139,** 571–580.

Stocklin J, Körner C. 1999. Interactive effects of elevated CO_2, P availability and legume presence on calcareous grassland: results of a glasshouse experiment. Functional Ecology **13,** 200–209.

Stonor RN, Smith SE, Manjarrez M, Facelli E, Smith F. 2014. Mycorrhizal responses in wheat: shading decreases growth but does not lower the contribution of the fungal phosphate uptake pathway. Mycorrhiza **24,** 465–472.

Syvertsen JP, Graham JH. 1999. Phosphorus supply and arbuscular mycorrhizas increase growth and net gas exchange responses of two *Citrus* spp. grown at elevated [CO_2]. Plant and Soil **208,** 209–219.

Treseder KK. 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO_2 in field studies. New Phytologist **164,** 347–355.

Treseder KK, Allen MF. 2000. Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO_2 and nitrogen deposition. New Phytologist **147,** 189–200.

Watts-Williams SJ, Jakobsen I, Cavagnaro TR, Grønlund M. 2015. Local and distal effects of arbuscular mycorrhizal colonization on direct pathway Pi uptake and root growth in *Medicago truncatula*. Journal of Experimental Botany **66,** 4061–4073.

Zinta G, AbdElgawad H, Domagalska MA, Vergauwen L, Knapen D, Nijs I, Janssens IA, Beemster GT, Asard H. 2014. Physiological, biochemical, and genome-wide transcriptional analysis reveals that elevated CO_2 mitigates the impact of combined heat wave and drought stress in *Arabidopsis thaliana* at multiple organizational levels. Global Change Biology **20,** 3670–3685.