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

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## 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 Vertosol with P addition varying from 0 to 16 mg P $kg^{-1}$.
***Results*:** Increasing P supply increased plant growth and total P uptake but not P concentration in shoot and root, 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. At 16 mg P $kg^{-1}$, nodule biomass had 46% greater response to $eCO_2$ than $aCO_2$, but no significant response in no-P treatments. Total P uptake was correlated with root length while N uptake correlated with nodule number and biomass regardless of $CO_2$ level. Elevated $CO_2$ did not alter nodule density and N-to-P ratio in plant. Elevated $CO_2$ did not change $NaHCO_3$-extractable inorganic P in the rhizosphere, while it, together with 16 mg P $kg^{-1}$ applied, increased the NaOH-extractable organic P by 92%. Field pea roots had higher P uptake per unit root length and N uptake per unit nodule biomass than chickpea.
***Conclusion:*** The increase in P uptake, nodule number and N uptake under $eCO_2$ resulted from the increased biomass production, rather than from changes in specific root-absorbing capability and specific nodule function. Elevated $CO_2$ appears to increase P immobilized by microorganisms in the rhizosphere.

***Key words*:** Free Air $CO_2$ Enrichment, FACE, $N_2$ fixation, nodulation, P acquisition, P fractions, rhizosphere

## 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. 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 when P supplied (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 meet their physiological requirements for increased biomass. Furthermore, P requirements may become even greater for $N_2$ fixing legumes (Israel, 1987; Bordeleau and Prevost, 1994), as P is required for nodule function and nodule development (Qiao et al., 2007). 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; Cambell 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).

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 Vertosol 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). 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. The two $CO_2$ levels were 1) ambient $CO_2$ (350 ppm) and 2) elevated $CO_2$ (550 ppm). Two grain legumes 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 P $kg^{-1}$ soil. The soil (Vertosol) was collected at a depth of approximately 10 to 30 cm from a roadside near Horsham, Victoria, Australia. 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 Soil FACE 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 1460 ml for each column during the experimental period.

*Measurements*

After 9 weeks of growth in the Soil FACE, 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 (Maschner 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, 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 $eCO_2$ ($P < 0.001$), and differed between the species with field pea producing a greater biomass than chickpea (Figure 1, 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 P $kg^{-1}$, and 32% with 16 mg P $kg^{-1}$, resulting in a significant P × $CO_2$ interaction ($P < 0.001$). There was also a significant P × Species interaction ($P < 0.05$), 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 (Figure 1B). There was no significant $CO_2$ × Species interaction ($P > 0.05$), indicating that the species did not difer in their response to $eCO_2$ (Figure 1C), nor was there any significant $CO_2$ × P × Species interaction (data not shown).

*Root growth and biomass allocation*

The response of root biomass depended on P supply, $CO_2$ and species. In contrast to the shoot biomass, chickpea had significantly greater root biomass than field pea with the difference being greater at high P than at no or low P ($P < 0.001$) (Table 1; Figure 1D). 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 (Figure 1F). Unlike the effect on shoots, there was no significant ($P > 0.05$) $CO_2$ × P interaction for roots (Table 1).

The root-to-shoot ratio markedly declined as P supply increased ($P < 0.001$), 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; Figure 1E). A significant P × Species interaction was found ($P < 0.001$), 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 ($P > 0.05$).

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 ($P < 0.001$). Compared to $aCO_2$, $eCO_2$ increased average root length by 14% for chickpea and by 12% for field pea ($P < 0.001$) (data not presented). However, there were no significant interactive effects on root length between any two treatments ($P > 0.05$) (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 ($P < 0.001$) but did not affect nodule size (single nodule mass) or N uptake per unit nodule biomass ($P > 0.05$) (Figure 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 ($P > 0.05$) (Figure 2G). 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 (Figure 2F).

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 ($P < 0.01$). 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 (Figure 2A). Similarly, the response in nodule biomass to $eCO_2$ by chickpea was 35 mg $plant^{-1}$, compared 5 mg $plant^{-1}$ by field pea (Figure 2C).

There were significant P × Species interactions on nodule biomass ($P < 0.001$), number ($P < 0.05$) and size ($P < 0.001$), nodule per unit root biomass ($P < 0.05$) and N uptake per unit nodule biomass ($P < 0.001$). 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 $kg^{-1}$ (Figure 2B, D, E and H). In contrast, with increasing P supply, N uptake per unit nodule biomass decreased more in field pea than in chickpea (Figure 2F).

Irrespective of $CO_2$ and P treatments, total N uptake was significantly correlated with nodule number ($P < 0.05$), nodule biomass ($P < 0.01$) and total biomass production ($P < 0.01$) for both species (Figure 3).

*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 ($P < 0.05$). Elevated $CO_2$, however, did not significantly affect the distribution of root biomass ($P > 0.05$) (Figure 4A). There was no significant $CO_2$ × P interaction on the distribution of root biomass for either species ($P > 0.05$).

The relative proportion of root length located in the top 10 cm of soil tended to decrease as the rate of P application increased ($P < 0.01$) 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 (Figure 4B). In general, chickpea had longer roots distributed deeper in the soil than field pea.

Increasing P application significantly decreased nodule number in the 0-10 cm of soil depth ($P < 0.05$) (Figure 4C). Elevated $CO_2$ did not affect the nodule distribution ($P > 0.05$). 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% ($P < 0.01$), but had no effect in field pea ($P > 0.05$). There was no P × $CO_2$ interaction on P concentration ($P > 0.05$).

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 ($P < 0.05$), with total P uptake increasing more under $eCO_2$ than $aCO_2$ as P application increased. Total P uptake correlated positively with root length ($P < 0.05$) and root biomass ($P < 0.05$) of both species (data not shown).

*Plant N concentration and uptake*

Increasing P application generally decreased N concentration in shoots ($P < 0.001$) 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 increase being greater under $eCO_2$ than under $aCO_2$, resulting in a significant $CO_2$ × P interaction ($P < 0.05$). The basis for this was 9% increase in N uptake with nil P, compared to the 24% with 16 mg P $kg^{-1}$. There was also a significant $CO_2$ × Species interaction ($P < 0.05$) 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 ($P < 0.001$) as the rate of P application increased, but it was not affected by $eCO_2$ (Figure 2I). 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 $CO_2 \times P$ interaction ($P < 0.05$). 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 $kg^{-1}$ (Table 3). Species differences included higher concentrations of $NaHCO_3$-Po and NaOH-Pi in the rhizosphere of field pea, compared with chickpea ($P < 0.05$). Increased P application and $CO_2$ concentration did not change the HCl-P or residual-P fractions in rhizosphere with averages of 15.9 and 83 mg P $kg^{-1}$, respectively (data not shown).

## Discussion

### *Plant growth*

The two $N_2$-fixing grain legumes grown in the P-deficient Vertosol soil required P addition to 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 P $kg^{-1}$) while no response to $eCO_2$ was observed when no P was added (Figure 1), and this resulted in the highly significant $P \times CO_2$ interaction for shoot growth (Table 1). This finding is consistent with the conclusion of BassiriRad et al. (2001) that the growth response of plants to $eCO_2$ depends on an adequate nutrient supply in soil, because deficiencies of N and P will limit photosynthesis, which is a key physiological process underpinning plant responses to $eCO_2$ (Conroy et al., 1992; Sinclair, 1992). Studies on non-legume species such as pine seedlings (*Pinus radiata* D. Don) and strawberry (*Fragaria virginiana* R.) also showed that the responses to $eCO_2$ were more pronounced under P-sufficient conditions than P-deficient conditions (Conroy et al., 1990; Whitehead et al., 1997).

Elevated $CO_2$ stimulated root growth in this study. The root biomass and total root length of both legume species increased significantly under $eCO_2$, irrespective of P treatments (Figure 1, Table 1). Thus, there was no $P \times CO_2$ interaction for root growth. Other work has reported similar root response 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, research on *Senecio vulgaris* by Berntson and Woodward (1992) showed that $eCO_2$ resulted in longer roots and increased root branching. 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 $eCO_2$ (Figure 1). Furthermore, there was no effect of $eCO_2$ on the distribution of roots in the soil profile (Figure 4). Thus, the effect of elevated $CO_2$ 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, 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*

Elevated $CO_2$ had no specific effect on any of the components of symbiotic $N_2$ fixation in this study. Increasing $CO_2$ concentration did not increase nodule density, nodule size or N uptake per unit nodule biomass, for either chickpea or field pea, regardless of the P treatment. Instead, the increase in total N uptake and total nodule biomass under $eCO_2$ were the consequence of the increased biomass of the host plant. This can be seen from the direct linear relationship between N uptake and total plant dry weight, which was unaffected by $eCO_2$. Similarly, $eCO_2$ had no effect the linear relationship between total nodule number and N uptake (Figure 3). 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 authors attributed the enhanced nodule function to increased nitrogenase activity under $eCO_2$. Several possibilities may explain these inconsistencies. For example, the duration of the exposure to $eCO_2$ varied from 16 days with soybean (Finn and Brun, 1982), to more than 50 days with acacia and mungbean (Schortemeyer et al., 2002; Srivastava et al., 2002); short-term exposure may not allow enough time for the $N_2$-fixing capacity to be up-regulated by the $eCO_2$. Another possibility was raised by Cernusak et al. (2011) suggesting that legume species with a greater nodule: root mass ratio such as *Ormosia macrocalyx,* have a greater capacity to up-regulate $N_2$ fixation under $eCO_2$, than those with little ratio like *Schizolobium parahyba*. The third possibility could be that the non-responding legume lacked the efficient bacterial symbiont, preventing 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. Not only did greater P supply increase the total number and biomass of nodules per plant (Figure 2) but increased P supply also increased nodule density, nodule size and nodule biomass per unit root biomass. 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 extremely low concentrations of C and N, 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 that occurred in the nil P treatment.

The importance of P supply for nodule formation and development has been highlighted in other studies where nodulation was restricted under P deficiency. For example, nodule number and size in soybeans under P deficiency were only 9% and 34% of that under sufficient P addition (Israel, 1987). Qiao et al. (2007) also reported that P deficiency impaired nodule development in soybean. 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 $CO_2$ resulted in increased P uptake by both legumes when sufficient P was supplied (Figure 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 $eCO_2$ (Figure 3). 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 exudation of sugars and organic acids 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 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). One additional mechanism might be that increased growth of mycorrhizal hyphae occurred in the rhizosphere under $eCO_2$ and this contributed to the increase in size of the NaOH-Po pool. Mycorrhizal hyphae have been estimated to contain

1000 mg P $kg^{-1}$ of dry matter (Hagerberg et al., 2005), and this microbial component in soil has been linked with NaOH-extractable P fractions (Khan et al., 2008). However, given that high concentrations of labile Pi in soil tend to suppress mycorrhizal infection and biomass in many plants (Stribley et al., 1980), this mechanism is less likely to have been responsible for the increase in the NaOH-Po pool size.

*Species differences on P and N uptake*

There were marked differences between the two legumes in their ability to take up P from the Vertosol. Initially, 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 (Figure 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 and Ho, 2005; 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 (Figure 2). However, compared with chickpea, N concentration in shoots and roots, and total N uptake were greater in field pea (Table 2). Furthermore, the N uptake per unit nodule mass was greater in field pea (Figure 2), suggesting that field pea nodules have a greater $N_2$-fixation capacity. In other words, each functional nodule in field pea can fix more $N_2$ than chickpea. Rennie and Dubetz (1986) also confirmed that field pea nodules were more efficient in $N_2$ fixation than chickpea when both legumes were inoculated with *Rhizobium leguminosarum*. Thus, it indicates that field pea could have an inheritent advantage on $N_2$ fixation compared to chickpea. The basis for this superior capacity requires further investigation.

**Conclusion**

The growth response of grain legumes to $eCO_2$ depended on soil P supply. Elevated $CO_2$ increased P demand by both legumes and the resulting increase in P uptake under $eCO_2$ resulted from increased greater 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.

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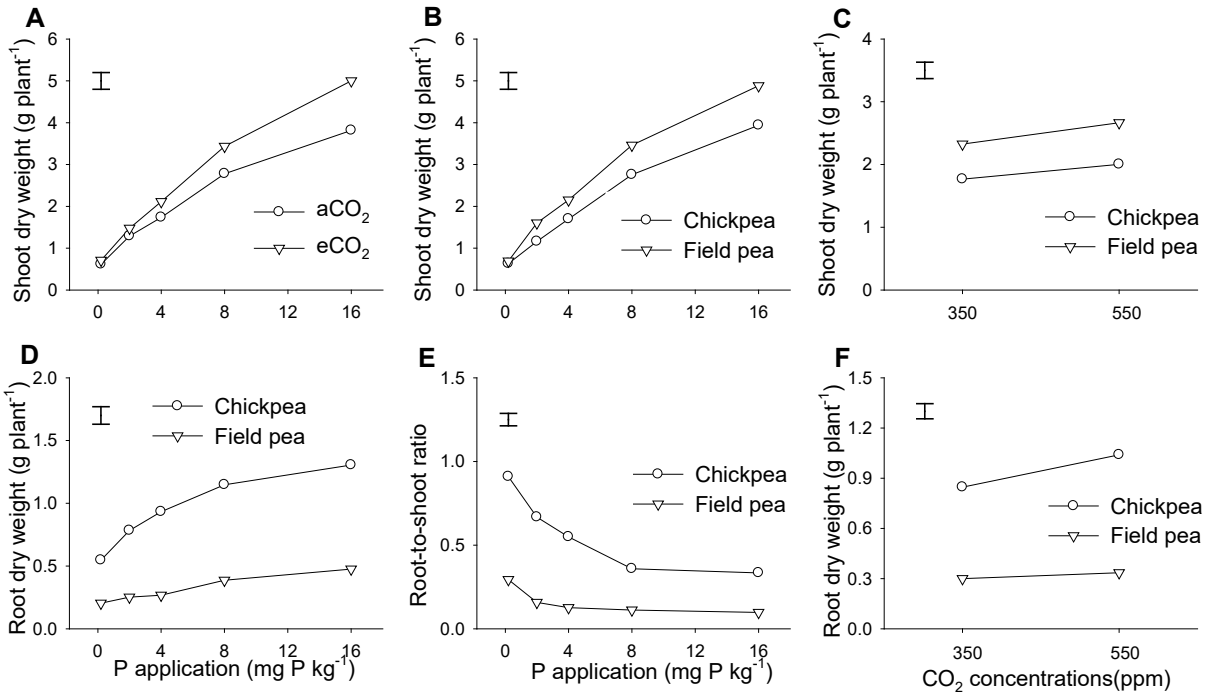


**Figure 1.** The effects of $CO_2$, P and species on shoot biomass (A, B and C), root biomass (D, F) and root-to-shoot ratio (E) of field pea and chickpea after plants were exposed to $eCO_2$ for 9 weeks in a P-deficient Vertosol supplied with 0 to 16 mg P $kg^{-1}$ soil. The vertical bar in each panel indicates the LSD ($P = 0.05$) for the $CO_2$ × P, P × species, or $CO_2$ × species interaction. The interactive effects were significant at $P < 0.05$, except for shoot dry weight.

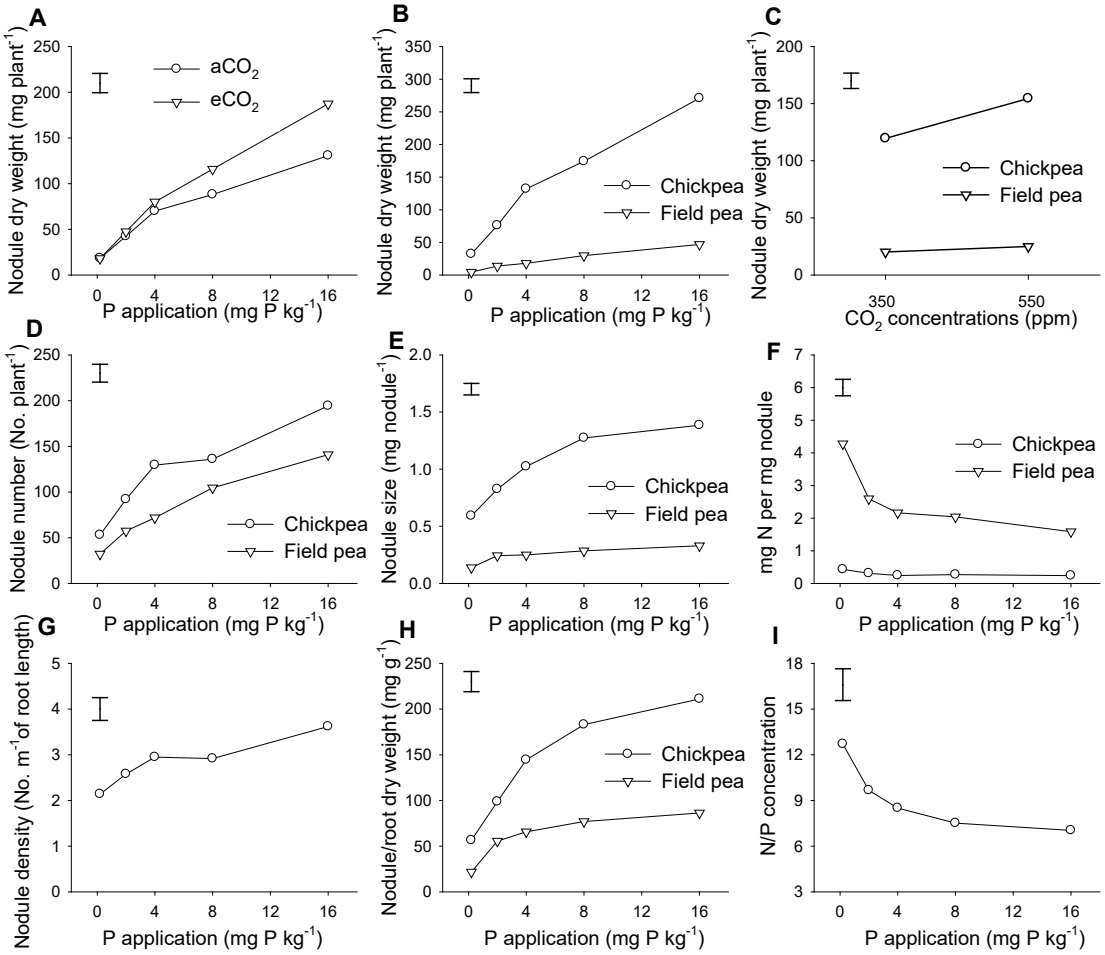


**Figure 2.** The effects of $CO_2$ × P (A), P × species (B), and $CO_2$ × species (C) on nodule biomass, the interaction of P × species on nodule number (D), nodule size (E), N uptake per mg nodule (F), the effect of P application on nodule density (G), the interaction of P × species on nodule/root dry weight (H), and the effect of P application on N/P concentration (I) after legumes were exposed to $eCO_2$ for 9 weeks in a P-deficient Vertosol 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, P × species, or $CO_2$ × species interaction.

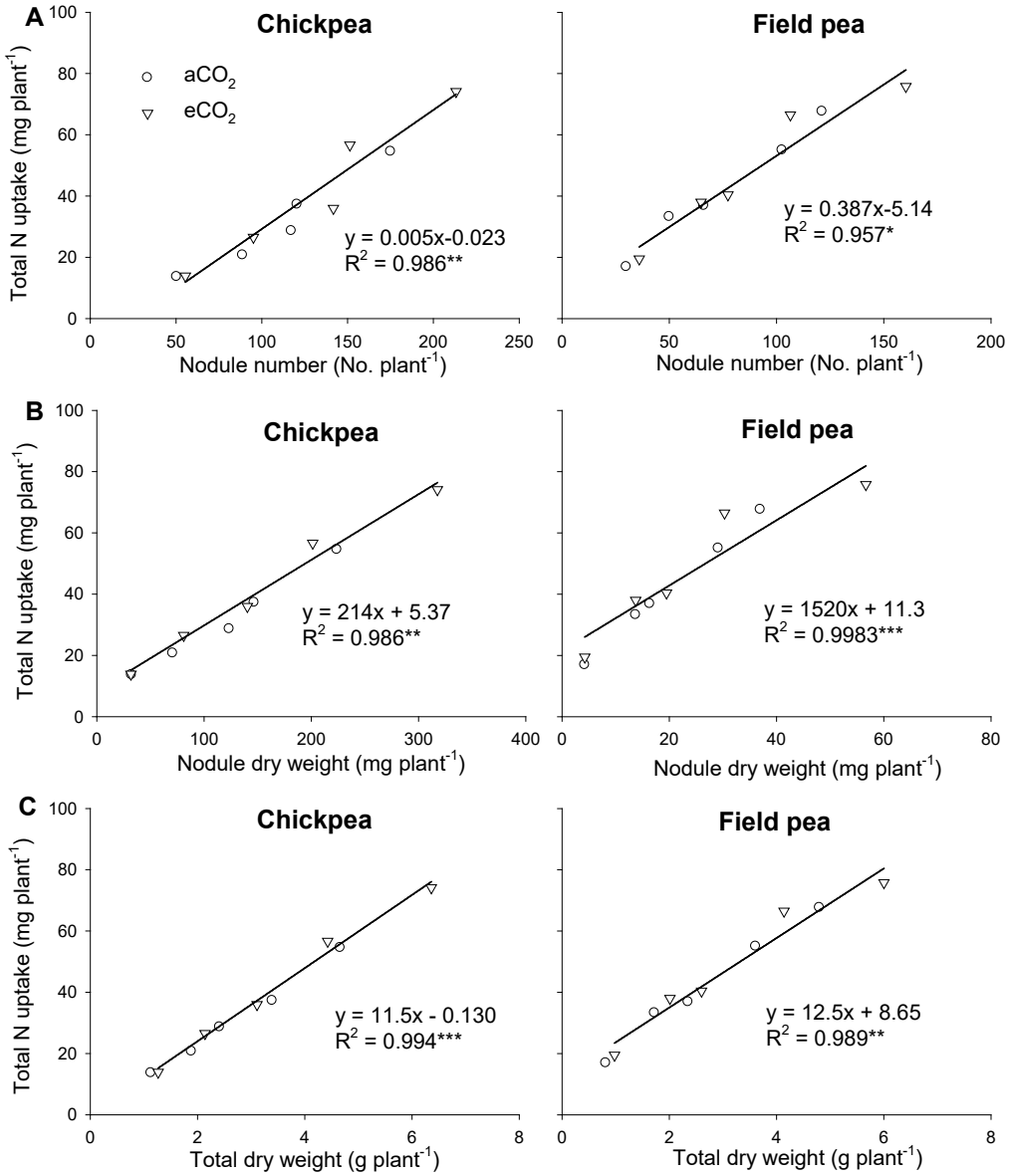


**Figure 3.** Relations of nodule number (A), nodule dry weight (B), and total plant dry weight (C) with total N content in chickpea and field pea supplied with 0-16 mg P $kg^{-1}$ soil under ambient (350 ppm) and elevated $CO_2$ (550 ppm). *, ** and *** indicate significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

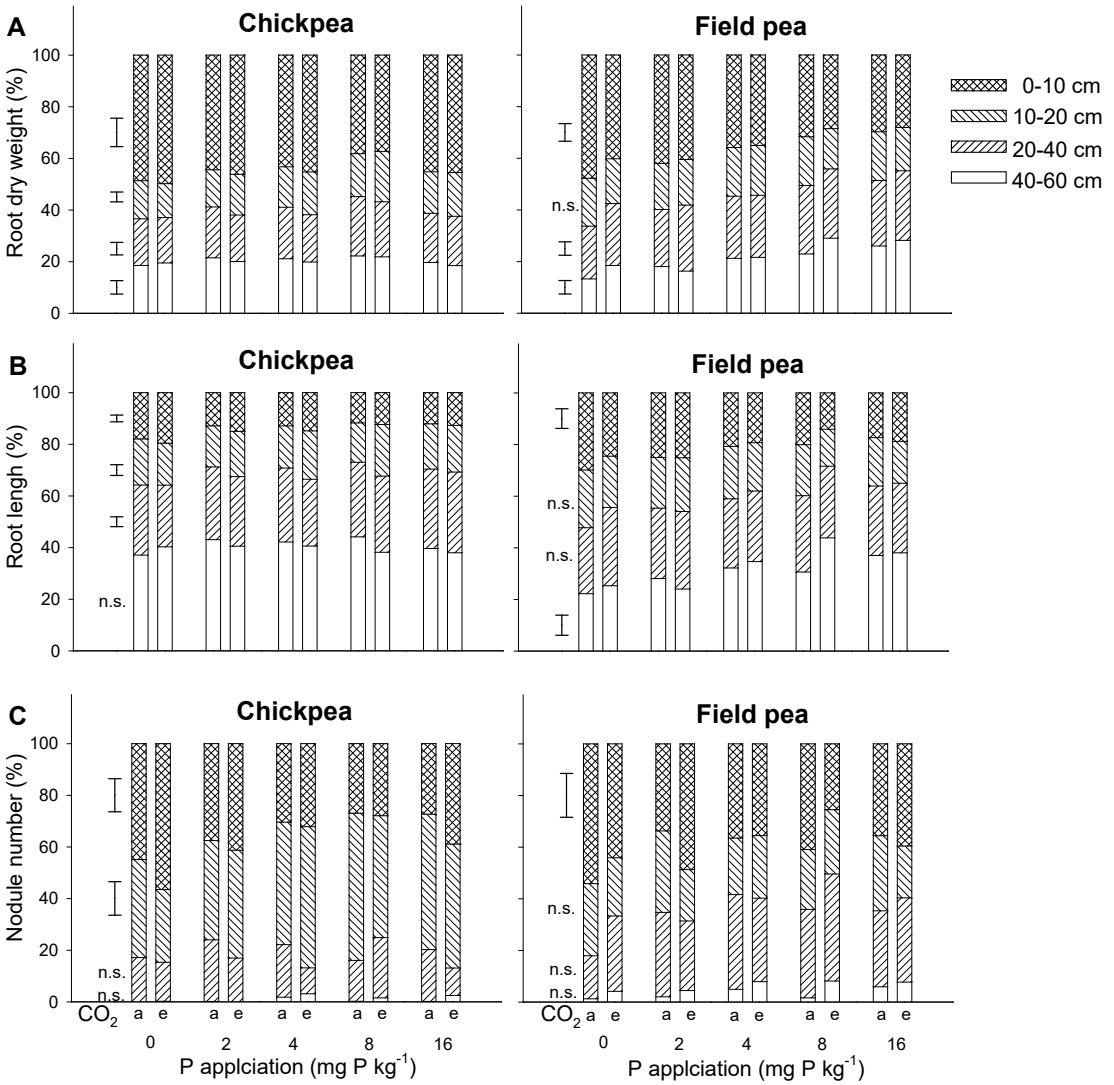


**Figure 4.** The distribution patterns at various soil depth of root biomass (A), root length (B)and nodule number (C) of chickpea and field pea grown for 9 weeks in a Vertosol supplied with 0-16 mg P $kg^{-1}$ soil under ambient (a) (350 ppm) and elevated $CO_2$ (e) (550 ppm). The vertical bars in 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 $P < 0.05$.

**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 number, dry weight and size, and N uptake per unit nodule mass.

| Factors | Shoot DW | Root DW | Root length | R/S | Nodule No. | Nodule DW | Nodule size | N uptake per mg of nodule |
|---|---|---|---|---|---|---|---|---|
| $CO_2$ | < 0.001 | < 0.001 | < 0.001 | 0.606 | < 0.001 | < 0.001 | 0.143 | 0.953 |
| P | < 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 | < 0.001 |
| $CO_2$×P | < 0.001 | 0.461 | 0.463 | 0.954 | 0.103 | 0.015 | 0.611 | 0.740 |
| $CO_2$× Species | 0.440 | 0.007 | 0.179 | 0.937 | 0.454 | 0.007 | 0.201 | 0.864 |
| P× Species | 0.029 | < 0.001 | 0.096 | < 0.001 | 0.020 | < 0.001 | < 0.001 | < 0.001 |

Data less than 0.05, 0.001 and more than 0.05 indicate $P < 0.05$, $P < 0.001$ and no significance, respectively.

**Table 2.** The concentrations of N and P in shoots, roots and nodules of chickpea and field pea grown for 9 weeks in a Vertosol 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 N (mg $g^{-1}$) | Root N (mg $g^{-1}$) | Total N (mg $plant^{-1}$) | | Shoot P (mg $g^{-1}$) | | Root P (mg $g^{-1}$) | | Total P (mg/plant) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ |
| Chickpea | 0 | 12.9 | - | - | - | 0.92 | | 0.79 | | 1.18 | |
| | 2 | 12.3 | - | - | - | 1.29 | | 0.90 | | 2.63 | |
| | 4 | 10.8 | - | - | - | 1.30 | | 0.98 | | 3.93 | |
| | 8 | 10.3 | - | - | - | 1.36 | | 1.20 | | 5.86 | |
| | 16 | 10.2 | - | - | - | 1.38 | | 1.27 | | 8.32 | |
| | Mean | 11.3 | 8.90 | 31.0 | 48.4 | 1.32 | 1.17 | 1.03 | | 4.38 | |
| Field pea | 0 | 20.8 | - | - | - | 1.850 | | 1.23 | | 1.51 | |
| | 2 | 18.7 | - | - | - | 1.952 | | 1.42 | | 3.52 | |
| | 4 | 15.1 | - | - | - | 1.892 | | 1.46 | | 4.65 | |
| | 8 | 14.1 | - | - | - | 1.928 | | 1.43 | | 7.40 | |
| | 16 | 12.9 | - | - | - | 1.946 | | 1.42 | | 10.4 | |
| | Mean | 16.3 | 18.3 | 41.9 | 48.1 | 1.921 | 1.907 | 1.39 | | 5.50 | |
| Across species | 0 | - | - | 15.3 | 19.5 | - | - | - | - | 1.27 | 1.42 |
| | 2 | - | - | 27.0 | 32.4 | - | - | - | - | 2.90 | 3.25 |
| | 4 | - | - | 32.7 | 38.2 | - | - | - | - | 3.97 | 4.60 |
| | 8 | - | - | 46.2 | 61.6 | - | - | - | - | 5.95 | 7.31 |
| | 16 | - | - | 61.1 | 75.0 | - | - | - | - | 8.06 | 10.7 |
| | Mean | - | - | 36.5 | 48.3 | - | - | 1.25 | 1.17 | 4.43 | 5.45 |
| | *LSD (P=0.05) (significance level)* | | | | | | | | | | |
| | $CO_2$ | n.s. | n.s. | 2.92 (***) | | 0.05 (***) | | 0.05 (**) | | 0.33 (***) | |
| | P | 1.01 (***) | n.s. | 4.62 (***) | | 0.07 (***) | | 0.08 (***) | | 0.52 (***) | |
| | Species | 0.63 (***) | 0.42 (***) | 2.93 (***) | | 0.05 (***) | | 0.05 (***) | | 0.33 (***) | |
| | $CO_2$×P | n.s. | n.s. | 6.54 (*) | | n.s. | | n.s. | | 0.73 (***) | |
| | $CO_2$×Species | n.s. | n.s. | 4.14 (*) | | 0.06 (**) | | n.s. | | n.s. | |
| | P×Species | 1.42 (***) | n.s. | n.s. | | 0.10 (***) | | 0.11 (***) | | 0.73 (**) | |

*, **, *** and n.s. indicate $P < 0.05$, $P < 0.01$, $P < 0.001$ and no significance, respectively.

**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 Vertosol supplied with 4 and 16 mg P $kg^{-1}$ soil for 9 weeks under ambient (350 ppm) and elevated $CO_2$ (550 ppm).

| | | $NaHCO_3$-Pi | $NaHCO_3$-Po | NaOH-Pi | | NaOH-Po | | Total P |
|---|---|---|---|---|---|---|---|---|
| P rate (mg P $kg^{-1}$) | 4 | 7.1 | - | - | | 5.4 | | 125 |
| | 16 | 8.6 | - | - | | 16.1 | | 139 |
| $CO_2$ | $aCO_2$ | - | - | - | | 8.1 | | |
| | $eCO_2$ | - | - | - | | 13.4 | | |
| Species | Chickpea | - | 1.01 | 12.3 | | - | | |
| | Field pea | - | 2.62 | 13.2 | | - | | |
| | | | | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | |
| P rate (mg P $kg^{-1}$) | 4 | - | - | - | - | 5.1 | 5.6 | |
| | 16 | - | - | - | - | 11.1 | 21.2 | |
| Species | Chickpea | - | - | 11.8 | 12.8 | - | - | |
| | Field pea | - | - | 13.5 | 12.9 | - | - | |
| | *LSD (P=0.05) (significance level)* | | | | | | | |
| | $CO_2$ | n.s. | n.s. | n.s. | | 4.57 (*) | | |
| | P | 0.66 (***) | n.s. | n.s. | | 4.57 (***) | | 19.3 (*) |
| | Species | n.s. | 1.31 (*) | 0.67 (*) | | n.s. | | |
| | $CO_2$×P | n.s. | n.s. | n.s. | | 6.47 (*) | | |
| | P× Species | n.s. | n.s. | n.s. | | n.s. | | |
| | $CO_2$× Species | n.s. | n.s. | 0.95 (*) | | n.s. | | |

*, *** and n.s. indicate $P < 0.05$, $P < 0.001$ and no significance, respectively.