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Free-air CO_2 enrichment (FACE) reduces the inhibitory effect of soil nitrate on N_2 fixation of *Pisum sativum*

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• **Background and Aims** Additional carbohydrate supply resulting from enhanced photosynthesis under predicted future elevated CO_2 is likely to increase symbiotic nitrogen (N) fixation in legumes. This study examined the interactive effects of atmospheric CO_2 and nitrate (NO_3^-) concentration on the growth, nodulation and N fixation of field pea (*Pisum sativum*) in a semi-arid cropping system.
• **Methods** Field pea was grown for 15 weeks in a Vertosol containing 5, 25, 50 or 90 mg NO_3^--N kg^{-1} under either ambient CO_2 (aCO_2; 390 ppm) or elevated CO_2 (eCO_2; 550 ppm) using free-air CO_2 enrichment (SoilFACE).
• **Key Results** Under aCO_2, field pea biomass was significantly lower at 5 mg NO_3^--N kg^{-1} than at 90 mg NO_3^--N kg^{-1} soil. However, increasing the soil N level significantly reduced nodulation of lateral roots but not the primary root, and nodules were significantly smaller, with 85 % less nodule mass in the 90 NO_3^--N kg^{-1} than in the 5 mg NO_3^--N kg^{-1} treatment, highlighting the inhibitory effects of NO_3^-. Field pea grown under eCO_2 had greater biomass (approx. 30 %) than those grown under aCO_2, and was not affected by N level. Overall, the inhibitory effects of NO_3^- on nodulation and nodule mass appeared to be reduced under eCO_2 compared with aCO_2, although the effects of CO_2 on root growth were not significant.
• **Conclusions** Elevated CO_2 alleviated the inhibitory effect of soil NO_3^- on nodulation and N_2 fixation and is likely to lead to greater total N content of field pea growing under future elevated CO_2 environments.

Key words: Nitrogen fixation, nitrate, carbon cycling, nitrogen cycling, free-air CO_2 enrichment, FACE, high atmospheric CO_2, ^{15}N, *Pisum sativum*, climate change.

INTRODUCTION

Elevated atmospheric CO_2 concentrations under future climate scenarios (IPCC, 2001) are predicted to produce major changes in the productivity and sustainability of agricultural cropping systems. It has been widely demonstrated that elevated CO_2 stimulates photosynthesis, reduces evapotranspiration and subsequently increases plant productivity when water and nutrients are not limiting (Ainsworth and Long, 2005; de Graaff *et al*., 2006; Luo *et al*., 2006; Leakey *et al*., 2009). The relative responses of different systems are highly dependent on plant species and soil properties.

Legumes generally produce greater responses to elevated CO_2 than non-legumes (Rogers *et al*., 2009). Additional carbohydrate supply under eCO_2 resulting from enhanced photosynthesis is likely to increase symbiotic N_2 fixation and fulfil any additional nitrogen (N) requirement (Cabrerizo *et al*., 2001; West *et al*., 2005). Field pea (*Pisum sativum*) is an economically important crop in many semi-arid cropping systems and is both mycorrhizal and a legume, and therefore may have a greater ability to avoid photosynthetic acclimation by diverting carbon (C) to its symbioses (Aranjuelo *et al*., 2013). The increase in N_2 fixation under eCO_2 could be derived from an increase in the number, size and/or activity of root nodules (Rogers *et al*., 2009). Lam *et al*. (2012) reported no change in number or mass of field pea root nodules under elevated CO_2 despite 44–51 % increases in N_2 fixation. In contrast, field pea grown under elevated CO_2 had greater nodule biomass but not a greater number of root nodules (Cabrerizo *et al*., 2001). However, few studies have examined the effects of elevated CO_2 on field pea.

The response of legumes to elevated CO_2 in Australian agricultural systems could be less predictable than other systems since they are frequently water limited and soil N levels are highly variable. Preceding cereal crops generally have poor N fertilizer use efficiency (Chen *et al*., 2008), and consequently high soil N levels during legume establishment could limit responses to elevated CO_2. Small amounts of soil N at the start of the growing season can enhance legume production, possibly due to bridging the interval between seed N utilization and N_2 fixation and the lower energy requirement of nitrate (NO_3^-) uptake and assimilation than of N_2 fixation (Jensen, 1986; Streeter and Wong, 1988; Peoples *et al*., 2012). Alternatively, plants supplied with small amounts of N may establish greater leaf area and photosynthesis during early growth stages that provides a greater capacity for subsequent nodulation and N_2 fixation, although this is not always the case (Peoples *et al*., 1995).

High levels of soil N, predominantly NO_3^- from fertilizers, are known to inhibit the initiation, development and function of legume symbioses. Nodule number is depressed in the presence of NO_3^-, possibly due to a reduction in root hair growth, signalling molecules (flavonoids) and subsequent expression of nodulation genes in *Rhizobium* that disrupt the infection process (Waterer and Vessey, 1993; Bollman and Vessey, 2006). Nodule formation and hence nodule number is often reported to be less sensitive to NO_3^- than nodule growth and activity (Streeter and Wong, 1988), although this is not always the case (Waterer and Vessey, 1993). For established nodules, NO_3^- is thought to retard nodule growth and activity by causing preferential diversion of photosynthate to NO_3^- assimilation, by reducing oxygen transport within nodules or via toxic effects once reduced to nitrite (NO_2^-), although these are highly debated (Streeter and Wong, 1988; Arrese-Igor *et al*., 1997). Moreover, the relative effect of NO_3^- on crop legumes differs widely between species and between cultivars (Cowie *et al*., 1990; Peoples *et al*., 2012).

The combined effects of elevated CO_2 and soil N level on the development and function of field pea root nodules is not clear. Previous studies on the mechanisms of NO_3^- inhibition of legumes under artificial and controlled conditions are not likely to reflect the response of plants grown in soil under field conditions. Naudin *at al*. (2010) showed that N_2 fixation of field pea intercropped with wheat could recover from NO_3^- inhibition at all growth stages. This could be due to temporal reduction of bulk soil N or to spatial depletion of rhizosphere NO_3^- that allows nodulation and N_2 fixation to proceed. Furthermore, greater C flow to the roots under elevated CO_2 could alter the response of field pea to the inhibitory effects of soil NO_3^-. This experiment aimed to quantify the interactive effects of soil NO_3^- concentration and CO_2 concentration on nodulation and N_2 fixation of field pea under field [free-air CO_2 enrichment (FACE)] conditions. We hypothesized that nodule establishment (nodule number), development (nodule mass) and function (nitrogenase activity, N derived from the atmosphere) would be progressively inhibited with increasing NO_3^- concentration, but these effects would be reduced under elevated CO_2 via enhanced N demand due to greater photosynthetic activity and plant biomass accumulation.

MATERIALS AND METHODS

Field site and sampling details

This study was carried out at the SoilFACE facility at the Department of Economic Development, Jobs, Transport and Resources (DEDJTR) Plant Breeding Centre, Horsham (36°44'57''S, 142°06'50''E), Victoria, Australia. The SoilFACE system is part of the Australian Grains FACE (AgFACE) facility in the Victorian Wimmera region (Mollah *et al*., 2009). A detailed description of SoilFACE is given in Butterly *et al*. (2015). Soil was collected in June 2011 by excavating small areas of a nearby roadside (0–15 cm) following the removal of plant material and debris from the soil surface. Soil was sieved (<4 mm), thoroughly mixed and air-dried. This virgin soil was classified as a Vertosol (Isbell, 1996) or Vertisol (FAO/ISRIC/ISSS, 1998), respectively, and had the following physiochemical properties: total C, 14 mg kg^{-1}; total N, 0·9 mg kg^{-1}; organic C, 9·4 mg kg^{-1}; Colwell P, 6 mg kg^{-1}; NH_4-N, 3 mg kg^{-1}; NO_3-N, 5 mg kg^{-1}; pH (1:5 in 0·01 M $CaCl_2$), 7·8; and clay, 50 % (Butterly *et al*., 2015).

Column experiment design and establishment

A column experiment was conducted using longitudinally sectioned PVC columns (10 cm ID × 60 cm high rejoined with silicon and PVC tape and fitted with PVC end caps at the base). Vertosol was mixed 1:1 (w/w) with triple-washed white sand (mean diameter 430 µm) to facilitate root sampling. Each column was filled with 5·5 kg of soil–sand mix containing basal nutrients (mg kg^{-1}) (KH_2PO_4, 70·4; K_2SO_4, 103; $CaCl_2$, 186; $MgSO_4$, 122; $MnSO_4$, 6; $ZnSO_4$, 8; $CuSO_4$, 6; $CoCl_2$, 0·4; $FeCl_3$, 0·6; $Na_2B_4O_7$, 1·6; and Na_2MoO_4, 0·4) at a bulk density of 1·2 g cm^{-3}. Columns also contained four NO_3-N levels; 5, 25, 50 and 90 mg N kg^{-1}, with 20, 20, 4 and 4 %, respectively, of the NO_3^- added as $Ca(^{15}NO_3)_2$ (20 % atom excess, Shanghai Research Institute of Chemical Industry, China). Each column was adjusted to 80 % field capacity ($\theta g = 0{\cdot}182$ g g^{-1}) using reverse osmosis (RO) water and allowed to equilibrate.

Columns were hand sown with field pea (*Pisum sativum* 'PBA Twilight') (six per column) on 27 June 2011 at a depth of 2 cm using uniform germinated seeds. Field pea was inoculated using a commercial Group E peat inoculum (*Rhizobium leguminosarum*). Columns were placed within one of the eight SoilFACE bunkers treated with either ambient CO_2 (aCO_2; 390 ppm) or elevated CO_2 (eCO_2; 550 ppm) concentration, replicated four times on 1 July 2011. After 3 weeks, field pea seedlings were thinned to two plants per column. Overall, the experiment consisted of a randomized split-plot design with 2 CO_2 concentrations (main plots) × 4 NO_3-N levels (subplots) × 4 replicates (32 columns in total). Wheat (*Triticum aestivum* L. 'Yitpi') (eight seeds were sown and seedlings thinned to three per column) was included as a non-legume reference plant in the same design but with only two NO_3-N levels (5 and 90 mg N kg^{-1}) (eight columns in total).

Column harvest and processing

Columns were removed from SoilFACE on 13 October, after 15 weeks of growth (late pod development; 50–60 % of pods at maximum size) and destructively sampled. Prior to removal, the youngest fully emerged leaf was cut from each plant, weighed and placed in liquid N (two leaves per column). Plant shoots were cut off at the soil surface, washed three times with RO water and dried at 70 °C for 3 d. One half of each PVC column was removed and the soil was sectioned into depths of 0–10 and 10–55 cm. Roots within each sample were carefully removed, washed free of soil and debris with RO water, and stored at 5 °C. The soil was thoroughly mixed, stored at 5 °C overnight, and soil N was extracted the following day on field moist soil as outlined below. The remaining soil samples were air-dried at 25 °C for subsequent analyses. After morphological measurements (within 4 d), root samples were dried at 70 °C for 3 d for subsequent analyses.

Nodulation and leghaemoglobin content

For surface (0–10 cm) roots, nodules on the main root and lateral roots were counted; the nodules were then removed using a scalpel and weighed, before being stored at –20 °C. Nodule mass and number of nodules in the subsoil (10–55 cm) were independently assessed by three individuals using a visual ranking system (1–5; lowest/smallest–greatest/largest).

The leghaemoglobin concentration in nodules collected from 0–10 cm was determined using the method of Riley and Dilworth (1985). Briefly, approx. 300 mg of nodule material was ground in 5 mL of 0·1 M sodium phosphate buffer (pH 6·8; 5 °C) using a pre-cooled mortar and pestle. The macerate was passed through muslin and centrifuged at 500 *g* for 10 min at 5 °C to remove coarse material. The supernatant was then centrifuged at 4400 *g* for 25 min at 5 °C, and the leghaemoglobin concentration was determined spectrophotometrically at 555 nm (Cary 50, UV-Visible Spectrophotometer, Agilent, USA) following conversion to pyridine haemochromogen (1:1 supernatant/reagent; containing 0·2 M KOH in 50 % pyridine) according to Paul *et al*. (1953).

Nitrate reductase activity

Nitrate reductase activity (NRA) in field pea leaves was determined using the approach of Kaiser *et al*. (2000) but with some modifications. Leaves (approx. 500 mg) were ground with 2 mL of extraction buffer [50 mM potassium phosphate buffer, pH 7·6; 1 mM dithiothreitol (DTT); 10 μM FAD; 10 mM $MgCl_2$] using a mortar and pestle pre-cooled with liquid N. Extracts were transferred to tubes and centrifuged at 16 000 *g* for 10 min at 4 °C. Aliquots (100 μL) were added to 900 μL of reaction buffer (50 mM potassium phosphate buffer, pH 7·6; 1 mM DTT; 10 μM FAD; 10 mM $MgCl_2$; 5 mM KNO_3; 9·2 mM NADH) and incubated for 3 min at 24 °C. The reaction was stopped using 125 μL of 0·5 M zinc acetate, and solutions were centrifuged as previously described. Initial and incubated extracts were mixed 1:1 with colour reagent [1·5 g L^{-1} NED (naphthylethylenediamine dihydrochloride); 2·5 g L^{-1} sulphanilamide; 1·13 N HCl), allowed to stand for 30 min, and nitrite concentrations were determined colorimetrically at 540 nm (Cary 50, UV-Visible Spectrophotometer, Agilent). The desalting of extracts prior to the assay performed by Kaiser *et al*. (2000) did not show any benefit and hence was omitted. Furthermore, potassium phosphate buffer was used instead of HEPES-KOH due to greater enzyme activity in preliminary tests (data not shown).

Soil and plant analyses

Soil texture was characterized by determining the particle size distribution using a Laser Particle Size Analyser (Malvern Mastersizer 2000, Worcestershire, UK) following dispersion of soil (approx. 10 g) with 10 mL of 0·164 M $Na_6P_5O_{18}$ (VWR, Australia) in 800 mL of RO water. Soil N extractions were performed on field moist soil using 25 g of soil (dry weight basis) with 2 M KCl (1:1) and shaking end-over-end for 1 h, centrifuging at 2000 *g* for 5 min and filtering through Whatman #1 filter papers (Whatman International, UK). Filtered extracts were frozen and later analysed for nitrate (NO_3^-) and ammonium (NH_4^+) using a QuickChem 8500 Flow Injection Analyser (Lachat Instruments, USA).

Dried plant samples were ground (<2 mm) using a Retsch ZM200 centrifugal mill (Retsch GmbH, Haan, Germany) to reduce sample volume. Sub-samples of both ground plant material and whole soil were then finely ground using a Retsch MM400 mixer mill (Retsch GmbH).

Isotope ratio mass spectrometry (IRMS) (Hydra 20-20; SerCon, Crewe, UK) was used to determine the total C and N concentration and ^{15}N abundance (atom% ^{15}N) of plant and soil samples. Total N content (mg N per column) and the amount of N derived from either the atmosphere or the fertilizer were calculated using combined N and ^{15}N in the whole plant and the entire soil column. The percentage of N derived from the atmosphere (% Ndfa) and fertilizer (% Ndff) was calculated according to McAuliffe *et al*. (1958) using wheat as the reference (Supplementary Data Table S1). The isotope dilution approach can overestimate % Ndfa as it does not account for N derived from soil. In the current study, the soil–sand mix contained 4 mg of $NO_3^- + NH_4^+$ kg^{-1} which constitutes an average error of approx. 6·3 %.

Statistical analyses

A two-way analysis of variance (ANOVA) using a split-plot design was used to test the effects of CO_2 concentration (main plots) and NO_3-N level (sub-plots) on plant and soil parameters. Differences between means were tested using the least significance difference (LSD) test at $P = 0{\cdot}05$.

RESULTS

Plant growth

Shoot growth of field pea was significantly ($P = 0{\cdot}016$) greater under eCO_2 than under aCO_2. Shoot biomass ranged from 11 to 15 g per column under aCO_2 and on average increased by 32 % under eCO_2 (Fig. 1A). Overall, shoot biomass was ten times greater than root biomass. Since the root biomass in the topsoil (0–10 cm) was small (15 % of the total root mass) and was not affected by the treatments, the pooled root biomass for each column was used for analyses. For root biomass, there was an apparent increase under eCO_2; however, this was not significant ($P = 0{\cdot}086$). Higher rates of NO_3^--N significantly ($P = 0{\cdot}038$) increased root mass. In particular, 25 mg NO_3^--N kg^{-1} soil had a lower root mass than 90 mg NO_3^--N kg^{-1} (Fig. 1B). No effect of CO_2 or N level on the root:shoot ratio was observed (Fig. 1C). For shoot and root biomass, no significant interactions between CO_2 and NO_3^--N concentration were observed.

Nodule number, mass and leghaemoglobin content

Nodulation of field pea was affected by NO_3^--N more than CO_2 concentration, and no interactions were observed (Table 1). For surface roots (0–10 cm), nodule numbers on primary and lateral roots were not different between aCO_2 and eCO_2. Under aCO_2, both the number and mass of nodules on lateral roots significantly ($P < 0{\cdot}05$) decreased with increasing

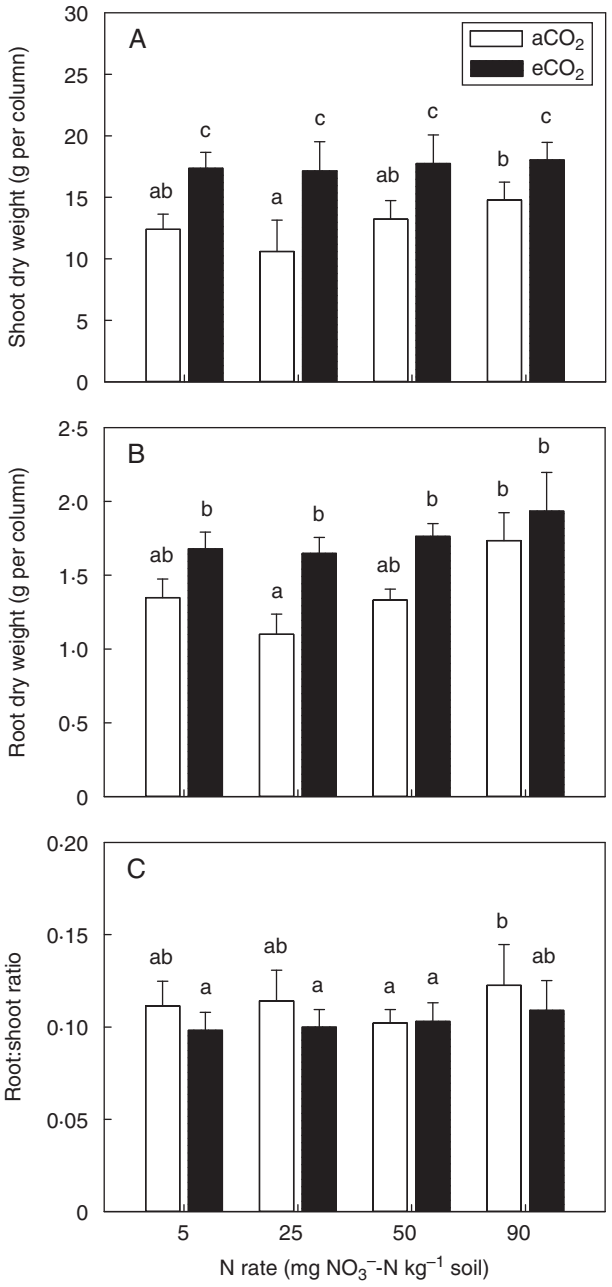

FIG. 1. Shoot (A) and root (B) biomass and root:shoot ratio (C) of field pea grown for 15 weeks under either an aCO_2 or eCO_2 concentration and with 5, 25, 50 or 90 mg NO_3^--N kg^{-1} soil. Root mass includes nodules. Error bars indicate the s.e.m. ($n = 4$). Means with the same lower case letter are not significantly ($P < 0{\cdot}05$) different.

N level; however, at eCO_2, this was only significant for 50 mg NO_3^--N kg^{-1}. The mass of field pea nodules on roots in the 0–10 cm layer was reduced by 85 % between 5 and 90 mg NO_3^--N under aCO_2. While the mass of nodules in the 0–10 cm layer was also reduced under eCO_2, the reduction was much lower in magnitude (72 %) and only significantly lower in the 90 mg NO_3^--N treatment. Hence, the decrease in nodule mass under eCO_2 was not as sharp when NO_3^--N was increased compared with aCO_2. The decrease in nodule mass with increasing NO_3^--N was largely due to a reduction in the size of nodules on the primary root. However, NO_3^--N and CO_2 concentration did not alter the leghaemoglobin concentration expressed per mass of nodule tissue (mmol g^{-1}) or normalized to account for nodule size (mmol per 1000 nodules) (Table 1). Due to the volume of roots in the deeper soil layer (10–55 cm), visual ranking was used to assess the number and size of root nodules. Consistent with surface soil, the number and size of nodules in the deeper layer were significantly reduced when the NO_3^--N concentration was increased (Table 1). In general, nodules in the subsoil were more numerous but much smaller in size than in the topsoil.

N concentration and ^{15}N abundance

The concentration of N in shoot and roots of field pea was not affected by CO_2 or N level (Table 2). Consequently, the C:N ratio of plant material was also not affected by the treatments. However, the N concentration of deeper roots (10–55 cm) was higher than that of surface roots (0–10 cm), resulting in lower C:N ratios. In contrast to overall N concentration, ^{15}N abundance (atom%) of plant material was significantly affected by CO_2 concentration ($P < 0{\cdot}05$) and N level ($P < 0{\cdot}001$). For shoot, ^{15}N abundance was greater under aCO_2 than eCO_2, except for 90 mg NO_3^--N kg^{-1}. In general, greater ^{15}N abundance under aCO_2 was observed for roots, except surface roots (0–10 cm) with 5 mg NO_3^--N kg^{-1} and deep roots (10–55 cm) with 5 and 90 mg NO_3^--N kg^{-1}.

Total N content and N derived from fertilizer

Total N content was significantly ($P = 0{\cdot}011$) greater under eCO_2; however, NO_3^--N concentration was less (Fig. 2A), and was only different between 25 and 90 mg NO_3^--N kg^{-1}. On average, total N content was 294 mg N per column under aCO_2, and was increased by 23–54 % under eCO_2. Fertilizer recovery was generally high (55 and 74 %) since inorganic N ($NO_x^- + NH_4^+$) (45·7 mg N per column) was relatively low compared with the amount of fertilizer added (495 mg N per column in the 90 mg kg^{-1} treatment) (data not shown). The percentage of N derived from fertilizer (% Ndff) showed a significant ($P = 0{\cdot}002$) $CO_2 \times N$ interaction (Fig. 2B). Specifically, field pea plants grown under aCO_2 derived a higher proportion of N from fertilizer and the maximal increase was 61 % for the 50 mg NO_3^--N kg^{-1} treatment.

N_2 fixation

Consistent with reduced fertilizer utilization, the percentage of N derived from the atmosphere (% Ndfa) was significantly ($P = 0{\cdot}011$) greater for field pea grown under eCO_2 than under aCO_2 (Fig. 3). An increase in Ndfa of 10 and 139 % under eCO_2 was observed for 5 and 90 mg NO_3^--N kg^{-1}, respectively. In contrast, a greater NO_3^- concentration strongly ($P < 0{\cdot}001$) reduced Ndfa, indicating that N fixation was severely inhibited. At 90 mg NO_3^--N kg^{-1}, the mean Ndfa was five times lower than that measured at 5 mg. Thus, the relative effect of eCO_2

TABLE 1. *Number, mass and leghaemoglobin content of root nodules in the 0–10 cm layer ($Root_1$) and visual ranking (1–5; lowest to highest and smallest to largest) of root nodules in the 10–55 cm layer ($Root_2$) of field pea grown for 15 weeks under either an aCO_2 or eCO_2 concentration and with 5, 25, 50 or 90 mg NO_3^--N kg^{-1} soil*

Factor	N rate (mg kg^{-1})	Primary $root_1$ nodules (per plant)	Lateral $root_1$ nodules (per plant)	$Root_1$ nodule mass (mg per column)	$Root_1$ nodule size (mg per nodule)	$Root_2$ nodule ranking (1–5)		$Root_1$ leghaemoglobin concentration (μmol)	
						n	Size	g^{-1}	Per nodule
N rate (mg kg^{-1})	5	28·0	127·9	512	1·26	3·4	4·0	628	1·66
	25	27·2	103·8	327	1·29	3·1	3·5	575	2·42
	50	27·7	70·4	267	1·01	2·5	2·8	557	2·27
	90	25·8	93·4	108	0·42	1·2	1·5	616	2·77
CO_2 level	aCO_2	27·1	97·7	272	0·97	2·4	2·9	567	2·5
	eCO_2	27·2	100·0	335	1·02	2·8	3·0	621	2·1
N rate		n.s.	*	*	***	**	***	n.s.	n.s.
LSD ($P = 0·05$)		–	31	236	0·32	0·38	0·73	–	–
CO_2 level		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
LSD ($P = 0·05$)		–	–	–	–	–	–	–	–

No CO_2 × N interactions were observed ($P > 0·05$).
n.s., $P > 0·05$; *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.
–, not applicable; LSD, least significant difference.

TABLE 2. *N concentration, C:N ratio and ^{15}N abundance in shoot and root of field pea grown for 15 weeks under either an aCO_2 or eCO_2 concentration and with 5, 25, 50 or 90 mg NO_3^--N kg^{-1} soil*

Factor	N rate (mg kg^{-1})	N concentration (g kg^{-1})			C:N			^{15}N (atom%)		
		Shoot	$Root_1$	$Root_2$	Shoot	$Root_1$	$Root_2$	Shoot	$Root_1$	$Root_2$
N rate (mg kg^{-1})	5	20·7	24·3	29·3	20·4	16·3	14·0	1·6	1·2	2·5
	25	20·3	24·3	29·5	20·8	16·1	14·1	6·0	4·1	7·4
	50	21·9	22·3	29·3	20·7	16·9	14·1	2·6	2·0	2·9
	90	20·5	24·2	29·2	20·7	16·4	13·6	3·1	2·4	3·3
CO_2 level	aCO_2	20·8	24·1	30·1	20·1	16·0	13·4	3·8	2·7	4·3
	eCO_2	20·9	23·4	28·5	21·3	16·8	14·6	2·9	2·2	3·8
N rate		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	***	***
LSD ($P = 0·05$)		–	–	–	–	–	–	1·08	0·32	0·63
CO_2 level		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	*
LSD ($P = 0·05$)		–	–	–	–	–	–	0·72	0·30	0·30

Roots from 0–10 cm ($root_1$) and 10–55 cm ($root_2$) were analysed separately.
No CO_2 × N interactions were observed ($P > 0·05$).
n.s., $P > 0·05$; *$P < 0·05$; ***$P < 0·001$.
–, not applicable; LSD, least significant difference

was greater at the higher N rate than the lower one, despite a much lower Ndfa overall.

Nitrate reductase activity

Nitrate reductase activity was determined in the youngest fully expanded leaves of field pea. There was an apparent increase in NRA between 5 and 50 mg NO_3^--N kg^{-1} and then a sharp drop in NRA at 90 mg NO_3^--N kg^{-1}. However, the data obtained for NRA was highly variable, and consequently no effects of NO_3^- and CO_2 concentration were observed ($P > 0·05$) (data not presented).

Soil inorganic N

Inorganic N ($NO_x^- + NH_4^+$) in the soil was pooled since NH_4^+ concentration was low (<4 % of NO_3^-) most probably due to the neutral pH. The inorganic N concentration in the soil was significantly ($P < 0·001$) affected by the N rate (Fig. 4). The inorganic N concentration increased with increasing rate of NO_3^-; however, except for the 90 mg NO_3^--N kg^{-1} treatment, all other N rates were not significantly different ($P > 0·05$). In particular, the 90 mg NO_3^--N kg^{-1} treatment had approx. 310 mg inorganic N per column, which was between six and 43 times more than the other treatments. While the inorganic N concentration of soil appeared to be lower under eCO_2, this was not significant ($P > 0·05$).

DISCUSSION

Effects of CO_2 and N level on plant growth and nodulation

Shoot and root biomass of field pea grown under aCO_2 was lower at 25 mg N kg^{-1} than at 90 mg N kg^{-1} (Fig. 1). The relative difference between these treatments was probably a function of contrasting N source and C allocation by plants.

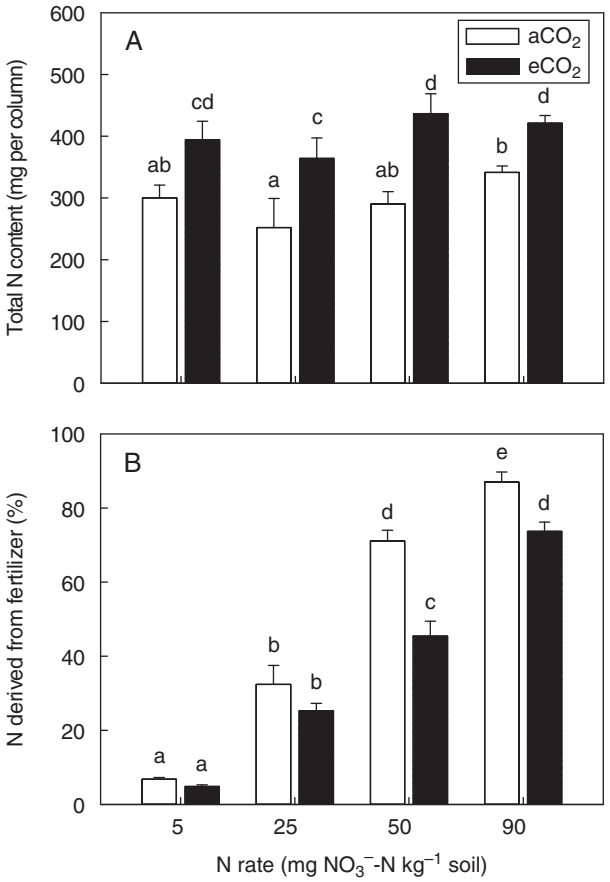

FIG. 2. Total N content (A) and percentage of N derived from the fertilizer (B) for field pea grown for 15 weeks under either an aCO_2 or eCO_2 concentration and 5, 25, 50 or 90 mg NO_3^--N kg^{-1} soil. Bars indicate the s.e.m. ($n = 4$). Means with the same lower case letter are not significantly ($P < 0{\cdot}05$) different.

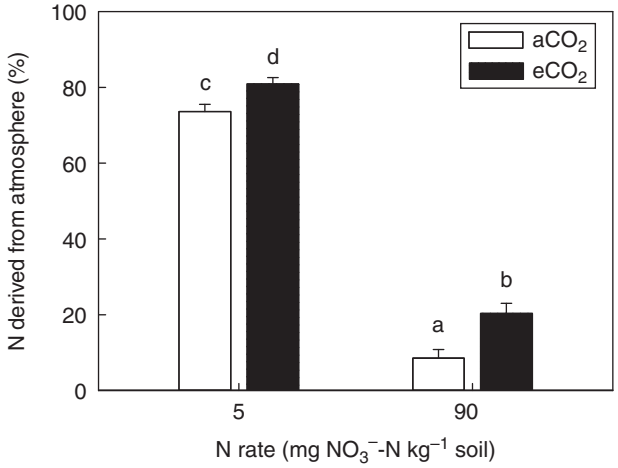

FIG. 3. Percentage of N derived from the atmosphere for field pea grown for 15 weeks under either an aCO_2 or eCO_2 concentration and 5 or 90 mg NO_3^--N kg^{-1} soil. Bars indicate the s.e.m. ($n = 4$). Means with the same lower case letter are not significantly ($P < 0{\cdot}05$) different.

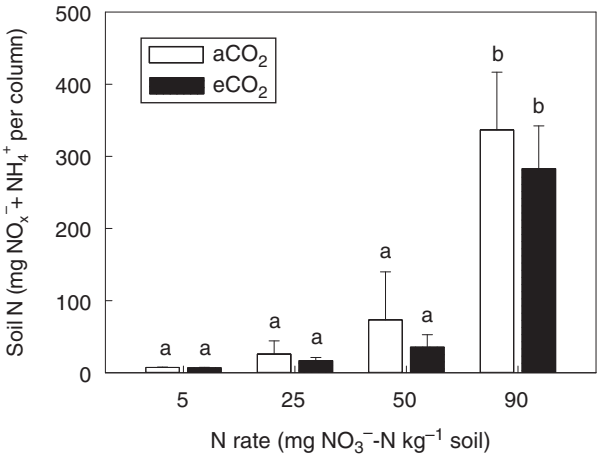

FIG. 4. Inorganic N ($NO_x^- + NH_4^+$) in soil following harvest of field pea grown for 15 weeks under either an aCO_2 or eCO_2 concentration and 5, 25, 50 and 90 mg NO_3^--N kg^{-1} soil. Bars indicate the s.e.m. ($n = 4$). Means with the same lower case letter are not significantly ($P < 0{\cdot}05$) different.

Moderate (approx. 25 mg N kg^{-1}) levels of soil NO_3^- are known to inhibit legume symbiosis and nodule function (Streeter and Wong, 1988; Cowie *et al*., 1990; Naudin *et al*., 2011), resulting in insufficient N acquisition to meet the plant's requirement. At high (approx. 90 mg N kg^{-1}) levels of soil NO_3^-, field pea derived less N from N_2 fixation (Fig. 3) but still maintained greater biomass due to greater availability of soil N. Photosynthates are known to be preferentially diverted to nodule morphogenesis rather than growth, particularly during the early growth stages (Voisin *et al*., 2002*a*). Therefore, lower biomass of the 25 mg N kg^{-1} than the 90 mg N kg^{-1} treatment could be attributed to either lower levels of photosynthate C or greater allocation of C to the production and maintenance of root nodules. This phenomenon has been used to explain greater biomass production, particularly of roots, in NO_3^--fed field pea plants (Aranjuelo *et al*., 2013). In the current study, the N level did not alter the root:shoot ratio, which is similar to the findings other studies (Voisin *et al*., 2003*a*; Aranjuelo *et al*., 2013), and the low root biomass (root:shoot 0·6–0·7) is consistent for this species (Nuruzzaman *et al*., 2006) (Fig. 1C).

The inhibitory effects of NO_3^- on nodule formation and development observed in the current study are consistent with previous reports (Tang *et al*., 1999; Bollman and Vessey, 2006; Aranjuelo *et al*., 2013). Although the numbers of nodules on the primary roots were the same for all treatments, nodulation of lateral roots and total nodule mass in the topsoil (0–10 cm) were significantly reduced as the NO_3^- level was increased (Table 1). The reduction in nodule number on the lateral roots but not the primary root may be due to spatial and temporal depletion of soil NO_3^-. It is likely that primary root nodules of high N treatments were formed but their development was delayed until later stages of growth once the NO_3^- concentration in the rhizosphere soil was sufficiently depleted. The reduction in nodule mass with increased NO_3^- level, predominantly due to lower primary root nodule mass, supports the idea of an inhibition of nodule growth and development. In contrast, lateral roots that are much finer and constantly grow into new areas of soil may not have been able to deplete the soil NO_3^- concentration sufficiently to allow nodulation and/or nodule function.

Reductions in the number and size of root nodules with increasing NO_3^- level in the 10–55 cm soil layer were consistent with field pea nodulation in the topsoil (0–10 cm) (Table 1).

Field pea plants grown under eCO_2 had greater biomass (approx. 32 %) than those grown under aCO_2 and were not affected by the soil NO_3^- level (Fig. 1). Therefore, field pea grown under eCO_2 achieved the same biomass irrespective of N source. While eCO_2 appeared to alleviate the inhibitory effects of NO_3^- on nodule number and mass (as indicated by a greater % Ndfa, discussed later), these effects were not significant (Table 1). Changes in nodulation and nodule morphology were expected under eCO_2, particularly for the 25 mg N kg^{-1} treatment that showed the largest gain in biomass production. Other studies have shown increased mass and activity of field pea nodules under eCO_2 (Cabrerizo *et al*., 2001; Aranjuelo *et al*., 2014). Enhanced nodule function is likely to have contributed to productivity gains under eCO_2, although the concentration of leghaemoglobin in nodules, used here as an indicator of nodule activity, did not support this (Table 1). However, nodule activity is known to decrease during reproductive growth due to greater allocation of assimilates to grain (Voisin *et al*., 2002*b*). Hence, the leghaemoglobin concentrations observed may be a poor indicator of actual nodule activity during the growing season. Furthermore, primary root nodules dominated the nodule biomass, and the greater activity of more numerous but smaller lateral root nodules under eCO_2 may have been masked. Importantly, the parameters used to assess treatment effects on field pea nodules were not related to overall nodule function (Ndfa), as discussed later.

Enhanced productivity of field pea under eCO_2 was probably facilitated by greater availability of photosynthates, particularly to root nodules. Using a $^{13}CO_2$ pulse-labelling approach, Voisin *et al*. (2003*b*) showed that C allocated to field pea roots increased with atmospheric CO_2 concentration due to enhanced net photosynthesis. Similarly, field pea nodules are a major C sink and are generally substrate limited under aCO_2 conditions (Aranjuelo *et al*., 2013). Furthermore, the additional N demand of field pea grown under eCO_2 could have resulted in a more rapid depletion of soil N and subsequently allowed the establishment of N_2 fixation earlier than plants grown under aCO_2. Since field pea has a relatively small root system and excretes less carbohydrates and enzymes compared with other crop legumes (Nuruzzaman *et al*., 2006), the contribution of microbial N immobilization to reductions in rhizosphere NO_3^- concentration are expected to be small compared with plant N demand. Nodulation rapidly commences once the NO_3^- concentration falls below a critical level (Voisin *et al*., 2002*b*), but this recovery is dependent on C availability to nodules (Naudin *et al*., 2011). Evidence suggests that C supply to field pea roots is independent of the N source and extent of nodulation; however, nodulated roots have lower C use efficiency due to greater respiration losses (Voisin *et al*., 2003*a*, *b*). In the current study, the relative increase in biomass of field pea grown under eCO_2 was greater for 25 mg N kg^{-1} than for 90 mg N kg^{-1}, and this could be due to the lower C use efficiency of well-nodulated plants in the lower N aCO_2 treatment. Alternatively, photosynthetic acclimation or downregulation of photosynthesis could have occurred in the high NO_3^- treatment due to lower sink strength for photosynthate C (Gavito *et al*., 2000; Aranjuelo *et al*., 2013).

Effects of CO_2 and N level on legume performance

The current study highlighted that the total N content of field pea grown under eCO_2 was enhanced (Fig. 2A). As expected, the N concentration and C:N ratio of shoots and roots were not affected by either treatment (Table 2) and, subsequently, the total N content was a function of plant biomass. Generally, the N concentration of legumes is maintained under eCO_2 unless environmental or nutritional limitations to N_2 fixation, such as phosphorus, exist (Jin *et al*., 2012; Lam *et al*., 2012). Greater N content was primarily achieved by N_2 fixation since N fertilizer recovery (data not shown) and the soil N concentration at harvest (Fig. 4) did not differ between the CO_2 treatments. The % Ndfa values for field pea grown at the low N level (74 % aCO_2, 81 % eCO_2) were consistent with other studies (average $\geq$80 %) (Voisin *et al*., 2002*b*; Hauggaard-Nielsen *et al*., 2010) but higher than commonly observed in southern Australia (average 68 %) (Unkovich *et al*., 1997). Accordingly, the amount of N_2 fixed under aCO_2 and eCO_2 ranged from 263 to 376 mg N per column at the low N level and from 49 to 111 mg N per column for the high N level, respectively. However, it is unclear whether specific increases in N_2 fixation efficiency occurred since this process was not directly quantified. The N concentration did not affect the rate of N_2 fixation of field pea but simply delayed the onset of N_2 fixation (Voisin *et al*., 2002*b*). Similarly, greater Ndfa of white clover under eCO_2 was due to greater nodule mass rather than specific nodule activity (Zanetti *et al*., 1998). However, NO_3^- added after legume establishment can reduce both nodule biomass and specific activity (Naudin *et al*., 2011).

Importantly, this study showed that Ndfa was greater under eCO_2 irrespective of N level. In the current study, Ndfa decreased and was inversely proportional to Ndff as the N level increased (Fig. 3). This substitution of N_2 fixation with soil N has been well established (Streeter and Wong, 1988; Naudin *et al*., 2011). In fact, Voisin *et al*. (2002*b*) suggest that Ndfa can be predicted across the growing season from initial mineral N at sowing. Results obtained in the current study conform with the relationship between N level and Ndfa reported by Jensen (1986) (data not shown). In contrast to other studies, N_2 fixation was less inhibited at the highest N level (equivalent to 630 kg N ha^{-1}) and may be due to the fact that fertilizer was mixed to a greater depth (uniformly through the 55 cm soil columns). Greater inhibition of N_2 fixation by inorganic N in other studies could also to be due to high existing soil N pools (Lam *et al*., 2012) or the use of $NH_4NO_3^-$ as the N fertilizer source (Voisin *et al*., 2002*b*). NH_4^+ has been shown to reverse the inhibitory effects of NO_3^- in artificial growth media (Bollman and Vessey, 2006). However, in soil and when added at the start of the growing season in the absence of living roots, NH_4^+ is likely to be converted to NO_3^-, thereby underestimating the actual NO_3- concentration.

Conclusions

Using FACE combined with a ^{15}N isotope dilution approach, this study showed for the first time that eCO_2 reduced the inhibitory effects of soil NO_3^- on N_2 fixation of field pea. Under eCO_2, the N content of field pea was enhanced via greater biomass production and higher % Ndfa, although the physiological

(number, size and activity of nodules) differences were not evident at the time of sampling. It is possible that eCO_2 increased the critical concentration at which N_2 fixation of field pea was inhibited and/or field pea was more efficient at switching between fertilizer N and N_2 fixation under eCO_2 due to more rapid depletion of the rhizosphere NO_3^- concentration. The current results indicate that field pea may perform well in semi-arid agricultural systems under future CO_2 concentrations irrespective of soil N status, and subsequent gains in N input via enhanced N_2 fixation will be important for maintaining the N fertility of cropping systems.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Table S1: biomass, N concentration, C:N ratio and ^{15}N abundance in shoot and root of wheat grown under either aCO_2 or eCO_2 concentrations and with 5 or 90 mg NO_3^--N kg^{-1} soil.

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