

PART OF A SPECIAL ISSUE ON PLANTS AND CLIMATE CHANGE

Phosphorus application and elevated CO₂ enhance drought tolerance in field pea grown in a phosphorus-deficient vertisol

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Received: 24 April 2014 Returned for revision: 1 September 2014 Accepted: 12 September 2014 Published electronically: 26 November 2014

- **Background and Aims** Benefits to crop productivity arising from increasing CO₂ fertilization may be offset by detrimental effects of global climate change, such as an increasing frequency of drought. Phosphorus (P) nutrition plays an important role in crop responses to water stress, but how elevated CO₂ (eCO₂) and P nutrition interact, especially in legumes, is unclear. This study aimed to elucidate whether P supply improves plant drought tolerance under eCO₂.
- **Methods** A soil-column experiment was conducted in a free air CO₂ enrichment (SoilFACE) system. Field pea (*Pisum sativum*) was grown in a P-deficient vertisol, supplied with 15 mg P kg⁻¹ (deficient) or 60 mg P kg⁻¹ (adequate for crop growth) and exposed to ambient CO₂ (aCO₂; 380–400 ppm) or eCO₂ (550–580 ppm). Drought treatments commenced at flowering. Measurements were taken of soil and leaf water content, photosynthesis, stomatal conductance, total soluble sugars and inorganic P content (Pi).
- **Key Results** Water-use efficiency was greatest under eCO₂ when the plants were supplied with adequate P compared with other treatments irrespective of drought treatment. Elevated CO₂ decreased stomatal conductance and transpiration rate, and increased the concentration of soluble sugars and relative water contents in leaves. Adequate P supply increased concentrations of soluble sugars and Pi in drought-stressed plants. Adequate P supply but not eCO₂ increased root length distribution in deeper soil layers.
- **Conclusions** Phosphorus application and eCO₂ interactively enhanced periodic drought tolerance in field pea as a result of decreased stomatal conductance, deeper rooting and high Pi availability for carbon assimilation in leaves.

Key words: Climate change, crop nutrition, drought tolerance, free air CO₂ enrichment, FACE, P nutrition, pea, *Pisum sativum*, root length distribution, stomatal conductance, water-use efficiency.

INTRODUCTION

In the scenario of climate change, drought may become more frequent, intensive and erratic in some regions (Robredo *et al.*, 2007; Allen *et al.*, 2010). In Western Australia, for example, rainfall has decreased by 15–20 % compared with the 1970s (Petroni *et al.*, 2010), which may be partly driven by anthropogenic climate change (Cai *et al.*, 2005; van Ommen and Morgan, 2010). A consequence of this reduced rainfall will be reduced growth and yield of many dryland crops (Araus *et al.*, 2002; Volaire, 2003).

As a fact of an increasing atmospheric CO₂ concentration during climate change (Calzadilla *et al.*, 2013; Wheeler and von Braun, 2013), elevated CO₂ (eCO₂) has been reported to be able to mitigate the impact of drought stress in many legume species. In soybean, for example, eCO₂ enhanced drought tolerance by lowering stomatal conductance and maintaining photosynthesis at the seed-filling stage (Li *et al.*, 2013). In alfalfa, eCO₂ improved water relations, and thereby enhanced photosynthetic rate and yield by alleviating drought stress (Erice *et al.*, 2006). The increased tolerance to drought under eCO₂ was attributed to changes in concentrations and/or composition of soluble carbohydrates in leaves that mediate osmotic adjustments and plant water potential (ψ_w) (Tyree and

Alexander, 1993; Seneweera *et al.*, 2001; Allen *et al.*, 2011). This physiological strategy enables plants to reduce stomatal and canopy conductance, and lower soil water consumption.

Applying phosphorus (P) to P-deficient soils reportedly stimulates growth responses to eCO₂, particularly for legumes (Edwards *et al.*, 2005; Jin *et al.*, 2012, 2013), and plays an important role in drought tolerance (Graciano *et al.*, 2005). The question arises as to whether P application could improve drought tolerance under eCO₂. Increasing P supply has been shown to improve the tolerance of white clover and soybean to dry soil conditions (Singh *et al.*, 2000; Jin *et al.*, 2006). The reasons for this improved tolerance include increasing root hydraulic conductivity, maintaining leaf water potential (Radin and Eidenbock, 1984; Singh *et al.*, 1997) and increasing root access to more soil water in deep soil layers (Jin *et al.*, 2005). Increasing P application is also likely to enhance the synthesis of the osmotically active carbohydrates in the leaf cells responsible for maintenance of leaf water potential under drought conditions because inorganic P (Pi) plays a key role in translocation of triose sugars out of chloroplasts (Abel *et al.*, 2002; Rychter and Rao, 2005; Lambers *et al.*, 2006). These assumptions on how P supply mediates the effect of

eCO₂ on drought tolerance, however, need to be experimentally tested.

This study aimed to elucidate whether increasing P supply in P-limiting soil and/or eCO₂ would affect drought tolerance of the legume species field pea via changes in water-use efficiency, leaf water relations and altered root growth in soil profiles. We hypothesized that increasing P supply would enhance the tolerance of field pea to soil water stress (drought) and this tolerance would be greater under eCO₂ than under ambient CO₂ (aCO₂), due to stimulations in root growth, increasing carbohydrate synthesis and maintenance of a higher relative water content (RWC). Furthermore, eCO₂ would enhance drought tolerance by reducing stomatal conductance and maintaining leaf water status. We anticipate that optimizing P application may become one strategy that helps in minimizing the impact of water stress in future eCO₂ climates.

MATERIALS AND METHODS

Experimental design and plant growth

The experiment had a split-plot design with CO₂ as the main plot, and P application and drought as sub-plot treatments. Ambient CO₂ (380–400 ppm) and eCO₂ (550–580 ppm) levels were achieved using the free air CO₂ enrichment (SoilFACE) facility in Horsham, Victoria, Australia (36° 42'S, 142° 11'E) (Mollah *et al.*, 2011). There were four FACE rings (four replicates) for each CO₂ concentration. Phosphorus was applied as KH₂PO₄ at two rates: 15 (P15) and 60 mg P kg⁻¹ (P60) mixed evenly throughout the soil. These P application rates were designed to provide deficient and adequate P nutrition, respectively, of the field pea. Field pea (*Pisum sativum* 'OzP0601') was used as the test species. Two soil water treatments, well-watered and periodic drought, commenced at the initial flowering stage (lasting for 21 d). The P and water treatments were replicated in all of eCO₂ and aCO₂ plots.

Soil was collected near the SoilFACE site (36° 42'S, 142° 11'E). The soil type used is known as Vertisol (FAO–UNESCO, 1976). Relevant soil properties are as follows: organic C of 7.8 mg g⁻¹ (Rayment and Higginson, 1992), 2 M KCl-extractable NO₃-N of 4.2 mg kg⁻¹ and NH₄-N of 1.0 mg kg⁻¹, total P of 114 mg kg⁻¹, Colwell P of 5 mg kg⁻¹ (Colwell, 1963) and a pH (1:5 in 0.01 M CaCl₂) of 7.7. This Colwell P level is considered to be severely deficient for the growth of crops (Richardson *et al.*, 2009). After air-drying and sieving through a 4 mm sieve, the soil was mixed with siliceous sand (w/w = 1:1) to facilitate root washing at harvest. Each column (60 cm long, 15 cm in diameter) contained 13 kg of experimental soil in total, and the soil was mixed with the following basal nutrients (mg kg⁻¹): K₂SO₄, 147; MgSO₄·7H₂O, 122; CaCl₂, 186; CuSO₄·5H₂O, 6; ZnSO₄·7H₂O, 8; MnSO₄·5H₂O, 6; FeCl₃, 0.6; CoCl₂, 0.4; NaMoO₄·2H₂O, 0.4; and NaB₄O₇, 1.6, and the required amount of P for each treatment.

Eight uniform germinated seeds were sown in each column and inoculated with rhizobia (Group E[®] *Rhizobium leguminosarum* for field pea) on 15 June 2012. All columns were allocated into underground bunkers that have been built in the SoilFACE. The seedlings were thinned to four plants per column 3 weeks after sowing (at the V1 stage). Temperatures, radiation and rainfall during the experimental period are shown

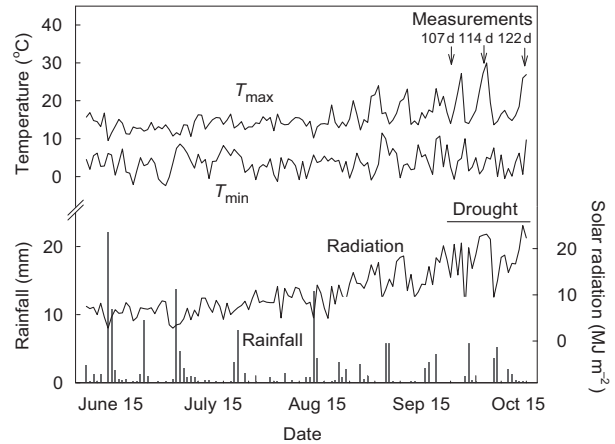


Fig. 1. Daily rainfall, solar radiation, and minimal (T_{\min}) and maximal (T_{\max}) temperatures during the experimental period from 15 June to 15 October 2012 near the experimental site. Three rounds of measurement on water status were at the initial-phase drought (63–70 % FWC) (Day 107 after sowing), mid-phase drought (52–57 % FWC) (Day 114) and final-phase drought (43–46 % FWC) (Day 122), respectively.

in Fig. 1. These meteorological data were obtained from Horsham Airport, located 6.6 km away from the SoilFACE site. Drought stress was imposed at the initial flowering stage (102 d after sowing). During the drought treatment, all columns were covered just above the soil surface to exclude rainfall. Watering was withheld in the drought treatments until the soil reached 43 % of field water capacity (FWC), the permanent wilting point (which occurred after 3 weeks). The well-watered treatments were maintained at 80 % of FWC by weighing and watering every 2 d throughout the experimental period. The total amount of water applied for each treatment was recorded.

Measurements

Soil water content was recorded every 2 or 3 d during the period of drought treatment both by weighing the columns (to measure water loss) and via a Theta probe (ML2X, DELTA-T DEVICES, Cambridge, UK). Parameters on water status were determined when the soil water content in the drought treatment had dropped to 66 % (63–70 %, $n=16$) (initial-phase drought, Day 107 after sowing), 55 % (52–57 %) (mid-phase drought, Day 114) and 45 % (43–46 %) of FWC (final-phase drought, Day 122) as showed in Fig. 1. Using a portable photosynthesis system (Li-Cor, Lincoln, NE, USA), stomatal conductance (g_s), transpiration rate (E) and photosynthetic rate (P_n) were measured on the second or third youngest fully expanded leaves through the drought treatment period. Measurements were taken between 0900 and 1200 h on days with full sunlight and a temperature of 22–25 °C. Measurements were performed in duplicate on two plants in each column and from replicate to replicate across the treatments. This procedure was performed throughout the three phases during the period of drought treatment. The conditions inside the leaf chamber such as photosynthetically active radiation and reference CO₂ concentration were hold constant across

all samples. Instantaneous transpiration efficiency (ITE) was calculated by dividing P_n by E (Robredo *et al.*, 2007).

Immediately after measuring photosynthesis, the same leaves in each replicate were then sampled and used for measurement of RWC, total soluble sugars (TSS) and Pi. The samples for TSS and Pi measurements were weighed, frozen in liquid nitrogen and stored in a -80 °C freezer for later measurements. For RWC analysis (Conroy *et al.*, 1988), the fresh leaves were weighed and floated on distilled water for 4 h at 25 °C under full sunlight. The turgid weight of these leaves was then recorded, and the dry weight was determined after drying at 70 °C for 72 h. The RWC was then calculated using the following formula:

$$\text{RWC} = \left[\frac{(\text{fresh weight} - \text{dry weight})}{(\text{turgid weight} - \text{dry weight})} \right] \times 100.$$

Samples for TSS analysis were crushed in 95 % (v/v) ethanol. The insoluble fraction of the extract was washed twice with 70 % ethanol, followed by 10 min of centrifugation at 3500 g to collect soluble fractions. An aliquot of 0.1 mL of combined supernatants was reacted with 3 mL of freshly prepared anthrone [150 mg of anthrone + 100 mL of 72 % (w/w) H₂SO₄] and put in a boiling water bath for 10 min. After cooling, the absorbance at 625 nm was determined with a spectrophotometer (Irigoyen *et al.*, 1992).

For the measurement of leaf Pi concentration, frozen leaves were ground in distilled water, before centrifuging at 5000 g for 10 min. The supernatant was boiled at 100 °C for 7 min, and filtered through a 0.45 µm filter to remove debris (Mimura *et al.*, 1996). The concentrations of Pi in the extract were colorimetrically measured using malachite green (Motomizu *et al.*, 1980).

At the final harvest (123 d after sowing), shoots were removed at ground level, washed with 0.1 M HCl and then rinsed twice in deionized water to remove any adhering dust. Each soil column was opened vertically and was separated into three soil layers, i.e. 0–20, 20–40 and 40–60 cm. Roots in each layer were recovered by carefully sieving with a 2 mm sieve. The root system was rinsed with tap water, and then soaked in 0.01 M CaCl₂ solution for 5 min to desorb nutrients on the root surface. Root length was determined using the WinRhizo Pro version 2003b program (Régent Instruments Inc., Québec, Canada).

Roots and shoots were dried at 70 °C for 72 h, weighed and then ground. Sub-samples of 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 the digests were colorimetrically measured using malachite green.

The stress tolerance index (STI) was calculated as

$$\text{STI} = (\text{DW}_w \times \text{DW}_s) / \overline{\text{DW}}_w^2$$

where DW_w and DW_s were the dry weights under well-watered and drought conditions, respectively, and $\overline{\text{DW}}_w$ was the mean dry weight under well-watered conditions (Fernandez, 1992).

Plant water-use efficiency (WUE) was estimated as total dry weight divided by water use, where water use equals the amounts of rain plus water added to a column plus the difference in total column water mass between the beginning and end of the experiment (Jones *et al.*, 2005).

Statistical analysis

All data were analysed using GenStat 10. Analysis of variance (ANOVA) tests were undertaken and least significant difference (LSD) calculated to assess the differences between treatment means (Steel and Torrie, 1980). The data for leaf water status, plant biomass, root morphology, concentrations of sugars and Pi in leaves, plant P concentrations and total P uptake were statistically analysed by factorial ANOVA to determine the effects of P, CO₂ and drought (42–45 % of FWC), and their interactions.

RESULTS

Plant growth

Drought markedly decreased shoot dry weight (by 31 %), but the drought-stressed plants exhibited the same response to CO₂ and P application of shoot dry weight as did well-watered plants, resulting in an insignificant CO₂ × P × drought interaction ($P > 0.05$) (Table 1). However, under drought conditions, eCO₂ and adequate supply of P resulted in significantly greater shoot dry weight (11.5 g plant⁻¹) than aCO₂ + P15. Both P application and eCO₂ significantly increased shoot dry weight (Tables 1 and 2). Increasing the P application rate from 15 to 60 mg kg⁻¹ increased shoot dry weight from 8.5 to 12.8 g plant⁻¹ (51 % increase) under aCO₂ (Fig. 2A). When the plants were grown under eCO₂, there was a further 9 and 17 % increase in shoot dry weight under P15 and P60, respectively,

TABLE 1. Significant levels of main effects and interactions of CO₂, P and drought

Factors	CO ₂	P	Drought	CO ₂ × P	CO ₂ × drought	P × drought
Shoot dry weight	***	***	***	*	n.s.	n.s.
Root dry weight	***	***	*	n.s.	n.s.	**
Water-use efficiency	***	***	***	*	**	n.s.
Stress tolerance index	***	***	—	**	—	—
Nodule number	***	***	***	**	n.s.	*
Nodule dry weight	***	***	***	*	n.s.	n.s.
N concentration	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N uptake	***	***	***	***	n.s.	n.s.
Shoot P concentration	n.s.	***	***	n.s.	n.s.	*
Root P concentration	**	***	**	n.s.	n.s.	n.s.
P uptake	***	***	***	***	n.s.	n.s.
Stomatal conductance	***	***	***	n.s.	***	***
Transpiration efficiency	***	***	n.s.	n.s.	*	**
Relative water content	**	n.s.	***	n.s.	***	n.s.
Total soluble sugars	***	*	***	n.s.	***	n.s.
Leaf Pi	n.s.	**	***	n.s.	n.s.	n.s.

The CO₂ × P × drought interaction was not significant for any of the measurements.

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

TABLE 2. Average responses (%) to main treatments relative to the corresponding controls at the final measurements/harvest

Factors	eCO ₂	P60	Drought
Shoot dry weight	22	59	-31
Leaf area	21	61	-36
Root dry weight	26	26	-9
Water-use efficiency	30	33	21
Nodule number	39	133	-48
Nodule dry weight	27	106	-39
N concentration	4	2	2
N uptake	21	70	-13
Shoot P concentration	-3	61	16
Root P concentration	-6	40	7
P uptake	22	145	-17
Stomatal conductance	-26	-20	-87
Transpiration efficiency	34	32	5
Relative water content	5	-2	-21
Total soluble sugars	5	20	60
Leaf Pi	-6	27	36

leading to a significant CO₂ × P interaction ($P < 0.05$) (Table 1). Similarly, eCO₂ and increasing P application increased leaf area by 21 and 61 %, respectively, while drought decreased leaf area by 36 % (Table 2).

Similar to the effects on shoot dry weight, eCO₂ and P60 also increased root dry weight compared with their respective controls under both well-watered and drought conditions, but no significant CO₂ × P × drought interaction was observed ($P > 0.05$). Elevated CO₂ increased root dry weight by an average of 26 % (Table 2). Increasing P supply also increased root dry weight by an average of 26 %; this increase was most pronounced in deeper soil layers (45 %) than in the topsoil (10 % increase) (Fig. 2B; Table 2).

Water-use efficiency and stress tolerance index

On average, P60 and eCO₂ increased WUE by 33 and 30 %, respectively (Table 2). Elevated CO₂ increased WUE by 33 % at P60 supply, whereas the response to eCO₂ was only 26 % when P was deficient (Fig. 2C). This contributed to a significant CO₂ × P interaction ($P < 0.05$) (Table 1).

Alleviating P deficiency increased the STI by 123 %. This trend was greater when plants were grown under eCO₂, resulting in a 175 % increase (Fig. 3). A significant CO₂ × P interaction effect ($P < 0.01$) was observed for the STI (Table 1).

Spatial root length in soil profiles

Drought decreased overall root length, but did not interact with P supply or CO₂ treatment. Although there was no significant effect of P × CO₂ interaction on root length, each treatment alone had a significant effect. Plants had greater root length in soil profiles supplied with P60 than in those supplied with P15 (Fig. 4A). Compared with P15, P60 resulted in a 43 % increase in the root length in the bottom soil layer, but only a 17 % increase in the top soil layer. Elevated CO₂ significantly increased root length regardless of P and drought treatments. Compared with P15, P60 significantly increased the distribution of root length in the bottom soil layer, resulting in an increase from 47 % at P15 to 53 % at P60 (Fig. 4B). However, eCO₂

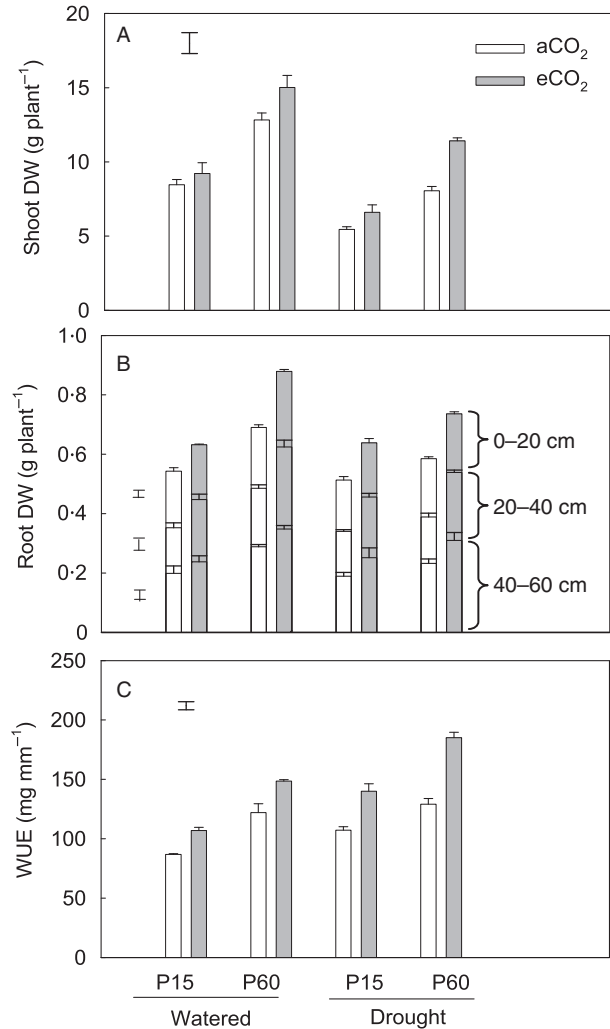


Fig. 2. The effects of CO₂, P and water regime on dry weight (DW) of shoots (A) and roots in the 0–20, 20–40 and 40–60 cm soil layers (B), and water-use efficiency (WUE) (C) of field pea. Plants were exposed to ambient (aCO₂) or elevated CO₂ (eCO₂) treatments for 123 d in a P-deficient vertisol soil supplied with 15 (P15) or 60 mg P kg⁻¹ (P60) soil, and drought-stressed plants had water withheld until the soil reached the permanent wilting point in the last 3 weeks of the experiment. Columns are means of four replicates ± s.e. The vertical bars indicate the the LSD ($P = 0.05$).

and drought did not affect the root length distribution within the soil profile.

Nodulation and N uptake

On average, drought decreased the nodule number by 48 % irrespective of P or CO₂ treatments. Plants formed 133 % more nodules at P60 than at P15 (Table 2). The effect of CO₂ concentration on nodulation depended on P supply; eCO₂ increased nodule number by 21 % at P15 but increased it by 48 % at adequate P supply compared with aCO₂ (Fig. 5A; Table 1). Nodule dry weight exhibited the same trend as nodule number (Fig. 5B; Table 2).

The concentration of N in shoot was not affected by P or CO₂ treatment and reached 23 mg g⁻¹ (Fig. 5C) which is in

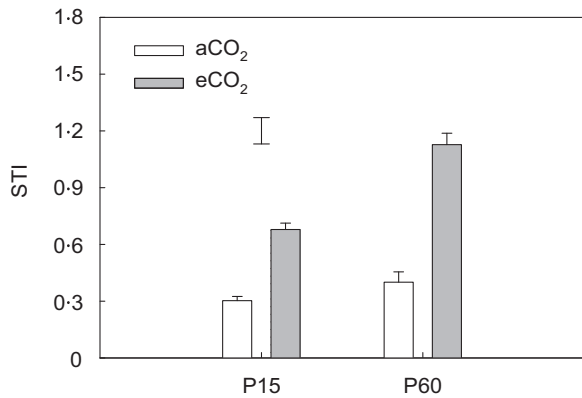


FIG. 3. The effects of CO₂ and P water regime on the stress tolerance index (STI) of field pea. Plants were exposed to ambient (aCO₂) or elevated CO₂ (eCO₂) treatments for 123 d in a P-deficient vertisol soil supplied with 15 (P15) or 60 mg P kg⁻¹ (P60) soil, and drought-stressed plants had water withheld until the soil reached the permanent wilting point in the last 3 weeks of the experiment. Columns are means of four replicates ± s.e. The vertical bars indicate the the LSD (*P* = 0.05).

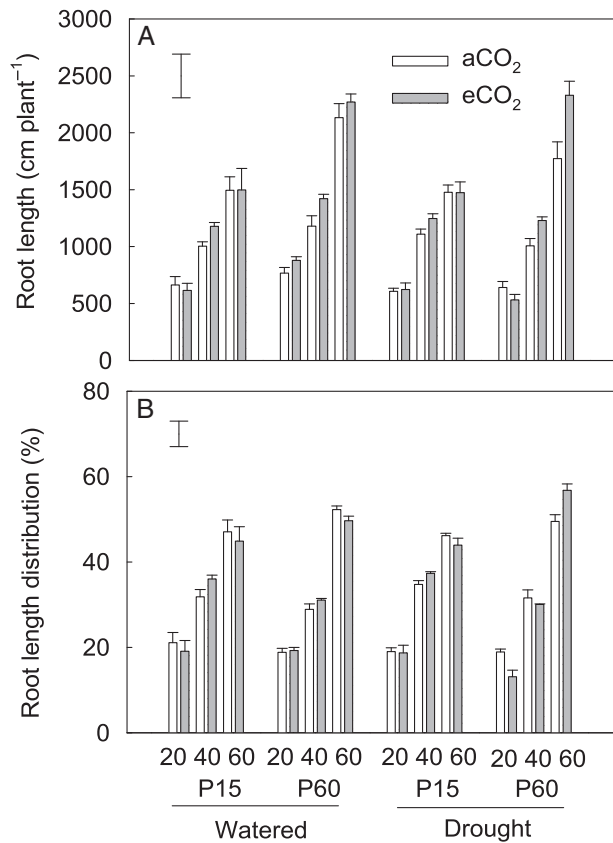


FIG. 4. The effects of CO₂, P and water regime on root length (A) and root length distribution of field pea in the 0–20 cm (20), 20–40 cm (40) and 40–60 cm (60) soil layers (B). Plants were exposed to ambient (aCO₂) or elevated CO₂ (eCO₂) treatments for 123 d in a P-deficient vertisol supplied with 15 (P15) or 60 mg P kg⁻¹ (P60) soil, and drought-stressed plants had water withheld until the soil reached the permanent wilting point in the last 3 weeks of the experiment. Columns are means of four replicates ± s.e. The vertical bars indicate the the LSD (*P* = 0.05).

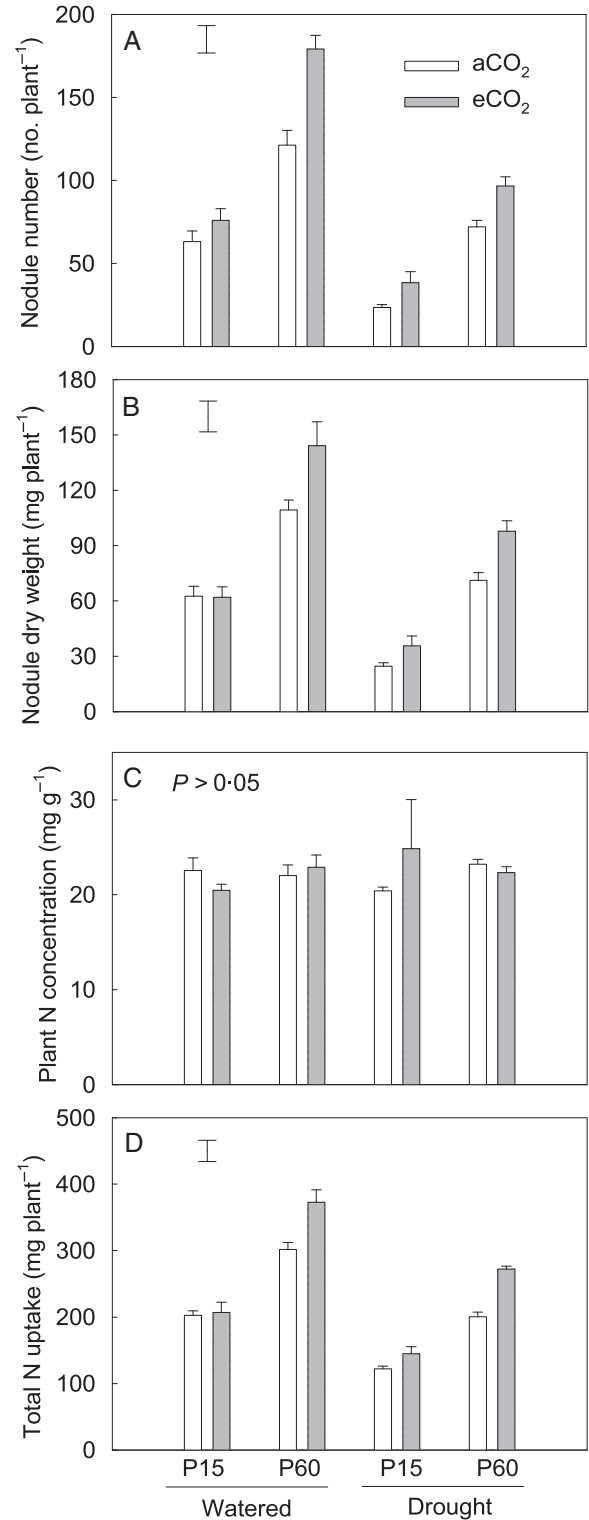


FIG. 5. The effects of CO₂, P and water regime on root nodule number (A), nodule dry weight (B), plant N concentration (C) and total N uptake (d) of field pea. Plants were exposed to ambient (aCO₂) or elevated CO₂ (eCO₂) treatments for 123 d in a P-deficient vertisol supplied with 15 (P15) or 60 mg P kg⁻¹ (P60) soil, and drought-stressed plants had water withheld until the soil reached the permanent wilting point in the last 3 weeks of the experiment. Columns are means of four replicates ± s.e. The vertical bars indicate the the LSD (*P* = 0.05).

the adequate range (Reuter and Robinson, 1997; Deibert and Utter, 2004). Both P supply and eCO₂ increased total N uptake by an average of 70 and 21 %, respectively (Table 2). Elevated CO₂ resulted in greater plant N content when P supply was adequate than when it was deficient (Fig. 5D) ($P < 0.01$) (Table 1).

Plant P status

Drought increased P concentration in shoot by 16 % and in roots by 7 %, but decreased total P uptake by 17 % across P

and CO₂ treatments compared with well-watered plants (Fig. 6; Table 2). Irrespective of drought treatment, increasing P application significantly increased P concentrations in shoot and roots, and also increased total P uptake (Table 2). However, eCO₂ did not affect P concentrations in either shoot or roots under either P or drought treatments (Fig. 6; Table 1). Elevated CO₂ increased total P uptake by 22 %, and this increase was greater at P60 than at P15. Thus, a significant CO₂ × P interaction effect ($P < 0.001$) on total P uptake was observed (Table 1).

Water relations

Both eCO₂ and supplying an adequate level of P decreased stomatal conductance by 26 and 20 %, respectively, compared with their respective controls (Fig. 7A; Table 2), but no CO₂ × P interaction was observed (Table 1). Compared with well-watered treatments, drought decreased stomatal conductance by 9 % during the initial drought phase and by 83–87 % during the mid and final phases. The effect of eCO₂ or increasing P application on stomatal conductance was less in drought than in well-watered plants (Table 1), resulting in a significant CO₂ × drought or P × drought interaction ($P < 0.001$).

Elevated CO₂ significantly increased the ITE across P and drought treatments (Table 2). Increasing P application also increased the ITE at the mid and final phase of drought, with a rise of 28 % on average. Drought did not affect the ITE until the final drought phase (Fig. 7B). The ITE at P60 was higher during the final phase of drought than the respective well-watered control, whereas no difference was observed at P15 (Table 1). Interestingly, the ITE of plants supplied with P60 under eCO₂ reached 9.6 μmol CO₂ mmol⁻¹ H₂O during the final drought phase, which was the highest among all the treatments.

The initial- and mid-phase drought only had a significant effect on RWC when soil P supply was low, resulting in a 6 % decrease compared with the respective well-watered control (Fig. 8A). Compared with the mid-phase drought, the final phase of drought treatment led to a greater decrease in RWC regardless of P treatment. However, this drought-induced decrease in RWC at the final phase was less severe under eCO₂ than that under aCO₂, resulting in a significant CO₂ × drought interaction ($P < 0.001$) (Table 1). There was no CO₂ × P × drought interaction observed ($P > 0.05$).

The imposition of drought treatments initially (phase 1) had no effect on TSS concentration except for the treatment with P60 + eCO₂, where it significantly increased. As the drought continued, however (mid and final phases), the TSS concentration increased significantly, with this increase being greater under P60 and eCO₂ than under other treatments (Fig. 8B; Tables 1 and 2).

Leaf Pi concentration was higher under P60 than under P15, but was not affected by eCO₂ (Fig. 8C; Table 2). Drought at the initial and mid phases increased leaf Pi only when P supply was low, but, as the drought treatment continued (final phase), leaf Pi was significantly greater compared with their respective well-watered controls regardless of P supply. At the final phase of drought, the leaf Pi concentration was 27 % higher at P60 than that at P15. Leaf Pi concentration was positively correlated with TSS under drought conditions ($r = 0.84$, $P < 0.05$).

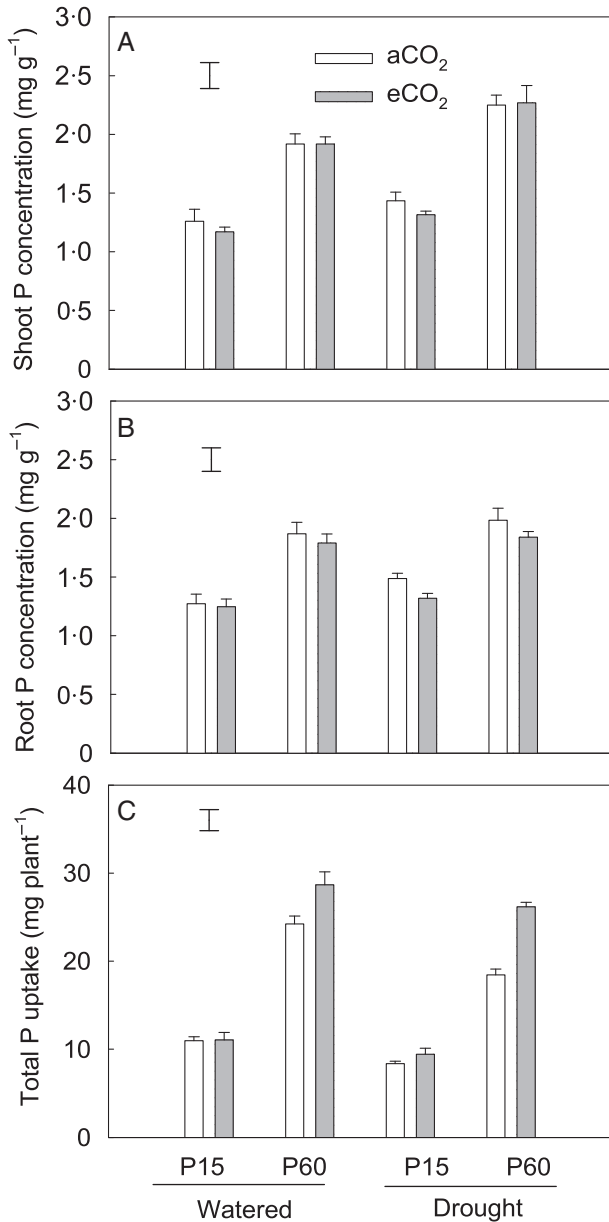


FIG. 6. The effects of CO₂, P and water regime on P concentration in shoot (A) and roots (B), and total P uptake (C) of field pea. Plants were exposed to ambient (aCO₂) or elevated CO₂ (eCO₂) treatments for 123 d in a P-deficient vertisol supplied with 15 (P15) or 60 mg P kg⁻¹ (P60) soil, and drought-stressed plants had water withheld until the soil reached the permanent wilting point in the last 3 weeks of the experiment. Columns are means of four replicates ± s.e. The vertical bars indicate the the LSD ($P = 0.05$).

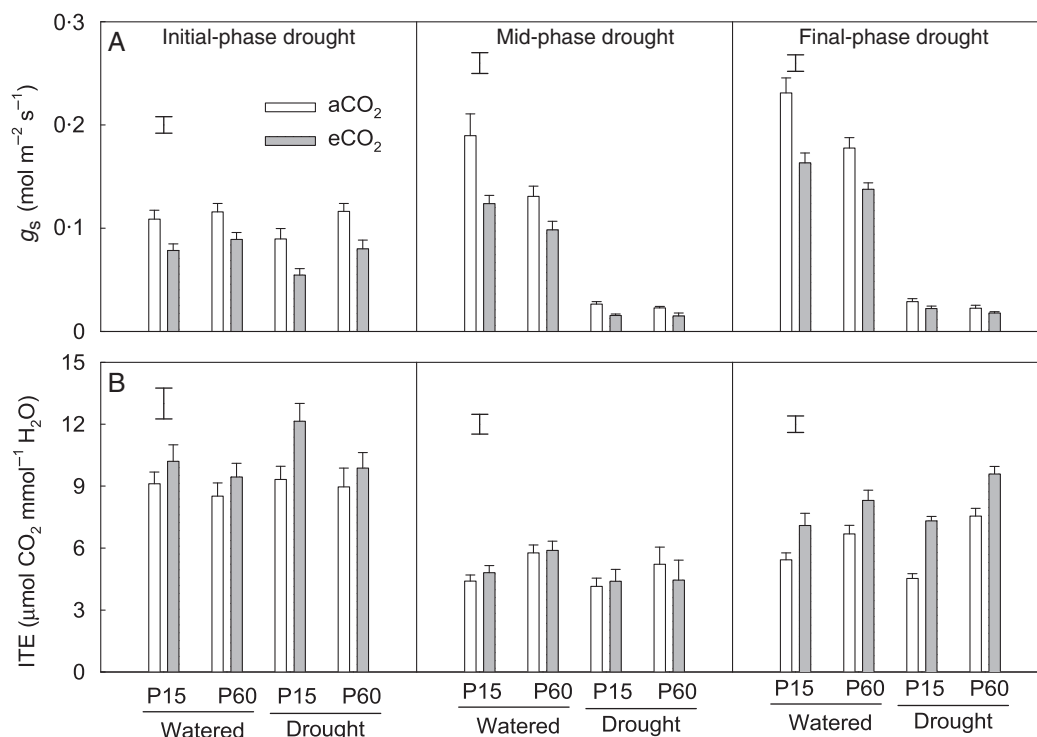


FIG. 7. The effects of CO₂, P and water regime on stomatal conductance (g_s) (A) and instantaneous transpiration efficiency (ITE) (B) of field pea at the flowering stage. Plants were exposed to ambient (aCO₂) or elevated CO₂ (eCO₂) treatments for 123 d in a P-deficient vertisol supplied with 15 (P15) or 60 mg P kg^{-1} (P60) soil, and drought-stressed plants had water withheld to generate 63–70 % of field water capacity (FWC) (initial-phase drought at Day 107), 52–57 % of FWC (mid-phase drought at Day 114) and 43–46 % of FWC (final-phase drought at Day 122) during the last 3 weeks of the experiment. Columns are means of four replicates \pm s.e. The vertical bars indicate the LSD ($P = 0.05$).

DISCUSSION

This study demonstrated for the first time that eCO₂ and P application interactively improved drought tolerance of field pea. This is best exemplified by the finding that the eCO₂-induced increase in the STI was greater in plants grown with a sufficient P supply than in those with an inadequate P supply (Fig. 3). Furthermore, the same trend was found for WUE, where eCO₂ coupled with sufficient P supply resulted in the highest WUE when drought stress was imposed (Fig. 2). Consequently, in future eCO₂ environments, increasing P supply may help to reduce the impact of drought on plant growth, while drought is predicted to occur more frequently in some environments (Allen *et al.*, 2010). Previous studies have shown that changes in either CO₂ or P can alter drought tolerance. For example, soybean, alfalfa and barley can utilize water more efficiently and are more tolerant to drought under eCO₂ than under a CO₂ (Eric *et al.*, 2006; Robredo *et al.*, 2007; Li *et al.*, 2013). Similarly, alleviating P deficiency can also reduce water stress in white clover, soybean and cotton (Radin, 1984; Singh *et al.*, 1997; Jin *et al.*, 2005). Shen *et al.* (2013) also stated that appropriately manipulating P supply can enhance plant growth, nutrient uptake and the ability to resist various stresses, including water deficit.

The greater drought tolerance exhibited by field pea under eCO₂ in this current experiment appears to be due to decreased stomatal conductance and associated reductions in water loss via transpiration which consequently increased instantaneous

transpiration efficiency under drought stress (Fig. 7). The net effect was to increase RWC in the canopy of field pea (Fig. 8A). Many studies also found that the enhanced tolerance of plants to drought under eCO₂ is consistent with a lower stomatal conductance and lower transpiration rate (Bunce, 1998; Morgan *et al.*, 2004; Robredo *et al.*, 2007). This reduction of stomatal conductance was the consequence of partial closure of the stomata, which was probably attributable to increased intercellular CO₂ concentration (C_i) under eCO₂ (Robredo *et al.*, 2007). In this present study, even though the leaf area was greater under eCO₂ than aCO₂ (data not shown), the greater stomatal closure and the resulting lower transpiration rate led to a greater conservation of soil water as observed in other FACE studies (Manderscheid *et al.*, 2014), and subsequently greater plant adaptability to soil water deficit.

Furthermore, in dry soils, eCO₂ favoured an accumulation of soluble sugars in leaf cells (Fig. 8B), which in turn contributed to the flux of water into the leaf cells to maintain cell volume during drought (Seneweera *et al.*, 2001; Spardouli and Moustakas, 2012). The increased ITE under eCO₂ (Fig. 7B) can explain the higher sugar accumulation in drought-stressed plants, indicating that the increased C_i by eCO₂ enabled plants to assimilate more C during photosynthesis whilst using less water and thus minimizing drought-induced stress. It has also been suggested that the greater availability of sugars under eCO₂ lowers osmotic potential at full turgor, allowing osmotic adjustment (Wullschlegel and Norby, 2001) and thereby

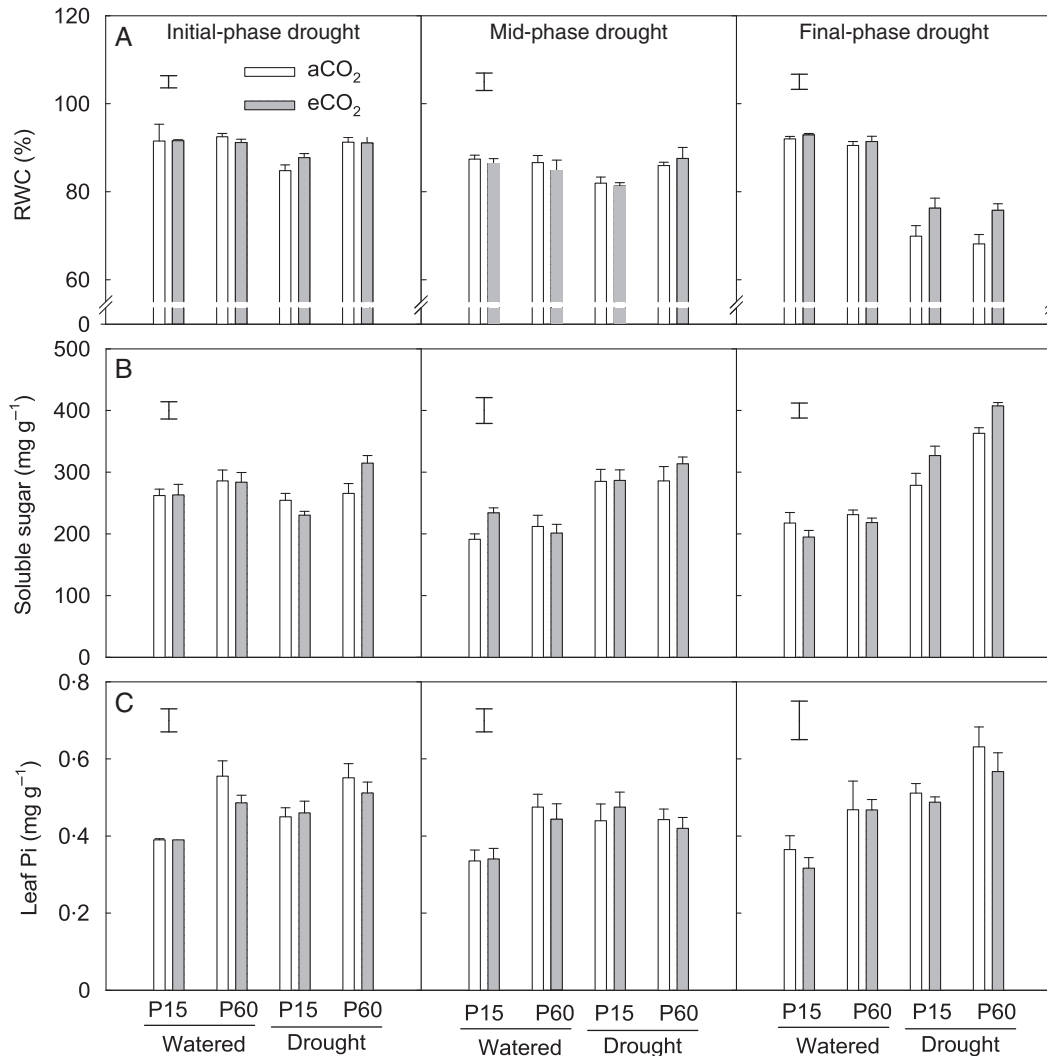


FIG. 8. The effects of CO₂, P and water regime on relative water content of leaf (RWC) (A), and concentrations of total soluble sugars (B) and inorganic P (Pi) (C) in leaves of field pea at the flowering stage. Plants were exposed to ambient (aCO₂) or elevated CO₂ (eCO₂) treatments for 123 d in a P-deficient vertisol supplied with 15 (P15) or 60 mg P kg⁻¹ (P60) soil, and drought-stressed plants had water withheld to generate 63–70 % of field water capacity (FWC) (initial-phase drought at Day 107), 52–57 % of FWC (mid-phase drought at Day 114) and 43–46 % of FWC (final-phase drought at Day 122) during the last 3 weeks of the experiment. Columns are means of four replicates \pm s.e. The vertical bars indicate the LSD ($P=0.05$).

maintaining a high ψ_w (Tyree and Alexander, 1993) and RWC (Fig. 8A).

The beneficial effect of increasing P application on improving the tolerance of field pea to drought stress was greater at eCO₂ than at aCO₂. Increasing P application improved the water status during severe drought, resulting from the significant increase of TSS (Fig. 8B). This change appeared to result from the higher concentrations of leaf Pi that were recorded under sufficient P supply facilitating the accumulation of soluble sugars in leaves under eCO₂ in the final phases of drought (Fig. 8C). Since low soil moisture inhibits P diffusion in soil through increasing the tortuosity (Barber and Wiley, 1995) as well as in plant tissues, the high leaf Pi would help to maintain energy-metabolic processes whilst the plant experienced temporary water stress (Peuke and Rennenberg, 2004). The significant relationship between Pi and TSS under drought conditions ($r=0.84$, $P<0.05$) indicates that the high Pi facilitated the

translocation of triose sugars from the chloroplast, thereby enhancing the sugar status of plant tissue (Abel *et al.*, 2002; Rychter and Rao, 2005; Lambers *et al.*, 2006). Increasing P application in eCO₂ environments is likely to enhance this positive effect further. Thus, large numbers of osmotically active molecules were synthesized (Wahid and Close, 2007; Farooq *et al.*, 2009), which improved osmotic adjustment and maintained turgor under drought stress (Graciano *et al.*, 2005). Therefore, the reduction of stomatal conductance and the increase of ITE under sufficient P supply and eCO₂ (Fig. 7B) slowed water depletion and enhanced WUE.

The improved drought tolerance under eCO₂ at adequate P supply was partly attributable to increased rooting depth. Although eCO₂ did not alter the root distribution in soil profiles, it significantly increased root biomass (Fig. 2B) and length (Fig. 4A). Increasing P application led to both an overall increase in root growth and a greater proportion of these roots

being distributed in deep soil layers, so that eCO₂ combined with an adequate P supply resulted in the highest root length being recorded in the deepest soil layer (Fig. 4). This would possibly be attributable to higher P availability and/or greater P diffusion in the deep soil layer, which favoured the deep rooting, when the topsoil was dried out under the drought conditions. Consequently, deep root systems are potentially able to obtain greater amounts of soil water (Singh and Sale, 1998; Duursma et al., 2011), and improve drought adaptation (Zhou et al., 2008; Vadez et al., 2012; Kong et al., 2013). An additional contributor to the drought adaptation is the potential increases of root hydraulic conductance with higher P application (Al-Karaki et al., 1995; Singh et al., 2000). Alternatively, the enhanced water-extracting capability of deep roots is likely to maintain photosynthetic function when soils are dry. Significant relationships of deep rooting to ITE ($r=0.87$, $P<0.01$) and WUE ($r=0.74$, $P<0.05$) observed in this study also support this view.

This study showed that field pea plants supplied with adequate P exhibited decreased stomatal conductance compared with P-deficient plants (Fig. 7A). Shubhra et al. (2004) also found that P supply increased ψ_w in cluster bean under both water-sufficient and deficit conditions. Because xylem sap pH and abscisic acid have been considered as signals to modulate the stomatal behaviour in response to water deficit (Rodrigues et al., 2008), it is speculated that these signals may be enhanced by P supply under drought conditions, and thus lead to the reduction of stomatal conductance. However, this assumption needs further investigation. In contrast, many previous studies reported that P addition did not affect stomatal conductance, osmotic potential or transpiration rate in plants (Nelsen and Safir, 1982; Graciano et al., 2005). The discrepancy between the findings of the present study and those of these other published studies may be explained first by interspecific variation in sensitivity of stomatal conductance to P application. For example, P addition did not alter stomatal conductance in *Eucalyptus* but significantly increased stomatal conductance of common bean (*Phaseolus vulgaris* L.) (Graciano et al., 2005; dos Santos et al., 2006). Secondly, the response to P supply may differ with the duration of the experiment. Most of the previous studies on P-induced drought tolerance were conducted for periods of <70 d (Nelsen and Safir, 1982; Fitter, 1988; Graciano et al., 2005), while the present study lasted until the pod setting stage (116 d post-emergence). Physiology-related water relations can change markedly at different growth stages (Jin et al., 2005).

Although the original level of available N in this vertisol soil was low for plant growth, the field pea did not exhibit N limitation in this experiment, with plant N concentrations being above the deficient level (Reuter and Robinson, 1997; Deibert and Utter, 2004). An obvious reason for this is that the inoculated plant offset this limitation by fixing N₂, as observed in a previous study (Jin et al., 2012). Thus, the low availability of mineral N in soil is unlikely to have restricted the plant response to eCO₂, P and drought treatment in this study. However, it is worth noting that increasing P application increased nodulation and subsequent N uptake in the low-N vertisol, especially under eCO₂ (Fig. 5; Table 1), which in turn may favour plant photosynthesis and stress tolerance (Jin et al., 2012; Devi and Sinclair, 2013).

Conclusions

Elevated CO₂ increased the STI of field pea, especially when an adequate level of P was supplied to the plant, via decreased stomatal conductance, increased concentration of soluble sugars and maintenance of higher RWCs of leaves under drought stress. Increasing P supply under eCO₂ increased root growth in the deep soil layer. The increased leaf Pi under sufficient P supply is likely to facilitate further the accumulation of soluble sugars in leaves under eCO₂ and drought stress. These results imply that pulse crops supplied with sufficient levels of P may better withstand periodic drought stress in future eCO₂ environments.

ACKNOWLEDGEMENTS

This research was supported by an Australian Research Council Linkage Project (LP100200757), and utilized the SoilFACE facility of the Department of Environment and Primary Industries, Victoria at Horsham. We thank Dr Saman Seneweera (The University of Melbourne) for valuable discussions during this experiment.

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