

Increased C availability at elevated carbon dioxide concentration improves N assimilation in a legume

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ABSTRACT

Plant growth is typically stimulated at elevated carbon dioxide concentration ([CO₂]), but a sustained and maximal stimulation of growth requires acquisition of additional N in proportion to the additional C fixed at elevated [CO₂]. We hypothesized that legumes would be able to avoid N limitation at elevated [CO₂]. Soybean was grown without N fertilizer from germination to final senescence at elevated [CO₂] over two growing seasons under fully open-air conditions, providing a model legume system. Measurements of photosynthesis and foliar carbohydrate content showed that plants growing at elevated [CO₂] had a c. 25% increase in the daily integral of photosynthesis and c. 58% increase in foliar carbohydrate content, suggesting that plants at elevated [CO₂] had a surplus of photosynthate. Soybeans had a low leaf N content at the beginning of the season, which was a further c. 17% lower at elevated [CO₂]. In the middle of the season, ureide, total amino acid and N content increased markedly, and the effect of elevated [CO₂] on leaf N content disappeared. Analysis of individual amino acid levels supported the conclusion that plants at elevated [CO₂] overcame an early-season N limitation. These soybean plants showed a c. 16% increase in dry mass at final harvest and showed no significant effect of elevated [CO₂] on leaf N, protein or total amino acid content in the latter part of the season. One possible explanation for these findings is that N fixation had increased, and that these plants had acclimated to the increased N demand at elevated [CO₂].

Key-words: amino acids; carbohydrate; elevated CO₂; free air CO₂ enrichment (FACE); legumes; ureides.

INTRODUCTION

The atmospheric [CO₂] has risen by 35% in the last 250 years. It is higher than at any time in the past 25 million

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years and is predicted to increase an additional 50% by 2050 (Albritton *et al.* 2001). This extremely rapid rise in [CO₂] portends critical uncertainty over both C uptake by terrestrial ecosystems and food production. Although photosynthesis and growth are typically stimulated when plants are grown at elevated [CO₂] (Norby *et al.* 1999; Long *et al.* 2004; Nowak, Ellsworth & Smith 2004; Ainsworth & Long 2005), the stimulation often disappears with time. The observed (Diaz *et al.* 1993; Oechel *et al.* 1994; Stitt & Krapp 1999; Oren *et al.* 2001) and modelled (Thornley & Cannell 2000) attenuation are nearly always associated with the inability of plants to acquire sufficient N, resulting in the commonly observed reduction in leaf N content in plants grown at elevated [CO₂] and the subsequent N limitation of C acquisition (Drake, Gonzalez-Meler & Long 1997; Peterson *et al.* 1999; Hungate *et al.* 2003; Luo *et al.* 2004).

Extensive evidence that N limits the growth response at elevated [CO₂] has been obtained from experiments conducted in controlled environmental chambers, greenhouses, transparent field enclosures and open-topped chambers (Stitt & Krapp 1999; Poorter & Pérez-Soba 2001). Despite concerns that some observations may have been an artefact of growth in a limited rooting volume (Arp 1991; Long & Drake 1992; Ainsworth *et al.* 2002), these results have been corroborated under fully open-air field conditions in free air CO₂ enrichment (FACE) experiments (Ainsworth & Long 2005).

A number of mechanisms have been proposed to explain the N limitation of productivity at elevated [CO₂], including exacerbated N limitation due to faster growth (Farage, McKee & Long 1998; Stitt & Krapp 1999), sink limitation due to a finite N supply (Rogers *et al.* 1996; Long *et al.* 2004), restricted rooting volume (Stitt & Krapp 1999; Long *et al.* 2004), increased microbial immobilization of N, or N sequestration in plant biomass (Diaz *et al.* 1993; Zak *et al.* 2000; Hungate *et al.* 2003; Luo *et al.* 2004), and increased competition for reductant that inhibits nitrate assimilation (Bloom *et al.* 2002; Shaw *et al.* 2002).

Legumes may be able to avoid N limitation at elevated [CO₂] because they form a symbiotic association with the N-fixing bacteria of the *Rhizobiaceae*, and the additional C fixed at elevated [CO₂] could be available for exchange with

the bacterial symbiont to enhance N fixation (Udvardi & Day 1997). Although evidence of stimulation in the growth of legumes, and also of N fixation, at elevated $[\text{CO}_2]$ is common (Zanetti *et al.* 1996; Hungate *et al.* 1999; Dakora & Drake 2000; Lee, Reich & Tjoelker 2003), the response of legumes to elevated $[\text{CO}_2]$ is not clear and appears species dependent, sensitive to drought, and dependent on the availability of N and other nutrients (Serraj, Sinclair & Purcell 1999; Hungate *et al.* 2004; West *et al.* 2005). In contrast with legumes found in natural or seminatural plant communities, the life cycle of field-grown soybean is extremely well characterized (Ritchie *et al.* 1997). Of particular relevance to this study is the well-defined transition from utilizing soil-borne N sources to relying predominantly on fixed N and the ability to study the effects of elevated $[\text{CO}_2]$ on a legume grown throughout its entire life cycle at elevated $[\text{CO}_2]$. For these reasons, we chose to use field-grown soybean as a model legume system upon which to study the effect of elevated $[\text{CO}_2]$ on N assimilation and to ask the question, can legumes overcome the potential constraints of a limiting N supply during growth at elevated $[\text{CO}_2]$? To answer this question, we made measurements of key physiological and biochemical markers for C and N status that together can begin to provide an integrated view of N metabolism (Stitt & Krapp 1999; Stitt *et al.* 2002; Foyer, Parry & Noctor 2003; Rogers & Ainsworth 2006). In this study, soybean, grown in annual rotation with maize, was seeded without adding any fertilizer and was grown under fully open-air CO_2 enrichment over two complete life cycles using FACE technology (Miglietta *et al.* 2001). A genotype with an indeterminate growth form was used to minimize genetic limitation on the response to elevated $[\text{CO}_2]$ (Ainsworth *et al.* 2004) and is a form more representative of natural system legumes.

MATERIALS AND METHODS

Plant material

The study was conducted at the SoyFACE facility (Champaign, Illinois; 40°02'N, 88°14'W, 228 m above sea level). The experimental facility is situated on 32 ha of Illinois farmland. The experimental design consisted of four blocks, each containing two 20-m-diameter octagonal plots. Within each block, one plot was at current $[\text{CO}_2]$ of 370 $\mu\text{mol mol}^{-1}$, and one plot was fumigated throughout the season from sunrise to sunset to an elevated target $[\text{CO}_2]$ of 550 $\mu\text{mol mol}^{-1}$ using FACE technology (Miglietta *et al.* 2001). The actual delivered $[\text{CO}_2]$ was 552 $\mu\text{mol mol}^{-1}$, and 1 min averages of $[\text{CO}_2]$ were within 10% of the target 84% of the time. Soybean [*Glycine max* (L.) Merr.] cv. 93B15 (Pioneer Hi-Bred) was seeded in 0.38 m rows on 1 June 2002 and 27 May 2003. The crop emerged on 7 June 2002 and 5 June 2003. The soil is a Drummer/Flanagan type, and no fertilizer was added to the soybean crop according to standard regional agronomic practice for annual maize/soybean rotation. Samples of the lateral leaflets of the most recently fully matured trifoliate leaf were taken at midday throughout the

season, frozen immediately in liquid N and stored at -80°C until analysed. In 2002, leaf area was not determined during sampling, and data were expressed on a dry weight basis corrected for the mass of total non-structural carbohydrate (DW_{TNC}). Marked increases in foliar carbohydrate content are common at elevated $[\text{CO}_2]$, even when plants are free from artificial restriction of sink development (Long *et al.* 2004). This correction avoids any false impression caused by the dilution effect of increased carbohydrate content. In 2003, the leaf area was determined during sampling, and data were expressed on a leaf area basis.

Gas exchange and biochemical analysis

Photosynthesis was measured at 2 h intervals for 6 d during the 2002 season and 4 d during the 2003 season using a portable open gas-exchange system (LI-6400; Li-Cor, Lincoln, NE, USA) as described by Rogers *et al.* (2004). Frozen leaf samples were powdered in liquid N. For determination of N and ureide content, the powdered leaf material was dried to a constant mass before analysis. Leaf N content was determined by dry combustion with an elemental analyser (PE 2400 Series II CHN analyser; Perkin Elmer, CT, USA), and ureide content was assayed using a colourimetric assay (Vadez & Sinclair 2000). Glucose, fructose, sucrose, nitrate and amino acids were extracted from frozen powdered leaf material with sequential incubations in ethanol (Rogers *et al.* 2004). The leaf starch content remained in the residue from the ethanol extraction. This starch was converted to glucose by incubation with exo- and endoamylases (Geigenberger *et al.* 1996). The glucose resulting from starch degradation and the glucose, fructose and sucrose in the ethanolic extract were assayed using a continuous enzymatic substrate assay (Rogers *et al.* 2004). Total amino acid content was determined using a fluorogenic-based microplate assay (Bantan-Polak, Kassai & Grant 2001), and individual amino acid contents were determined using high-performance liquid chromatography (Geigenberger *et al.* 1996).

Statistical analysis

The effects of elevated $[\text{CO}_2]$ on all variables were assessed by a repeated measures mixed model analysis of variance (PROC MIXED, SAS v9.1; SAS Institute, Cary, NC, USA) with days after emergence (DAE), treatment and the DAE by treatment interaction as fixed effects. The means and 95% confidence intervals derived from these analyses are reported. The amino acid ratio was analysed using a mixed model analysis of variance. Years were analysed separately because of crop rotation altering field position. Assumptions of normality were met by transforming ureide (2002 and 2003) and TNC (2002); values and the best-fit variance/covariance matrices were chosen for each variable using Akaike's information criterion (Littell, Pendergast & Natarajan 2000). Treatment effects within DAE were assessed from a priori pair-wise comparisons ($\alpha = 0.05$). In all analyses, $n = 4$ replicate plots.

RESULTS

The daily integral of CO₂ assimilation (A') showed a c. 25% uniform and significant stimulation at elevated [CO₂] throughout the season ($P < 0.0001$ for both 2002 and 2003; Table 1). There was a highly significant ($P < 0.001$, 2002 & 2003) c. 58% increase in foliar carbohydrate content at elevated [CO₂] compared with current [CO₂]. Levels of foliar carbohydrate at both current and elevated [CO₂] were high at the beginning of the season, suggesting a higher demand for photosynthate later in development (Fig. 1).

Leaf N content per unit area and per unit mass, corrected for non-structural carbohydrate content, was significantly lower in leaves from soybeans grown at elevated [CO₂] (Fig. 2, $P < 0.01$). However, there was a significant interaction between growth stage and CO₂ treatment (2002, $P < 0.05$; 2003, $P < 0.1$). At the beginning of the season (before approximately 50 DAE), leaf N content was low at current [CO₂], and reduced on average by another 19% (2002) and 15% (2003) at elevated [CO₂]. In the middle of the season, leaf N content rose in both current and elevated [CO₂], and, with the exception of a transitory reduction in 2003 (62 DAE) leaf N content was no longer significantly ($P < 0.05$) lower at elevated compared with current [CO₂].

The developmental stages of agriculturally managed soybean, including changes in the supply of fixed N, are well understood (Ritchie *et al.* 1997). Under field conditions, root nodules containing *Bradyrhizobium* form shortly after emergence, but active N fixation does not begin until three trifoliolate leaves have developed. After this time, the number of root nodules formed and the amount of N fixed increases with time. Typically, N fixation increases dramatically when soybean is in full bloom, continues to rise throughout pod set and peaks during seed set. During the period of rapid seed growth, N fixation usually drops abruptly. Table 2 details the development of soybean in this study and indicates the number of DAE when the three key stages associated with the documented changes in N supply occur.

Measurements of metabolites corroborate this N-fixation profile for our system. Foliar ureide levels, the N-rich compounds derived from the root nodules, were measured as an indicator of the availability of fixed N. In both years, there was a significant effect of growth stage on ureide

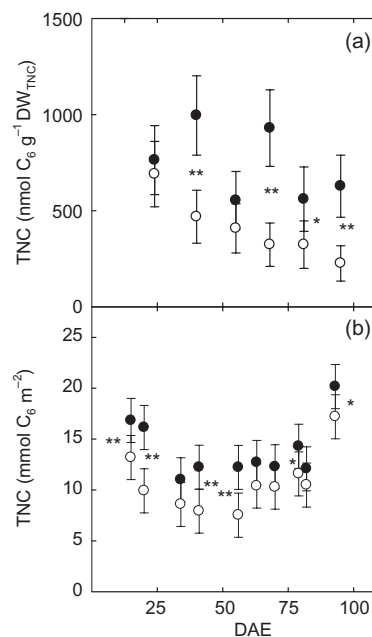


Figure 1. Total non-structural carbohydrate (TNC) content in soybean leaves sampled throughout crop development and grown in the field at current (open symbols) and elevated (filled symbols) [CO₂]. (a) Data collected from soybean harvested in 2002. Leaf area was not determined before harvest, and data were expressed on a dry weight basis corrected for the mass of total non-structural carbohydrate (DW_{TNC}). (b) Data collected from soybean harvested in 2003 and expressed per unit leaf area. Symbols show mean \pm 95% confidence intervals; $n = 4$ replicate free air CO₂ enrichment (FACE) plots (*, $P < 0.05$; **, $P < 0.001$). DAE, days after emergence.

content ($P < 0.001$). Ureide levels were low but increased markedly at one point in the middle of the season (40–60 DAE, Fig. 3). The observed increase in ureide content coincided with the mid-season increase in leaf N content (Fig. 2). There was no effect of elevated [CO₂] on ureide content ($P = 0.21$, 2002; $P = 0.22$, 2003). In 2003, we measured leaf nitrate content. Nitrate levels were high at the start of the season (23 DAE, c. 500 $\mu\text{mol m}^{-2}$) and fell ($P < 0.001$) to very low levels later in the season ($< 50 \mu\text{mol m}^{-2}$, 53 DAE onwards). There was no effect of

Table 1. The mean daily integral of CO₂ assimilation (A') for the 2002 and 2003 growth seasons

Year	[CO ₂]	A' (mol CO ₂ m ⁻¹ d ⁻¹)	Stimulation at elevated [CO ₂] (%)
2002	Current	0.98 \pm 0.02	23
	Elevated	1.21 \pm 0.02	
2003	Current	0.94 \pm 0.02	26
	Elevated	1.18 \pm 0.03	

Data are mean \pm SEM, $n = 4$ plots.
SEM, standard error of the mean.

Table 2. Days after emergence (DAE) to reach key developmental stages associated with marked changes in the supply of fixed N derived from *Bradyrhizobium* (Ritchie *et al.* 1997)

Year	[CO ₂]	DAE to reach developmental stage		
		Third trifoliolate	Full bloom	Seed set
2002	Current	16	46	84
	Elevated	16	43	90
2003	Current	15	50	94
	Elevated	15	48	98

DAE, days after emergence.

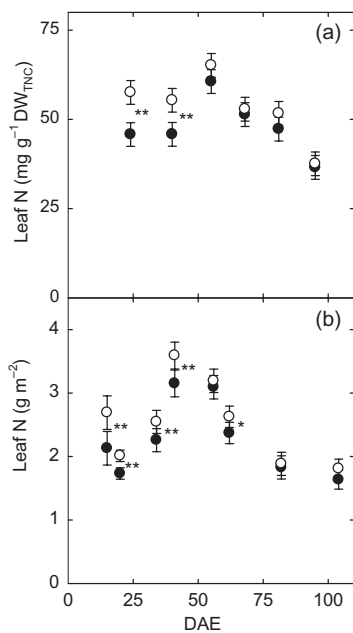


Figure 2. N content of soybean leaves sampled throughout crop development and grown in the field at current (open symbols) and elevated (filled symbols) [CO₂]. (a) Data collected from soybean harvested in 2002. (b) Data collected from soybean harvested in 2003. Symbols show mean \pm 95% confidence intervals; $n = 4$ replicate free air CO₂ enrichment (FACE) plots (*, $P < 0.05$; **, $P < 0.001$). DAE, days after emergence; DW_{TNC}, dry weight basis corrected for the mass of total non-structural carbohydrate.

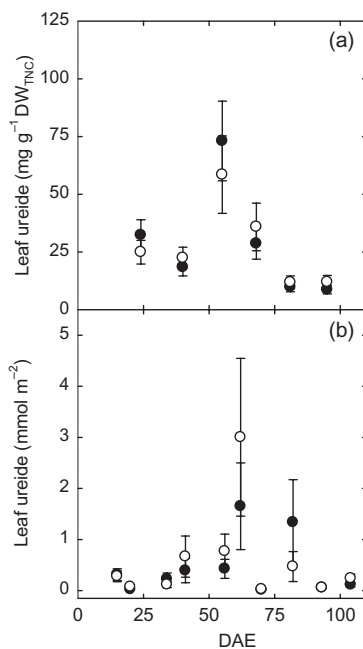


Figure 3. Ureide content of soybean leaves sampled throughout crop development and grown in the field at current (open symbols) and elevated (filled symbols) [CO₂]. (a) Data collected from soybean harvested in 2002. (b) Data collected from soybean harvested in 2003. Symbols show mean \pm 95% confidence intervals; $n = 4$ replicate free air CO₂ enrichment (FACE) plots. DAE, days after emergence; DW_{TNC}, dry weight basis corrected for the mass of total non-structural carbohydrate.

elevated [CO₂] on leaf nitrate content (data not shown, $P = 0.84$).

At current [CO₂], the total amino acid content was low early in the season, and increased markedly later in the season ($P < 0.001$, 2002 & 2003; Fig. 4). This rise coincides with the mid-season peak in ureide levels (Fig. 3), at a time when the demand for carbohydrate was highest (Fig. 1 and Rogers & Ainsworth 2006). There was no significant effect of elevated [CO₂] on total amino acid content ($P = 0.51$, 2002; $P = 0.88$, 2004; Fig. 4). Because the major amino acids comprise a large proportion of total amino acids, we analysed individual amino acid levels to reveal any trends that might be masked by the response of a few major amino acids. Most of the major (Fig. 5a) and minor (Fig. 5b) amino acids were also lower in elevated than current [CO₂] early in the season. However, around the middle of the season (40–60 DAE), the situation reversed, with most of the major amino acids being higher (Fig. 5a) and some of the minor amino acids dramatically higher (Fig. 5b) in elevated [CO₂]. When pooled, the ratio of the sum of minor amino acids 55 DAE at elevated [CO₂] to that at current [CO₂] was significantly ($P < 0.05$) greater. Leaf soluble protein levels were significantly ($P < 0.001$) lower at elevated [CO₂] early in the season (Fig. 5c), but the effect disappeared later in the season.

DISCUSSION

Although leaf N content was lower at elevated [CO₂] at the

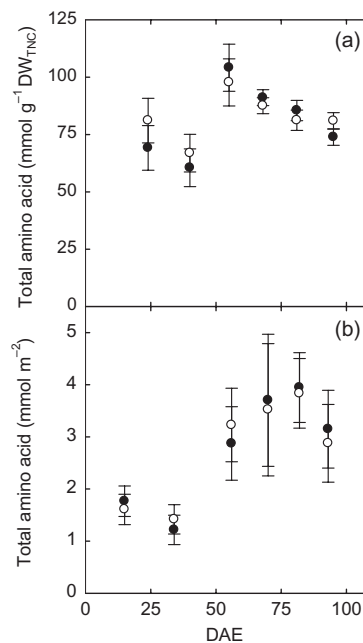


Figure 4. Total free amino acid content in soybean leaves sampled throughout crop development and grown in the field at current (open symbols) and elevated (filled symbols) [CO₂]. (a) Data collected from soybean harvested in 2002. (b) Data collected from soybean harvested in 2003. Symbols show mean \pm 95% confidence intervals; $n = 4$ replicate free air CO₂ enrichment (FACE) plots. DAE, days after emergence; DW_{TNC}, dry weight basis corrected for the mass of total non-structural carbohydrate.

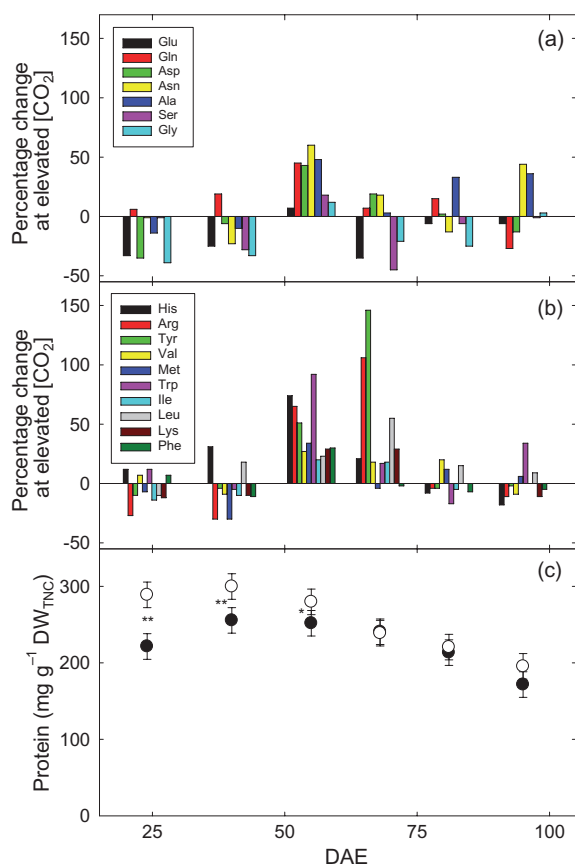


Figure 5. Individual amino acids and protein content. The percentage change in the content of individual amino acids in soybean foliage grown at elevated [CO₂] relative to the content at current [CO₂]. (a) The ratio for major amino acids and (b) for the minor amino acids and (c) the protein content in the same tissue at current (open symbols) and elevated (filled symbols) [CO₂]. Symbols show mean \pm 95% confidence intervals; $n = 4$ replicate free air CO₂ enrichment (FACE) plots (*, $P < 0.05$; **, $P < 0.001$). All data are from the 2002 season. DAE, days after emergence.

beginning of the season, soybean was able to overcome this exacerbated N limitation, and by the middle of the season, marked and significant reductions in leaf N content were absent. Despite a *c.* 16% increase in dry mass at the final harvest (Morgan *et al.* 2005), these plants showed no significant reduction in leaf N, protein or amino acid content, suggesting that soybean had acclimated to the increased N demand at elevated [CO₂]. This suggests that N fixation may have increased at elevated [CO₂] to match the increased C gain.

As expected, the daily integral of A' showed a uniform and significant stimulation at elevated [CO₂] throughout the season, providing *c.* 25% more C for growth at elevated [CO₂] (Rogers *et al.* 2004; Table 1). The increased foliar carbohydrate content at elevated [CO₂] suggests that carbohydrate formation at elevated [CO₂] exceeds utilization, even though a genotype with an indeterminate vegetative growth form was selected for this experiment. Evidence from an earlier crop of soybean grown at this experimental

site (Rogers *et al.* 2004) and a detailed study on the sink capacity of the 2002 soybean crop (Rogers & Ainsworth 2006) showed that sink strength peaks in the middle of the season. The carbohydrate data presented here (Fig. 1), and the work of Rogers *et al.* (2004) and Rogers & Ainsworth (2006) show that soybean plants grown at elevated [CO₂] have a surplus of C, particularly at the beginning of the season.

Amino acid synthesis requires reduced N and C skeletons, and represents the hub around which the processes of N assimilation and C metabolism revolve. Free amino acids are the product of N assimilation, and correlate well with N supply (Stitt & Krapp 1999; Foyer *et al.* 2003). Free amino acid levels were lower during the first part of the season. Because carbohydrate levels were high at the beginning of the season, the low amino acid levels probably reflect a low N supply rather than a shortage of C skeletons (Stitt *et al.* 2002; Foyer *et al.* 2003). Together, the low leaf N content and low amino acid content at the beginning of the season provide good evidence that soybean was N limited early in development (Figs 2 & 4). The leaf N content (Fig. 2) at the beginning of the season was even lower at elevated [CO₂]. This shows that the N limitation was exacerbated at elevated [CO₂], as has been seen in many other studies (see Introduction).

However, in the middle of the season, leaf N content rose, and the effect of elevated [CO₂] disappeared. Consistent with predicted increases in N fixation (Ritchie *et al.* 1997), the levels of ureides rose in the middle of the season (Fig. 3) and coincided with the peak in leaf N content (Fig. 2), a marked increase in amino acid content (Fig. 4) and the disappearance of a CO₂ effect on leaf N content. There was no effect of elevated [CO₂] on total amino acid content. However, amino acids involved in photorespiration (glycine, serine) make a large contribution to the total amino acid pool. These typically decrease at elevated [CO₂] as a consequence of the lower rates of photorespiration (Stitt & Krapp 1999). A similar pattern was seen in our experiments where we found lower glycine and serine levels in soybean leaves at elevated [CO₂] (Fig. 5a). The levels of the major (Fig. 5a) and minor amino acids (Fig. 5b) were lower at elevated [CO₂] at the beginning of the season but increased markedly in the middle of the season, indicating that soybeans grown at elevated [CO₂] were able to produce more minor amino acids in the middle of the season than soybeans grown at current [CO₂]. Leaf protein content in the same samples showed a trend similar to that of leaf N content. The disappearance of the CO₂-induced reduction in leaf protein content in the middle of the season suggests that the increased availability of minor amino acids at this time may have been used for additional protein synthesis at elevated [CO₂] (Fig. 5c). Nitrate levels were high at the beginning of the season but then decreased markedly, indicating that nitrate is readily available early in the season, probably from the fertilization of maize the year before, but that nitrate assimilation is unlikely to contribute to the recovery of total leaf N later in the season at elevated [CO₂]. These data indicate that N fixation not only

ameliorated the N limitation that was seen earlier in the growth of this vegetation in both ambient and elevated [CO₂], but was able to keep pace with increased growth at elevated [CO₂] later in the season.

This is consistent with measurements of above-ground primary production, where increases in biomass, plant height and node number due to growth at elevated [CO₂] were not apparent until the second half of the season (Morgan *et al.* 2005). By the end of season, above-ground dry mass in these plants had increased by 15 and 17% in 2002 and 2003, respectively (Morgan *et al.* 2005). Leaf N and total leaf amino acid content showed no sustained effect of elevated [CO₂] in the second half of the season, suggesting that a comparable *c.* 16% increase in N fixation at elevated [CO₂] would be required to match the gains in above-ground biomass. In addition, we were unable to detect a significant effect of CO₂ treatment on ureide content. Because the mass of the plants grown in elevated [CO₂] was greater, this also suggests that N fixation per plant may have increased proportionally.

As soybean develops, plants switch from deriving their N from soil-borne nitrate to ureides produced by their bacterial symbionts (Ritchie *et al.* 1997). The timing of this transition may explain why the deleterious effects of elevated [CO₂] on leaf N content occurred early in the season and were reversed later in the season. Assimilation of 1 M of nitrate into glutamate requires 10 mol of electrons. Growth at elevated [CO₂] stimulates the photosynthetic C reduction cycle that requires 4 mol of electrons for each mole of CO₂ assimilated. Therefore, it has been argued that increased photosynthesis at elevated [CO₂] will compete for available reductant with nitrate assimilation in the leaf (Bloom *et al.* 2002; Rachmilevitch, Cousins & Bloom 2004). A very different situation arises during N fixation, in which the high energetic costs occur in the root nodules. In the leaf, only 2 mol of electrons are required to degrade ureide and re-assimilate the released ammonium into glutamate (Vadez & Sinclair 2000). This could explain why, in contrast to nitrate assimilation, N fixation via the symbiotic association with N-fixing bacteria and utilization of ureides is promoted rather than depressed at elevated [CO₂] (Rachmilevitch, Cousins & Bloom 2004).

Even in the absence of N fertilization, soybean can sustain a large increase in net primary production and seed yield (Morgan *et al.* 2005), without any decline in tissue N content during the periods of peak production and seed development that occur in the second half of the season. Our data suggest that the increased acquisition of C under elevated [CO₂] may have enabled greater N assimilation through the trading of C for N with N-fixing bacteria, and enabled soybean to overcome the potential constraints of a limiting N supply during growth at elevated [CO₂]. Our findings provide a mechanistic basis for the observation that, in N-poor ecosystems, growth at elevated [CO₂] favours legumes that tend to avoid limitations of photosynthetic capacity and dominate non-leguminous species (Hanley, Trofimov & Taylor 2004; Winkler & Herbst 2004) and further suggests that in the absence of other major

limitations (Serraj *et al.* 1999; Almeida *et al.* 2000; Hungate *et al.* 2004), field-grown soybean, and legumes in general, will show a continued increase in productivity with rising [CO₂].

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