Plant Soil (2012) 358:91–104
DOI 10.1007/s11104-012-1270-z

REGULAR ARTICLE

Phosphorus supply enhances the response of legumes to elevated CO_2 (FACE) in a phosphorus-deficient vertisol

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Received: 2 December 2011 / Accepted: 24 April 2012 / Published online: 10 May 2012

Abstract
Background & aims Understanding the mechanism of how phosphorus (P) regulates the response of legumes to elevated CO_2 (eCO_2) is important for developing P management strategies to cope with increasing atmospheric CO_2 concentration. This study aimed to explore this mechanism by investigating interactive effects of CO_2 and P supply on root morphology, nodulation and soil P fractions in the rhizosphere.
Methods A column experiment was conducted under ambient (350 ppm) (aCO_2) and eCO_2 (550 ppm) in a free air CO_2 enrichment (FACE) system. Chickpea and field pea were grown in a P-deficient Vertisol with P addition of 0–16 mg P kg^{-1}.
Results Increasing P supply increased plant growth and total P uptake with the increase being greater under eCO_2 than under aCO_2. Elevated CO_2 increased root biomass and length, on average, by 16 % and 14 %, respectively. Nodule biomass increased by 46 % in response to eCO_2 at 16 mg P kg^{-1}, but was not affected by eCO_2 at no P supply. Total P uptake was correlated with root length while N uptake correlated with nodule number and biomass regardless of CO_2 level. Elevated CO_2 increased the NaOH-extractable organic P by 92 % when 16 mg P kg^{-1} was applied.
Conclusion The increase in P and N uptake and nodule number under eCO_2 resulted from the increased biomass production, rather than from changes in specific root-absorbing capability or specific nodule function. Elevated CO_2 appears to enhance P immobilization in the rhizosphere.

Keywords Free air CO_2 enrichment · FACE · N_2 fixation · Nodulation · P acquisition · P fractions · Rhizosphere

Responsible Editor: Hans Lambers.

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Introduction

The concentration of global atmospheric CO_2 has increased from around 270 μmol mol^{-1} prior to the Industrial Revolution to 384 μmol mol^{-1} in 2009 (Leakey et al. 2009). It is predicted that CO_2 will reach 550 μmol mol^{-1} by the middle of this century and climb up to 700 μmol mol^{-1} by the end of the century (de Graaff et al. 2006; Ainsworth et al. 2008). Elevated CO_2 (eCO_2) has significant effects on plant growth and physiology (Stöcklin and Körner 1999; von Felten et al. 2007). However, the response of plants to eCO_2

greatly depends on species and the availability of nutrients such as P (Conroy et al. 1992; Newbery et al. 1995). For example, the growth of legumes appears to be more responsive to eCO_2 than non-legumes, especially under high-P conditions (Stöcklin et al. 1998; Stöcklin and Körner 1999).

Phosphorus (P) is involved in various metabolic processes such as conserving and transferring energy in cell metabolism (Raghothama 1999; Abel et al. 2002). It is expected that plants grown under eCO_2 would require more P to maintain their physiological requirements due to increases in biomass normally associated with eCO_2. Stöcklin and Körner (1999) reported a 166 % increase of biomass under eCO_2 by *Hippocrepis comosa* when P was supplied but there was no response to eCO_2 without P application. Furthermore, P plays a specific role in nodulation in legumes (Israel 1987; Bordeleau and Prevost 1994), as more P is required for nodule development and nodule function than for the host plant growth (Qiao et al. 2007). Under eCO_2 environment, legumes exhibit stronger N_2 fixation, resulting in positive photosynthetic and growth responses (Lee et al. 2003; Rogers et al. 2009). However, knowledge of the demand for P in N_2-fixing legumes, and associated responses of N_2 fixation and growth to P supply under eCO_2 is limited.

Changes in root morphology and metabolism-driven rhizosphere processes occurring under eCO_2 are believed to favour P acquisition (Barrett et al. 1998; Campbell and Sage 2002). For example, eCO_2 has been shown to enhance root growth in *Senecio vulgaris*, *Festuca ovina* and *Nardus stricta* (Berntson and Woodward 1992; Fitter et al. 1996) and the formation of root hairs in *Arabidopsis thaliana* (Niu et al. 2011), which would, in turn, increase P uptake. In legumes, eCO_2 may intensify rhizosphere acidification through differential cation/anion uptake during N_2 fixation and hence benefit P mobilization (Tang et al. 2009). Also, the increased release of carbon-rich compounds under eCO_2 including organic acid anions and phosphatases into the rhizosphere (Richardson 2001; de Graaff et al. 2006) might attract and stimulate soil microorganisms to mineralize or directly mobilize soil P (George et al. 2002). However, direct evidence is lacking as to whether rhizosphere processes under eCO_2 facilitates P mobilization or immobilization.

Legume species differ markedly in their ability to take up P from soil. For example, chickpea has significantly higher root biomass and surface area than field pea (Srinivasarao et al. 2006; Erman et al. 2009). Chickpea also exudes large amounts of low-molecular weight carboxylates, which mobilize P by competing for the same adsorption sites in soil matrix (Gerke et al., 2000; Wouterlood et al. 2005; Veneklaas et al. 2003). In contrast, field pea, with a relatively small root system, secretes less carboxylates and phosphatases per root mass (Nuruzzaman et al. 2005), suggesting that field pea roots are less efficient in taking up P than chickpea roots.

In this study, a range of P application rates were added to two legume species grown in a P-deficient Vertisol within a free air CO_2 enrichment facility to investigate the effect of eCO_2 on P requirement, N uptake, and root and nodule characteristics. We hypothesized that eCO_2 would increase the P demand, and this increased demand could be met by a greater capacity for P acquisition by the root system, and by increasing P-regulated nodulation and N_2 fixation under eCO_2, than under ambient CO_2 (aCO_2).

Materials and methods

Experimental design and plant growth

A column experiment was conducted at a free air CO_2 enrichment (SoilFACE) facility at the Department of Primary Industries in Horsham, Victoria, Australia (36°42′S, 142°11′E) (Mollah et al. 2011). There were four FACE rings for elevated CO_2 (550 ppm) and four ambient CO_2 rings (350 ppm). The FACE array was engineered to achieve at least 80 % of the ring area with a CO_2 concentration at or above 90 % of the target concentration at the ring-centre for 80 % of the time. The CO_2 concentration in the elevated CO_2 FACE rings was in a range of 512 to 580 ppm (Mollah et al. 2011). The experiment consisted of two levels of CO_2, two leguminous species and five P levels in a split-plot design with CO_2 as the main plot, and legume species and P application as sub-plot treatments. Each treatment had four replicates and each replicate was randomly allocated into one FACE-ring. Two grain legume species were chickpeas (*Cicer arietinum* L. cv. Genesis 836) and field pea (*Pisum sativum* L. cv. OzP0601) which differ in root morphology and physiology. Phosphorus was applied as KH_2PO_4 at five rates, i.e. 0, 2, 4, 8 and 16 mg Pkg^{-1} soil. The

soil was collected at a depth of approximately 10 to 30 cm from a virgin site under native vegetation that had not previously been used for farming near Horsham, Victoria, Australia. The soil type was a Vertosol (Isbell 1996) or a Vertisol (FAO - UNESCO 1976). It had organic C of 7.8 mg g^{-1} (Rayment and Higginson 1992), 2 M KCl-extractable NO_3-N of 4.2 mg kg^{-1} and NH_4-N of 1.0 mg kg^{-1}, total P of 114 mg kg^{-1} (Guppy et al. 2000), Colwell P of 5 mg kg^{-1} (Colwell 1963) and a pH (1:5 in 0.01 M $CaCl_2$) of 7.7. The experimental soil was air-dried and sieved through a 4-mm sieve, then mixed with siliceous sand (w:w=1:1) to aid root washing and collecting rhizosphere soil at harvest.

Each column used in this experiment comprised of two equal halves of a vertically-split PVC pipe (60 cm long, 10 cm in diameter). The two halves of pipe were taped together with plumbing tape with a PVC cap placed at the bottom of the column. Each column contained 8 kg of experimental soil mixed with the following basal nutrients (mg kg^{-1}): K_2SO_4, 147; $MgSO_4.7H_2O$, 122; $CaCl_2$, 186; $CuSO_4.5H_2O$, 6; $ZnSO_4.7H_2O$, 8; $MnSO_4.5H_2O$, 6; $FeCl_3$, 0.6; $CoCl_2$, 0.4; $NaMoO_4.2H_2O$, 0.4; and NaB_4O_7, 1.6 (Vu et al. 2010) and the required amount of P for each treatment.

Nine uniform germinated seeds of each species were hand-sown at a depth of 2 cm in each column and inoculated with rhizobium (*Rhizobium ciceri* for chickpea and *Rhizobium leguminosarum* for field pea) on the 19th September, 2010. The seedlings were thinned to 2 plants per column 3 weeks after sowing. The average temperatures during plant growth were 25.1 °C in the day and 10.1 °C at night. The total rainfall during the experiment was 116.8 mm. These meteorological observations were taken from Horsham Airport which is 6.6 km away from the SoilFACE site. The soil moisture in column was adjusted to 80 % of field capacity every 3 days by weighing and watering with reverse osmosis water adding up to 1,460 ml for each column during the experimental period.

Measurements

After 9 weeks of growth in the SoilFACE, plant shoots were cut off at ground level. To remove dust, shoots were washed with 0.1 M HCl and then rinsed twice in deionized water (Tang et al. 1990). Each column was opened and the soil was separated vertically into 4 layers, namely 0–10, 10–20, 20–40 and 40–60 cm. Roots in each layer were carefully removed by sliding out the entire root mass. The soil adhering to the roots was shaken off as rhizosphere soil (Marschner et al. 2004). The root system was washed with tap water until free of soil, and then soaked in 0.01 M $CaCl_2$ solution for 5 min to desorb nutrients on root surface (Tang et al. 1990). Root nodules were counted and removed. The root morphology in terms of root length, surface area and diameter was determined by scanning roots on an EPSON EU-35 scanner (Seiko Epson Corp., Japan), and images were analysed using the Mac Rhizo Pro version 2003b programme (Régent Instruments Inc., Québec, CA).

All plant samples were dried at 70 °C for 72 h and then ground. Subsamples of ground shoots and roots were digested with a mixture of nitric and perchloric acid (4:1) (Yuen and Pollard 1954), and the concentrations of P in digests were colorimetrically measured using malachite green (Motomizu et al. 1980). The concentration of N in plant tissues was determined using an Elementar CNS analyser (Vario EL III, Elementar Analysensysteme GmbH, Germany).

Rhizosphere soil samples were mixed thoroughly, air-dried, and milled to <0.5 mm before further analysis. Phosphorus fractions were performed using the modified Hedley P fractionation scheme (Guppy et al. 2000). Total dissolved P including organic (Po) and inorganic P (Pi) in the bicarbonate ($NaHCO_3$) and hydroxide (NaOH) extracts were determined after digesting in an autoclave at a pressure of 103 kPa at 121 °C for 1 h using acid ammonium persulphate (Butterly et al. 2009). The Po in these two fractions was determined by subtracting the Pi from total P. The Pi in extracts was determined using the malachite green method (Motomizu et al. 1980).

Statistical analysis

Statistical analyses were performed on parameters using SAS Release 6.12 for Windows (SAS Institute 1997). Protected ANOVA tests of LSD were used to assess the differences between treatment means (Steel and Torrie 1980). The data of plant biomass, root morphology, P and N parameters were statistically analyzed by factorial ANOVA to determine the effects of P, CO_2, species and their interactions (Genstat, Version 13, VSN International software for bioscience).

Results

Shoot growth

Shoot biomass of the legumes increased significantly with added P and with eCO_2, and differed between the species with field pea producing a greater biomass than chickpea (Fig. 1a, Table 1). However, the relative shoot biomass response to eCO_2 depended on the P treatment. The response of shoot biomass of both species to eCO_2 was around 15 % with 0 mg Pkg^{-1}, and 32 % with 16 mg Pkg^{-1}, resulting in a significant $P \times CO_2$ interaction There was also a significant P × Species interaction, with chickpea having a 5.9-fold increase in shoot biomass when the P applied was increased from 0 to 16 mg kg^{-1} soil, compared to the 6.8-fold increase in field pea (Table 1). There was no significant CO_2 × Species interaction, indicating that the species did not differ in their response to eCO_2, nor was there any significant CO_2 × P × Species interaction (Table 1).

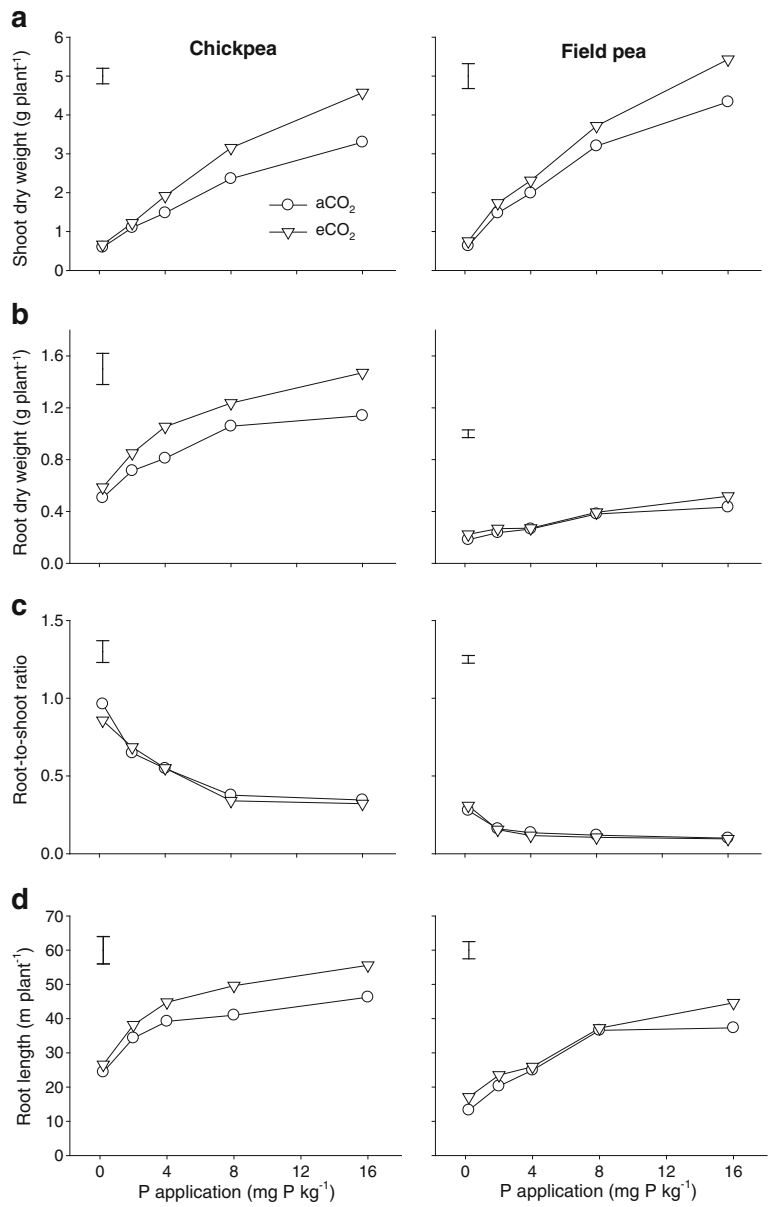

Fig. 1 The effects of CO_2, P and species on shoot biomass (**a**), root biomass (**b**), root-to-shoot weight ratio (**c**) and root length (**d**) of chickpea (*left*) and field pea (*right*) after plants were exposed to CO_2 treatments for 9 weeks in a P-deficient Vertisol supplied with 0 to 16 mg P kg^{-1} soil. The vertical bar in each panel indicates the LSD (P=0.05) for the CO_2 × P interaction

Table 1 Significant levels of main effects and interactions of CO_2, P application and species on dry weights (DW) of shoots and roots, root length, root-to-shoot ratio (R/S), nodule dry weight, number and size, nodule density (nodule number per unit root length) and N uptake per unit nodule mass

Factors	Shoot DW	Root DW	Root length	R/S	Nodule DW	Nodule No.	Nodule size	Nodule density	N uptake per mg of nodule
CO_2	<0.001	<0.001	<0.001	0.606	<0.001	<0.001	0.143	0.502	0.953
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Species	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.393	<0.001
CO_2 × P	<0.001	0.461	0.463	0.954	0.015	0.103	0.611	0.653	0.740
CO_2 × Species	0.440	0.007	0.179	0.937	0.007	0.454	0.201	0.725	0.864
P × Species	0.029	<0.001	0.096	<0.001	<0.001	0.020	<0.001	0.958	<0.001
CO_2 × P × Species	0.851	0.751	0.729	0.928	0.214	0.637	0.623	0.958	0.735

Root growth and biomass allocation

Unlike the effect on shoots, there was no significant CO_2 × P interaction for roots (Table 1), but both of these main effect treatments increased root biomass (Fig. 1b). Between species, chickpea had significantly greater root biomass than field pea with the difference being greater at high P than at no or low P (Table 1; Fig. 1b). Chickpea responded more to eCO_2 than field pea, increasing root biomass by 22 % when exposed to eCO_2, whereas field peas had only 10 % increase (Fig. 1b).

The root-to-shoot ratio markedly declined as P supply increased, but was not affected by CO_2 treatment. Irrespective of CO_2 treatment, chickpea had higher root-to-shoot ratios than field pea (Table 1; Fig. 1c). A significant P × Species interaction was found, with the root-to-shoot ratio of chickpea decreasing more than field pea as P application rate increased. However, there were no significant CO_2 × P, CO_2 × Species or CO_2 × P × Species interaction for the root-to-shoot ratio (Table 1).

Similar to the effects on root biomass, increasing P supply from 0 to 16 mg P kg^{-1} increased root length from 26.8 to 46.3 m $plant^{-1}$ for chickpea and from 13.3 to 37.3 m $plant^{-1}$ for field pea. Compared to aCO_2, eCO_2 increased average root length by 14 % for chickpea and by 12 % for field pea (Fig. 1d). However, there were no significant interactive effects on root length between any two treatments (Table 1).

Nodulation

Increasing P application increased nodule biomass, number and size but decreased N uptake per unit nodule biomass, while increasing CO_2 concentration increased the total nodule biomass and nodule number but did not affect nodule size (single nodule mass) or N uptake per unit nodule biomass (Fig. 2, Table 1). Nodule density (nodule number per unit root length) also increased with P application, but was not affected by eCO_2 across P treatments (Fig. 2d). Compared with field pea, chickpea on average produced a 6-fold greater nodule biomass and 49 % more nodules, and these nodules were 3 times larger. However, the plant N uptake per unit of nodule biomass was much lower in chickpea (Fig. 2e).

Although eCO_2 did increase total nodule biomass, the response varied with P rate and between two species, resulting in significant CO_2 × P, and CO_2 × Species interactions (Table 1). The basis for the former interaction was 46 % greater nodule biomass under eCO_2 with 16 mg P kg^{-1}, compared to the lack of any difference in nodule biomass with nil applied P (Fig. 2a). Similarly, the response in nodule biomass to eCO_2 by chickpea was 35 mg $plant^{-1}$, compared with 5 mg $plant^{-1}$ by field pea.

There were significant P×Species interactions on nodule biomass, number and size, and N uptake per unit nodule biomass (Table 1). Nodule biomass, number and size of chickpea increased more sharply than those of field pea as the rate of P application increased from 0 to 16 mg P kg^{-1} (Fig. 2a, b, and c). In contrast, with increasing P supply, N uptake per unit nodule biomass decreased more in field pea than in chickpea (Fig. 2e).

Irrespective of CO_2 and P treatments, total N uptake was positively correlated ($P<0.001$) with nodule number ($R^2=0.96$–0.99), nodule biomass ($R^2=0.99$) and total biomass production ($R^2=0.99$) for both species.

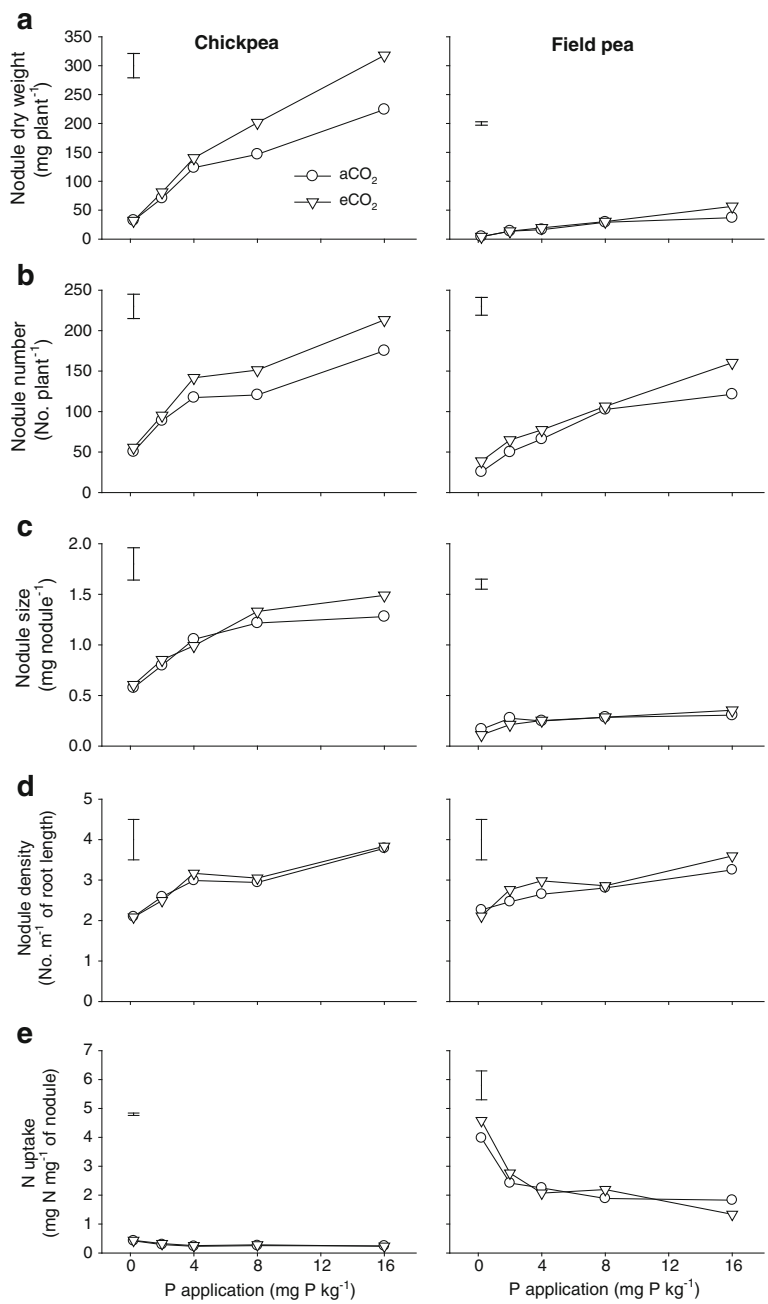

Fig. 2 The effects of CO_2, P and species on CO_2 nodule biomass (**a**), nodule number (**b**), nodule size (**c**), nodule density (**d**) and N uptake per mg nodule (**e**) of chickpea (*left*) and field pea (*right*) after plants were exposed to CO_2 treatments for 9 weeks in a P-deficient Vertisol supplied with 0 to 16 mg P kg^{-1} soil. The vertical bar in each panel indicates the LSD (P=0.05) for the CO_2 × P interaction

Root and nodule distribution in soil profiles

Chickpea distributed 43–49 % of the root biomass and field pea distributed 30–48 % in 0–10 cm of soil profile. Applying P significantly decreased the distribution of root biomass in top 10 cm of the soil. Elevated CO_2, however, did not affect the distribution of root biomass (Fig. 3a). There was no significant CO_2 × P interaction on the distribution of root biomass for either species.

The relative proportion of root length located in the top 10 cm of soil tended to decrease as the rate of P application increased but was not affected by CO_2. The distribution of root length throughout the soil profile varied with species, with chickpea having 9 % less root length in the topsoil than field pea (Fig. 3b). In general, chickpea had longer roots distributed deeper in the soil than field pea.

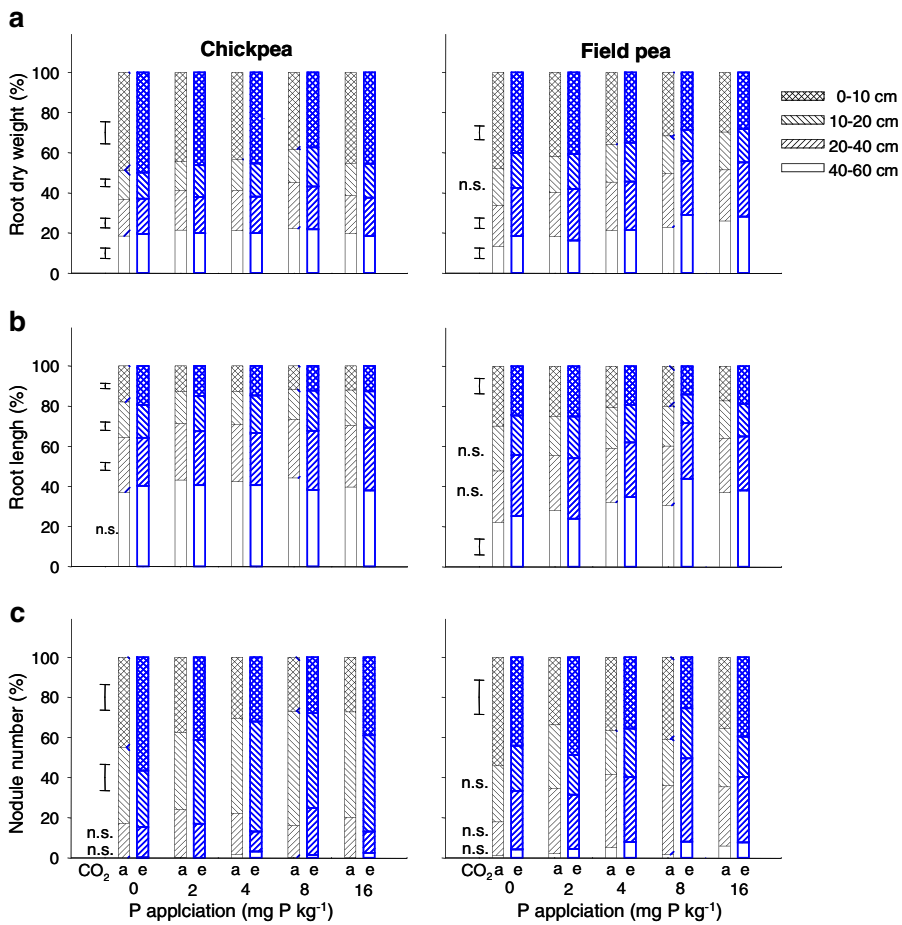

Fig. 3 The distribution patterns at various soil depths of root biomass (**a**), root length (**b**) and nodule number (**c**) of chickpea (*left*) and field pea (*right*) grown for 9 weeks in a Vertisol supplied with 0–16 mg P kg^{-1} soil under ambient (350 ppm) (**a**) and elevated CO_2 (550 ppm) (**e**). The vertical bars in each panel indicate the LSD (P=0.05) for individual layers (0–10 cm, 10–20 cm, 20–40 cm and 40–60 cm) if the treatment effect or interaction is significant. n.s. not significant at P<0.05

Increasing P application decreased nodule number in the 0–10 cm of soil depth (P<0.05) (Fig. 3c). Elevated CO_2 did not affect the nodule distribution. The two species differed in the distribution of nodule number (P<0.01), with chickpea having 40 % of its nodules in the 10–20 cm soil layer while field pea had only 20 % of its nodules in the same soil layer.

Plant P concentration and uptake

Phosphorus application and eCO_2 significantly affected the concentration of P in plants but this effect depended on the species (Table 2). Increasing P application generally increased P concentrations in shoots and roots of chickpea but not of field pea. On average, chickpea had higher tissue P concentrations than field pea. Elevated CO_2 decreased the P concentration in shoots of chickpea by 12 %, but had no effect in field pea. There was no P × CO_2 interaction on P concentration.

Total P uptake increased with increasing P application for both species but this increase was greater for field pea than chickpea. On average field pea had 29.5 % more total P than chickpea (Table 2). A significant CO_2×P interaction occurred on total P uptake, with total P uptake increasing more under eCO_2 than aCO_2 as P application increased. Total P uptake correlated positively with root length and root biomass of both species (data not shown).

Plant N concentration and uptake

Increasing P application generally decreased N concentration in shoots but not in roots (Table 2). Field pea had higher N concentrations in both shoots and roots than chickpea. However, CO_2 treatment did not affect N concentration of either species (Table 2).

Total N uptake was affected by P and CO_2 treatments (Table 2). Total N uptake increased as P application rate increased for both legume species with the

Table 2 The concentrations of N and P in shoots and roots, total P and N, and N/P ratio in the plant of chickpea and field pea grown for 9 weeks in a Vertisol supplied with 0–16 mg P kg^{-1} soil under ambient (350 ppm) and elevated CO_2 (550 ppm)

Species	P supply (mg P kg^{-1} soil)	Shoot P (mg g^{-1})		Root P (mg g^{-1})		Total P (mg $plant^{-1}$)		Shoot N (mg g^{-1})		Root N (mg g^{-1})		Total N (mg $plant^{-1}$)		N/P	
		aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
Chickpea	0	0.95	0.88	0.78	0.80	1.13	1.23	13.1	12.6	9.3	7.9	13.7	13.9	12.06	11.26
	2	1.37	1.21	0.92	0.88	2.54	2.72	11.2	13.3	8.2	8.2	20.7	26.6	8.15	9.77
	4	1.39	1.20	1.06	0.91	3.56	4.29	10.5	11.1	9.1	8.1	28.6	36.0	8.06	8.39
	8	1.42	1.31	1.26	1.15	5.06	6.66	9.4	11.2	10.0	9.9	37.3	56.7	7.36	8.51
	16	1.47	1.28	1.31	1.22	7.02	9.61	10.4	9.9	9.2	9.1	54.5	74.1	7.77	7.72
	Mean	1.32	1.17	1.07	0.99	3.86	4.90	10.9	11.6	9.2	8.6	31.0	48.4	8.69	9.14
Field pea	0	1.87	1.83	1.20	1.25	1.41	1.60	20.7	20.9	17.6	17.6	16.9	19.5	11.95	12.14
	2	1.96	1.95	1.46	1.39	3.26	3.77	19.4	17.9	16.6	18.6	33.3	36.4	10.19	10.08
	4	1.89	1.89	1.50	1.42	4.36	4.91	15.0	15.1	18.8	17.8	36.8	36.9	8.45	8.23
	8	1.94	1.92	1.47	1.38	6.84	7.96	14.5	13.7	18.8	18.1	55.0	59.7	8.04	8.36
	16	1.95	1.95	1.47	1.37	9.09	11.7	13.8	11.9	20.2	17.1	59.3	69.8	7.44	6.44
	Mean	1.92	1.91	1.42	1.36	4.99	5.99	16.7	15.9	18.4	17.8	41.9	48.1	9.23	9.05
	LSD (P=0.05) (significance level)														
	CO_2	0.05 (***)		0.05 (**)		0.33 (***)		n.s.		n.s.		2.92 (***)		n.s.	
	P	0.07 (***)		0.08 (***)		0.52 (***)		1.01 (***)		n.s.		4.62 (***)		1.00 (***)	
	Species	0.05 (***)		0.05 (***)		0.33 (***)		0.63 (***)		0.42 (***)		2.93 (***)		n.s.	
	CO_2 × P	n.s.		n.s.		0.73 (***)		n.s.		n.s.		6.54 (*)		n.s.	
	CO_2 × Species	0.06 (**)		n.s.		n.s.		n.s.		n.s.		4.14 (*)		n.s.	
	P × Species	0.10 (***)		0.11 (***)		0.73 (**)		1.42 (***)		n.s.		n.s.		n.s.	
	CO_2 × P × Species	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	

n.s., *, ** and *** indicate P>0.05, P<0.05, P<0.01 and P<0.001, respectively

increase being greater under eCO_2 than under aCO_2, resulting in a significant CO_2 × P interaction. The basis for this was 9 % increase in N uptake with nil P, compared to the 27 % with 16 mg P kg^{-1} (Table 2). There was also a significant CO_2 × Species interaction due to the greater N uptake response to eCO_2 in chickpea than in field pea.

The N-to-P concentration ratio in the plant significantly decreased as the rate of P application increased, but it was not affected by eCO_2 (Table 2). There was no P × CO_2 interaction for the N-to-P ratio.

P fractionation in rhizosphere

Phosphorus supply and CO_2 affected P pools in rhizosphere of the legumes. Increasing P application from 4 to 16 mg kg^{-1} significantly increased concentrations of both $NaHCO_3$-Pi and NaOH-Po (Table 3). However, eCO_2 only increased the NaOH-Po fraction, but this increase depended on P supply due to a significant CO_2 × P interaction. This resulted from an 11 % increase in NaOH-Po with eCO_2 at 4 mg P kg^{-1}, compared to a 92 % of increase with eCO_2 at 16 mg P kg^{-1}, irrespective of species (Table 3). Species differences included higher concentrations of $NaHCO_3$-Po and NaOH-Pi in the rhizosphere of field pea, compared with chickpea. Increased P application and CO_2 concentration did not change the HCl-P or residual-P fractions in rhizosphere with averages of 16 and 83 mg P kg^{-1}, respectively (data not shown).

Discussion

Plant growth

The two N_2-fixing grain legume species grown in the P-deficient Vertisol soil required P addition to

Table 3 The distribution of soil P fractionations (mg P kg^{-1} soil) in rhizosphere of chickpea and field pea grown in a P-deficient Vertisol supplied with 4 and 16 mg P kg^{-1} soil for 9 weeks under ambient (350 ppm) and elevated CO_2 (550 ppm)

Species	P supply (mg P kg^{-1} soil)	$NaHCO_3$-Pi		$NaHCO_3$-Po		NaOH-Pi		NaOH-Po		Total P	
		aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
Chickpea	4	6.92	7.27	1.09	0.74	11.6	12.7	6.1	6.6	118	114
	16	8.38	8.81	1.41	0.78	12.0	12.9	11.3	15.7	134	136
Field pea	4	7.41	6.80	1.23	2.54	13.7	12.8	4.1	4.7	125	120
	16	8.50	8.39	2.22	4.47	13.3	13.0	10.8	26.6	135	149
	LSD (P=0.05) (significance level)										
	CO_2	n.s.		n.s.		n.s.		4.57 (*)		n.s.	
	P	0.66 (***)		n.s.		n.s.		4.57 (***)		19.3 (*)	
	Species	n.s.		1.31 (*)		0.67 (*)		n.s.		n.s.	
	CO_2 × P	n.s.		n.s.		n.s.		6.47 (*)		n.s.	
	P × Species	n.s.		n.s.		n.s.		n.s.		n.s.	
	CO_2 × Species	n.s.		n.s.		0.95 (*)		n.s.		n.s.	
	CO_2 × P × Species	n.s.		n.s.		n.s.		n.s.		n.s.	

n.s., * and *** indicate $P>0.05$, $P<0.05$ and $P<0.001$, respectively

overcome the deficiency, before the shoot growth could respond to the eCO_2. The maximum response to eCO_2 occurred at the highest rate (16 mg Pkg^{-1}) while no response to eCO_2 was observed when no P was added (Fig. 1), resulting in a highly significant P × CO_2 interaction for shoot growth (Table 1). This finding is consistent with previous studies on pine seedlings (*Pinus radiata* D. Don) and strawberry (*Fragaria virginiana* R.), where responses to eCO_2 were more pronounced under P adequate conditions than when P was deficient (Conroy et al. 1990; Whitehead et al. 1997). This result appears due to the effect of P deficiency on photosynthesis, a key physiological process underpinning plant responses to eCO_2 (Conroy et al. 1992; Sinclair 1992; BassiriRad et al. 2001).

Root growth was enhanced by eCO_2, but biomass allocation to the root was not affected by CO_2 treatment in this study. The root biomass and total root length of both legume species increased significantly under eCO_2, irrespective of P treatments (Fig. 1, Table 1). Thus, there was no P × CO_2 interaction for root growth. Other work has reported similar root responses to eCO_2. Fitter et al. (1996) found that *Festuca ovina* and *Nardus stricta* had increases of 41 % and 48 %, respectively, in root dry weight in response to elevated CO_2. Rogers et al. (1992) demonstrated that CO_2 enrichment significantly increased the root mass, length and diameter of soybean roots. Similarly, Berntson and Woodward (1992) showed that eCO_2 resulted in longer roots and increased root branching in *Senecio vulgaris*. Thus, increased root growth is a widespread response to eCO_2 resulting from increased photosynthate supply to the roots (Pritchard and Rogers 2000; Laby et al. 2000). Although there was increased root mass and length under eCO_2, there was no effect on carbon partitioning between shoots and roots, as the root-to-shoot ratio did not change under eCO_2 (Fig. 1). Furthermore, there was no effect of eCO_2 on the distribution of roots in the soil profile (Fig. 4). Thus, the effect of eCO_2 concentration in this study stimulated overall root growth without affecting the allocation of photosynthate between roots and shoots, or between shallower and deeper roots. Other studies that examined shoot and root growth under eCO_2 reported different results. For example, root-to-shoot ratios increased under eCO_2 in carrots and radish (Rogers et al. 1983, 1996), and corn (Idso et al. 1988). It is possible that species differences in the C-sink strength in the roots are responsible for these differences (Niu et al. 2011).

Nodulation and N uptake

The increase in total N uptake and total nodule biomass under eCO_2 were the consequence of the increased biomass of the host plant, rather than specific effects on the components of symbiotic N_2

fixation. This can be seen from the direct linear relationship between N uptake and total plant dry weight (data not shown), which was unaffected by eCO_2. Similarly, eCO_2 had no effect the linear relationship between total nodule number and N uptake (data not shown). Studies on *Glycine max* showed a similar result, in that CO_2 enrichment did not influence specific nodule formation or nodule activity (Finn and Brun 1982). However, the N_2-fixing activity in nodules significantly increased under eCO_2 in other species such as alfalfa (Bertrand et al. 2007), mungbean (Srivastava et al. 2002), acacia (Schortemeyer et al. 2002) and *Ormosia macrocalyx* (Cernusak et al. 2011). These inconsistencies between studies may be attributed to (1) differences in the duration of eCO_2 exposure that enable the N_2-fixing capacity to be up-regulated (Srivastava et al. 2002); and/or (2) species variation in the nodule: root mass ratio determining the capacity to up-regulate N_2 fixation under eCO_2 (Cernusak et al. 2011); and/or (3) differences in rhizobial population affecting the N_2 fixation to respond to extra photosynthate supply under eCO_2 (West et al. 2005; Haase et al. 2007).

In contrast to eCO_2, the addition of P to soil enhanced nodule formation and nodule development in the two legumes species. Similar results were also found in *Stylosanthes humilis* and *Trifolium subterraneum* (Robson et al. 1981; Gates 1974). Although increased P supply markedly increased total amount of N per plant, in parallel to increase in plant biomass, it decreased N concentration and N/P concentration ratio in the plant. Since the soil used in the experiment had an extremely low N concentration, the majority of N in the plant would have been derived from N_2 fixation. Thus N_2 fixation in the legumes was not inhibited by the P deficiency.

The importance of P supply for nodule formation and development has been highlighted in other studies. For example, nodule number and size in soybeans under P deficiency were only 9 % and 34 % of that under sufficient P addition (Israel 1987). This effect of P supply on nodule formation is probably because P supply affects the production of root-exudates including flavonoids that trigger *nod*-gene expression to form nodules, and also plays a role in nodule cell metabolism that affects nodule development (Raghothama et al. 1999; Abel et al. 2002).

Although P supply increased nodulation in the legumes, it did not affect the functioning or the N_2-fixing capacity of the nodules. Reports in the literature on the effect of P on nodule function are inconsistent. Cassman et al. (1980) observed that increased P supply enhanced nodule function in *Stylosanthes humilis*, *Glycine max* and *Medicago truncatula* whereas Robson et al. (1981) found no effect of P supply on the N_2-fixing capacity of nodules on the roots of *Trifolium subterraneum*. The discrepancy could be due to different P requirements for N_2 fixation between species, as P supply in the nodule can regulate nitrogenase activity via ATP-dependent reactions (Sa and Israel 1991), and this regulation may differ between species.

P uptake by root system and its availability in rhizosphere

Elevated CO_2 increased P uptake by both legumes when sufficient P was supplied (Fig. 1), indicating that the P demand under eCO_2 increased significantly. This increase in total P uptake appeared to result from increased biomass production under eCO_2, rather than from any enhanced ability of the roots to acquire soil P (Table 2). This can be seen by the fact that the linear relationships between total root length and total P uptake were not affected by eCO_2 (data not shown). In addition, the P uptake per unit of root length or per unit of root surface area did not differ between eCO_2 and aCO_2 (data not shown), and the P concentration in the two legumes studied did not increase under eCO_2 (Table 2). Similar findings have been reported in other studies where there was a decrease or no change of P concentration in wheat (Wolf 1996; Fangmeier et al. 1999), *Eucalyptus grandis* (Conroy et al. 1992), *Calluna vulgaris* (Whitehead et al. 1997), *Lolium perenne* (Gentile et al. 2011) or *Agrostis capillaries* (Newbery et al. 1995), although eCO_2 did increase foliar P concentration of *Bouteloua eriopoda* (BassiriRad et al. 1997). Genetic differences in nutrient acquisition in response to eCO_2 may explain the discrepancy, because the *Bouteloua* species was observed to have a stronger root absorption capacity for nutrient uptake than other species (BassiriRad et al. 1997). Although P demand increased with the biomass response to eCO_2 in this study, we could not define the critical level of external and internal P concentrations, because maximum growth was not reached even at the highest P supply. Further research will be required to quantify the critical P concentrations in these species under eCO_2.

Although eCO_2 did not affect the P uptake capacity of the roots, it did alter P fractions in the rhizosphere of both legumes species. The effect was to increase the

NaOH-extractable Po pool size in the rhizosphere (Table 3). This fraction contains a range of organic P compounds such as phosphate monoesters, phosphate diesters and phosphonate, which are derived from soil microbes and organic matter (Beck and Sanchez 1994; Turner et al. 2007). As these compounds can potentially be mineralized into labile Pi, they are considered to be the moderately labile P. On the other hand, eCO_2 did not increase the $NaHCO_3$-extractable Pi or Po pools, irrespective of P application (Table 3), suggesting that there was a net flux of Pi into the NaOH-extractable Po pool. The fact that the NaOH-extractable Po pool size was greater when 16 mg P kg^{-1} was applied compared with 4 mg P kg^{-1} supports this view. Immobilization of Pi by soil microbes in the rhizosphere and the formation of moderately stable Po compounds would explain this observation.

There are a number of possible mechanisms whereby eCO_2 could increase the NaOH-extractable Po pool in the rhizosphere. The first is that root exudates could be increased under eCO_2 and this would enhance the activity of microorganisms in the rhizosphere (Richardson 2001; de Graaff et al. 2006). Increased root exudation could have a priming effect on soil organic matter decomposition, and transfer more complex organic P to the NaOH-extractable Po pool (Fontaine et al. 2004). In addition, the increased microbial activity would also enable microbes to compete for labile Pi forms and increase the microbial P pool size that is extractable in NaOH (Binkley et al. 2000; Achat et al. 2010; Richardson and Simpson 2011).

Species differences on P and N uptake

There were marked differences between the two legumes in their ability to take up P from the Vertisol. It was proposed that chickpea would be more efficient in P uptake than field pea, because chickpea has a larger root system (Gerke et al. 2000) and releases more P-mobilizing root exudates than field pea (Nuruzzaman et al. 2005, 2006). However, in this study, field pea was able to accumulate more P in shoots and roots than chickpea. Despite the smaller root system of field pea (Fig. 1), P uptake per unit root length was greater than chickpea irrespective of CO_2 treatments. Furthermore, P concentrations in the roots and shoots of field pea were higher than in chickpea, irrespective of CO_2 or P supply (Table 2). The soil $NaHCO_3$-extractable Po and NaOH extractable Pi concentrations in the rhizosphere were also higher with field pea than chickpea, indicating that the field pea roots could potentially mobilize more stable soil P pools into labile P. The explanation for the higher P acquisition efficiency of field pea may be due to its finer root system. Field pea roots had smaller diameters than chickpea roots (0.35 and 0.51 mm for field pea and chickpea, respectively). The field pea, therefore, produces more roots with lower tissue construction costs in energy and carbon, and this is likely to enable them to explore the soil with a lower metabolic investment, enabling the plant to take up P more efficiently (Lynch 2011).

The two legumes also differed in their ability to accumulate N in both shoots and roots. Nitrogen accumulation was greater for field pea, indicating a more efficient N_2-fixing symbiosis. It had smaller roots, fewer nodules and smaller nodules than chickpea, resulting in lower nodule biomass (Fig. 2). However, N concentration in plants, total N uptake and the N uptake per unit nodule mass were greater in field pea than chickpea (Table 2). Rennie and Dubetz (1986) also confirmed that field pea nodules were more efficient in N_2 fixation than chickpea when field pea was inoculated with *Rhizobium leguminosarum* strains of 175F1, 17SF2, 175F5 and 175F8, and chickpea with 27A2,27A7 and 27A9. The basis for this superior capacity of field pea requires further investigation.

Conclusion

Phosphorus addition is required for the two grain legume species studied to overcome P deficiency before the shoot growth could respond to the eCO_2. Elevated CO_2 increased P demand by both of these legumes and the resulting increase in P uptake under eCO_2 resulted from increased biomass rather than any enhanced P acquisition capacity in the roots. The study could not establish critical concentrations of P for plant growth and nodulation under eCO_2 because the maximum growth was not achieved at the highest level of P supply. When P is supplied under eCO_2, the increase in the size of the root system would enhance exploration of the soil for P, and nodulation which also benefits N uptake and consequent plant growth. However, the specific uptake of P and N by roots and nodules was not influenced by eCO_2. In the rhizosphere, eCO_2 increased the moderately labile Po pool, indicating an increase of microbial P immobilization.

Acknowledgment We thank Dr. Clayton Butterly and anonymous reviewers for reviewing the manuscript. This research was supported by an Australian Research Council *Linkage Project* (LP100200757), and utilised the SOILFACE facilities at DPI Horsham which were developed with funding by the Victorian Department of Primary Industries (VDPI), the University of Melbourne (UM), the Grains Research and Development Corporation (GRDC), the Federal Department of Agriculture, Fisheries and Forestry (DAFF) and the Australian Greenhouse Office (AGO).

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