Journal of Experimental Botany, Page 1 of 13
doi:10.1093/jxb/erl233

Journal of Experimental Botany

RESEARCH PAPER

Does elevated atmospheric [CO_2] alter diurnal C uptake and the balance of C and N metabolites in growing and fully expanded soybean leaves?

Elizabeth A. Ainsworth[1,2,*], Alistair Rogers[3,4], Andrew D. B. Leakey[2], Lindsey E. Heady[3], Yves Gibon[5], Mark Stitt[5] and Ulrich Schurr[6]

[1] *USDA/ARS Photosynthesis Research Unit and Department of Plant Biology, University of Illinois, Urbana, IL 61801, USA*

[2] *Institute for Genomic Biology, University of Illinois, Urbana, IL 61801, USA*

[3] *Department of Environmental Sciences, Brookhaven National Laboratory, Upton, NY 11973-5000, USA*

[4] *Department of Crop Sciences, University of Illinois, Urbana, IL 61801, USA*

[5] *Max Planck Institut für Molekulare Pflanzenphysiologie, 14476 Golm, Germany*

[6] *ICG-III, Juelich Research Center, D-52425, Juelich, Germany*

Received 19 June 2006; Accepted 16 October 2006

Abstract

Increases in growth at elevated [CO_2] may be constrained by a plant's ability to assimilate nutrients needed for new tissue in sufficient quantity to match the increase in carbon fixation and/or the ability to transport those nutrients and carbon in sufficient quantity to growing organs and tissues. Analysis of metabolites provides an indication of shifts in carbon and nitrogen partitioning due to rising atmospheric [CO_2] and can help identify where bottlenecks in carbon utilization occur. In this study, the carbon and nitrogen balance was investigated in growing and fully expanded soybean leaves exposed to elevated [CO_2] in a free air CO_2 enrichment experiment. Diurnal photosynthesis and diurnal profiles of carbon and nitrogen metabolites were measured during two different crop growth stages. Diurnal carbon gain was increased by *c.* 20% in elevated [CO_2] in fully expanded leaves, which led to significant increases in leaf hexose, sucrose, and starch contents. However, there was no detectable difference in nitrogen-rich amino acids and ureides in mature leaves. By contrast to mature leaves, developing leaves had high concentrations of ureides and amino acids relative to low concentrations of carbohydrates. Developing leaves at elevated [CO_2] had smaller pools of ureides compared with developing leaves at ambient [CO_2], which suggests N assimilation in young leaves was improved by elevated [CO_2]. This work shows that elevated [CO_2] alters the balance of carbon and nitrogen pools in both mature and growing soybean leaves, which could have downstream impacts on growth and productivity.

Key words: Amino acids, elevated [CO_2], FACE, *Glycine max*, hexose, starch, sucrose, ureide.

Introduction

Atmospheric CO_2 concentration ([CO_2]) is now higher than it has been at any time in the past 20 million years and continues to rise at an unprecedented rate (Prentice *et al.*, 2001). Increased photosynthesis (A) at elevated [CO_2] commonly leads to increased plant growth. However, maximum exploitation of a CO_2-rich atmosphere can only be achieved when a plant has sufficient capacity to use the increased supply of carbon (C) available at elevated [CO_2], and this is often limited by the availability of nitrogen (N) (Stitt and Krapp, 1999; Oren *et al.*, 2001;

* To whom correspondence should be addressed. E-mail: ainswort@uiuc.edu

Abbreviations: A, net leaf CO_2 uptake rate (μmol m^{-2} s^{-1}); A', daily integral of CO_2 fixation (mmol C m^{-2} d^{-1}); AA, amino acid; C, carbon; c_i, intercellular [CO_2]; DOY, day of year; FACE, free air CO_2 enrichment; g_s, stomatal conductance to H_2O vapour (mol m^{-2} s^{-1}); LMA, leaf mass per unit area (g m^{-2}); N, nitrogen; PPFD, photosynthetic photon flux density; Pro, protein.

Hungate *et al*., 2003; Luo *et al*., 2004). Investigation of this mechanism has mainly been conducted in mature (fully expanded) leaves (Matt *et al*., 2001; Ellsworth *et al*., 2004). However, the stimulation of productivity by elevated [CO_2] will depend on the metabolic status of growing tissue. Growing leaves are particularly important since greater growth at elevated [CO_2] will lead to a larger leaf area and possibly compound gains in productivity. In addition to its agronomic importance, soybean (*Glycine max* Merr.) is an interesting species to investigate in elevated [CO_2] because it has an association with N-fixing bacteria (*Rhizobiaceae*) that increases N availability to the plant. Soybean has both a large sink capacity (Walsh *et al*., 1987) and the ability to match its N supply to C supply at elevated [CO_2] (Rogers *et al*., 2006). Therefore, indeterminate soybeans are expected to escape the limitation of sink capacity that other species experience at elevated [CO_2].

Determining the presence of an N limitation at elevated [CO_2] is a challenge since many of the ecosystem-level parameters that might provide evidence of N limitation are not very sensitive, and many years or decades may be required to detect significant effects (Luo *et al*., 2004). Measurements of internal metabolite pools could provide a far more sensitive indicator, providing diagnostic metabolites can be identified that rise or fall in N-limited material. Understanding the source–sink balance of a plant is equally important since the regulatory role of carbohydrates is well known. Of particular relevance to elevated [CO_2] is the role of carbohydrates in down-regulating photosynthetic capacity (Long *et al*., 2004; Rogers and Ainsworth, 2006). Therefore, there is increasing interest in determining whether metabolite pools can be used as a measure of metabolic states like the N or the C/N status of plants (Stitt and Krapp, 1999; Matt *et al*., 2001; Foyer *et al*., 2003; Kruse *et al*., 2003; Jeong *et al*., 2004). Metabolite profiles may give an indication of shifts in C and N partitioning due to rising atmospheric [CO_2] and can help identify if bottlenecks in C utilization occur.

There is evidence that metabolite balance in growing leaves is impacted by growth at elevated [CO_2] (Geiger *et al*., 1998; Matt *et al*., 2001). Nielsen and Stitt (2001) carried out a detailed analysis of fluxes in fully expanded and developing tobacco leaves. Whereas the former synthesize predominantly carbohydrates, the latter make small amounts of carbohydrate but large amounts of amino acids and also incorporate newly fixed CO_2 into protein and cell wall material. There have been very few reports of the balance of C and N metabolites in growing (sink) leaves exposed to elevated [CO_2], particularly under field conditions. Data are emerging that correlate changes in leaf growth at elevated [CO_2] with altered metabolite pool sizes. Taylor *et al*. (2003) elegantly showed that the pattern of leaf expansion was altered in *Populus*×*euramericana* leaves exposed to elevated [CO_2] in the field, and hypothesized that C status of the leaf plays an important regulatory role. Walter *et al*. (2005) similarly showed that diel expansion patterns were perturbed by elevated [CO_2] in *Populus deltoides*; in this system, a transient decrease in glucose pools accompanied altered leaf expansion patterns (Walter *et al*., 2005). In young tobacco leaves, biosynthesis and growth dominated metabolism, whereas in mature leaves, assimilation and export dominated (Masclaux *et al*., 2000). Growing leaves therefore have a higher demand for C relative to N compared with mature leaves because protein synthesis requires more C per N than protein export (Foyer and Noctor, 2002). If C export is increased in elevated [CO_2] in mature leaves, then the limitation of biosynthesis by C supply may be alleviated in growing leaves.

In this study, leaf C and N metabolite content in fully expanded and growing soybean leaves exposed to elevated [CO_2] were investigated using free air CO_2 enrichment (FACE). These experiments were conducted twice during the growing season to further assess how the developmental stage of the crop altered C and N metabolic profiles. The aim of the present study was to identify metabolic imbalance which might constrain soybean exploitation of a future high-CO_2 environment, by measuring C and N availability in source and sink soybean leaves grown under elevated [CO_2] in the field. Diurnal courses of gas exchange of young and mature leaves were measured to investigate changes in C flux under elevated [CO_2]. Leaf carbohydrate and amino acid pools were measured throughout the diurnal course to investigate if elevated [CO_2] altered the carbohydrate and amino acid profiles in developing and mature leaves. Previous work at this site suggested that mature leaves would have a higher carbohydrate content, but that the N-fixing ability of soybean would not result in dilution of N metabolites in the leaves (Rogers *et al*., 2006).

Materials and methods

Experimental site

Soybeans cv. 93B15 (Pioneer Hi-Bred) were grown under ambient and elevated [CO_2] at the SoyFACE facility, located in Champaign, IL, USA (40°02′N, 88°14′W, 228 m a.s.l.). The field was planted on 28 May 2004. Measurements were made on 7–8 July 2004 [day of year (DOY) 189] when the crop was in the vegetative 6 (V6) growth phase and on 11–12 August 2004 (DOY 223) when the crop was in the reproductive 5 (R5) growth phase. The SoyFACE experimental design was a randomized complete block design with four blocks. Each block contained two 20-m-diameter octagonal plots, one at current ambient [CO_2] (*c*. 375 μmol mol^{-1}) and one fumigated from sunrise to sunset to an elevated target [CO_2] of 550 μmol mol^{-1}, using the FACE design of Miglietta *et al*. (2001). In 2004, the actual elevated [CO_2] averaged across the growing season was 550 μmol mol^{-1} and 1 mi averages of [CO_2] in the fumigated plots were within ±20% of the target 93% of the time (T Mies, personal communication). The SoyFACE experimental facility is described in detail elsewhere (Ort *et al*., 2006).

Gas exchange measurements

The diurnal course of gas exchange of young (4–5 cm long) and the most recently fully expanded (10–13 cm long) middle leaflets was measured from dawn to dusk on 7 July and 11 August 2004. Both the young and mature leaves were located at the top of the soybean canopy on both sampling dates, and therefore were not shaded. Four portable, open gas exchange systems (LI-COR 6400; LI-COR, Lincoln, NE, USA) were used simultaneously at intervals of *c*. 2 h from early morning to sunset. Three plants were measured in each plot at each time interval. Measurements of gas exchange parameters on all plants were made at growth [CO2] and at ambient air temperature and photosynthetic photon flux density (PPFD). The gas exchange systems were rotated between blocks, and started in different [CO2] treatments at each time point to ensure that measurements were not biased by differences in microclimate over time or by different gas exchange systems (Leakey *et al*., 2004; Rogers *et al*., 2004). For the overall comparison of A and g_s between trifoliates and [CO2] treatments over the diurnal period, a mixed model was fitted to repeated measures in time. Each day was analysed independently, and leaf age and [CO_2] were considered fixed effects, while block was a random effect. Statistics were performed on plot means using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA). The total daily CO_2 uptake (A') was calculated by integrating under the area of the diurnal curve of photosynthesis.

Leaf CO_2 assimilation rate (A) was determined in response to changes in the intercellular [CO_2] (c_i) for developing and fully expanded leaves in June and July 2006, using a portable infrared gas exchange system (LI-6400). Leaves were sampled pre-dawn and kept at low light prior to measurement in order to avoid transient decreases in water potential, decreases in chloroplast inorganic phosphate concentration, or decreases in maximum photosystem II efficiency (Bernacchi *et al*., 2005). Photosynthesis was initially induced at growth [CO_2], then the [CO_2] entering the chamber was reduced stepwise to a lower concentration of 50 μmol mol^{-1}, and then increased stepwise to an upper concentration of 1000 μmol mol^{-1}. Leaf temperature was maintained at 25 °C and PPFD was 1250 μmol m^{-2} s^{-1}. Photosynthetic parameters were calculated by fitting the equations of Farquhar *et al*. (1980) and by maximum likelihood regression (Sigmaplot, Jandel Scientific, Erkrath, Germany).

Leaf metabolite measurements

Directly after making photosynthetic measurements in the field, three leaf discs from each plot and each developmental stage were sampled for biochemical analysis. Samples for leaf carbohydrates were taken at four time points: pre-dawn, approximately solar noon, dusk, and the following dawn. Samples for total soluble protein and total amino acids were taken at midday. Each disc (*c*. 1.8 cm^2) was removed from the middle leaflet while avoiding the midrib, wrapped in foil, plunged immediately into liquid N and stored at −80 °C until analysis. Five discs per plot were also sampled at midday for ureide content and C and N elemental content. These samples were dried to a constant mass prior to analysis.

Foliar contents of carbohydrates, protein, and amino acids were extracted from ground leaf tissue in 80% (v/v), buffered (2 mM HEPES, pH 7.8) ethanol at 80 °C. Four 20 min incubations were needed to recover the soluble metabolites. Glucose, fructose, and sucrose were determined using a continuous enzymatic substrate assay (Jones *et al*., 1977). For protein and starch determination, pellets of the ethanol extraction were solubilized by heating to 95 °C in 0.1 M NaOH. Protein content was determined using a commercial kit (a protein assay kit; Pierce, IL, USA) with BSA as a standard. The NaOH solution was then acidified to pH 4.9 and starch content was determined from glucose equivalents (Rogers *et al*., 2004). Total amino acid contents were determined using a fluorogenic-based microplate assay (Bantan-Polak *et al*., 2001). Individual amino acids were measured from ethanol/water extracts using high performance liquid chromatography as in Geigenberger *et al*. (1996). Leaf N and C content were 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 colorimetric assay (Vadez and Sinclair, 2000). For the comparison of metabolites, a repeated-measures mixed model ANOVA was performed with trifoliate and [CO_2] considered as fixed effects and block as a random effect.

Results

Elevated [CO_2] increased *in situ* rates of photosynthesis when measured in the field in fully expanded leaves (Fig. 1a, b), despite decreased stomatal conductance (Fig. 1c, d; Table 1). The daily integral of carbon uptake (A') was 24% higher in mature leaves grown under elevated [CO_2] on DOY 189 and 16% higher on DOY 223 (Fig. 2). However, a significant stimulation of diurnal C uptake in developing leaves was not detected on either day of measurement during vegetative growth (DOY 189) or reproductive growth (DOY 223) (Figs 1a, b, 2; Table 1). Stomatal conductance was significantly lower in young leaves grown at elevated [CO_2], and intercellular [CO_2] was 12–15% higher in developing leaves compared with fully expanded leaves in both ambient and elevated [CO_2] (Fig. 1e, f). The A/c_i response curves (Fig. 3) showed that fully expanded leaves had higher photosynthetic capacity than developing leaves, but there was no effect of [CO_2] treatment on the shape of the A/c_i response curve.

Elevated [CO_2] increased leaf carbohydrate contents in fully expanded leaves, but not in developing leaves (Fig. 4). Leaf hexose, sucrose, and starch contents were all significantly increased by growth at elevated [CO_2] in fully expanded leaves over the diel cycle during the first time point (Fig. 4; Table 1). There was a clear diel pattern of carbohydrate accumulation during the day and use at night on DOY 189; however, the pattern was not clear on DOY 223, when sucrose and starch accumulated in the leaves, particularly in mature, elevated [CO_2]-grown leaves (Fig. 4).

Developing leaves had significantly lower leaf mass per unit area (LMA) than fully expanded soybean leaves (Fig. 5a, b), and were in the exponential phase of expansion in both ambient and elevated [CO_2] (data not shown). Elemental analysis of leaf C and N revealed that developing leaves had the same N content as fully expanded leaves early in the growing season (Fig. 5c) and slightly lower N content later in the growing season (Fig. 5d), when measured on an area basis. However, during both time points, developing leaves had lower C:N ratios (Fig. 5e, f) than fully expanded leaves in both ambient and elevated [CO_2]. As expected from the photosynthesis measurements, total chlorophyll content was significantly

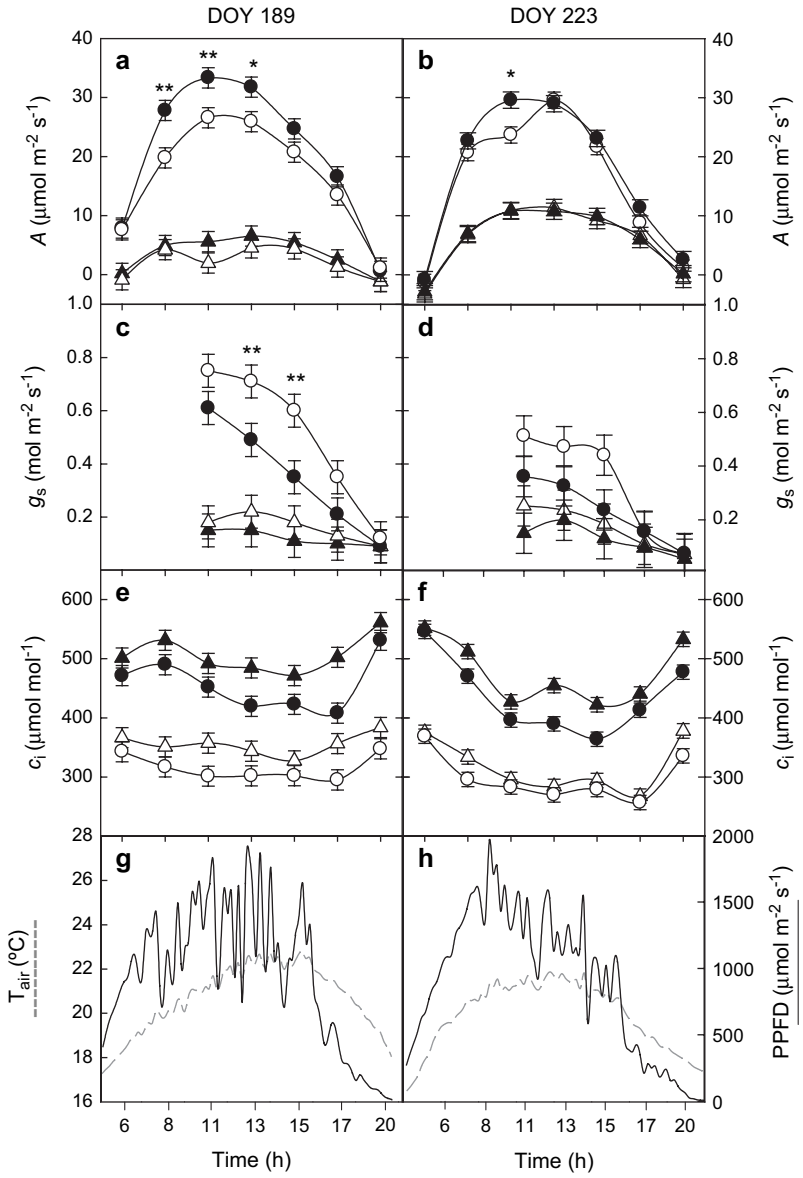

Fig. 1. Diurnal course of photosynthesis (A; a, b), stomatal conductance (g_s; c, d) and intercellular [CO_2] (c_i; e, f) on 7 July 2004 (DOY 189) and 11 August 2004 (DOY 223) in fully expanded (circles) and developing (triangles) soybeans exposed to ambient [CO_2] (open symbols) and elevated [CO_2] (closed symbols). Diurnal courses of photosynthesis (A) and stomatal conductance (g_s) were measured *in situ* at growth [CO_2], and at ambient light and temperature (g, h) conditions. Data are least square means ±1 standard error of the difference of means. The results of the statistical analysis of A and g_s are shown in Table 1. Significant differences between elevated and ambient means in fully expanded leaves within time points are marked with asterisks: *P <0.05; **P <0.01; ***P <0.001.

lower in developing leaves (Fig. 5g, h). Total protein content was not affected by [CO_2] treatment or developmental stage on DOY 189 (Fig. 5i), but was significantly lower in developing leaves on DOY 223 (Fig. 5j). Free amino acid content was higher in developing leaves compared with fully expanded leaves (Table 2; Fig. 5k, l), and was significantly higher in young developing leaves grown at elevated [CO_2] on DOY 189 (Table 2; Fig. 5k). Ureide levels, measured as allantoin, were significantly and markedly higher in developing leaves on both days (Fig. 6). There was no effect of elevated [CO_2] on ureide levels in fully expanded leaves but, in developing leaves, elevated [CO_2] resulted in a significant reduction in ureide content (Fig. 6).

Individual amino acids were measured over the course of the day on DOY 223. Elevated [CO_2] did not significantly affect amino acid content (Table 3; Figs 7–9). Individual amino acid content was highly dependent upon the developmental stage of the leaf. Glu (Fig. 7a), Gln (Fig. 7b), Gly (Fig. 8a), Asn (Fig. 9a), and Ala (Fig. 9c) were all higher in developing leaves than fully expanded leaves (Table 3). The content of most amino

Table 1. *Statistical analysis of diurnal photosynthesis (A), stomatal conductance (g_s), intercellular [CO_2] (c_i), leaf hexose, sucrose, and starch content*

A mixed model was fitted to repeated measures in time for overall comparisons, with [CO_2] treatment ([CO_2]) and leaf developmental age (Age) considered fixed effects, and time a repeated measure. Each day was analysed independently. Significant results (F, P) from the ANOVA are shown in bold.

	A	g_s	c_i	Hexose	Sucrose	Starch
DOY 189						
[CO_2]	**17.17**, **<0.001**	**6.62**, **0.012**	**553.94**, **<0.001**	**22.26**, **0.003**	**8.43**, **0.062**	**21.20**, **0.019**
Age	**598.97**, **<0.001**	**137.21**, **<0.001**	**51.93**, **<0.001**	**15.18**, **<0.001**	**142.21**, **<0.001**	**282.41**, **<0.001**
[CO_2]×Age	3.87, 0.053	1.82, 0.181	0.67, 0.416	**11.40**, **0.002**	**7.23**, **0.010**	**13.61**, **<0.001**
Time	**63.54**, **<0.001**	**68.84**, **<0.001**	**10.43**, **<0.001**	**8.83**, **<0.001**	**8.51**, **<0.001**	**24.63**, **<0.001**
[CO_2]×Time	1.37, 0.235	1.86, 0.099	1.91, 0.089	0.24, 0.865	0.33, 0.802	1.83, 0.156
[CO_2]×Age×Time	**12.99**, **<0.001**	**7.78**, **<0.001**	0.68, 0.763	0.40, 0.878	**2.42**, **0.043**	**2.40**, **0.044**
DOY 223						
[CO_2]	**6.22**, **0.015**	0.78, 0.411	**597.02**, **<0.001**	**15.19**, **<0.001**	0.94, 0.369	**17.01**, **<0.001**
Age	**340.46**, **<0.001**	**14.58**, **<0.001**	**43.35**, **<0.001**	0.03, 0.869	**291.84**, **<0.001**	**253.05**, **<0.001**
[CO_2]×Age	**5.16**, **0.026**	2.95, 0.090	**6.03**, **0.016**	**5.52**, **0.023**	0.64, 0.430	**20.27**, **<0.001**
Time	**154.79**, **<0.001**	**32.08**, **<0.001**	**68.49**, **<0.001**	2.64, 0.060	**7.60**, **<0.001**	**41.73**, **<0.001**
[CO_2]×Time	0.32, 0.924	1.54, 0.175	**4.62**, **<0.001**	**3.25**, **0.030**	**3.75**, **0.018**	0.34, 0.798
[CO_2]×Age×Time	**11.95**, **0.001**	1.61, 0.107	1.18, 0.315	**2.66**, **0.026**	**6.49**, **<0.001**	**4.70**, **<0.001**

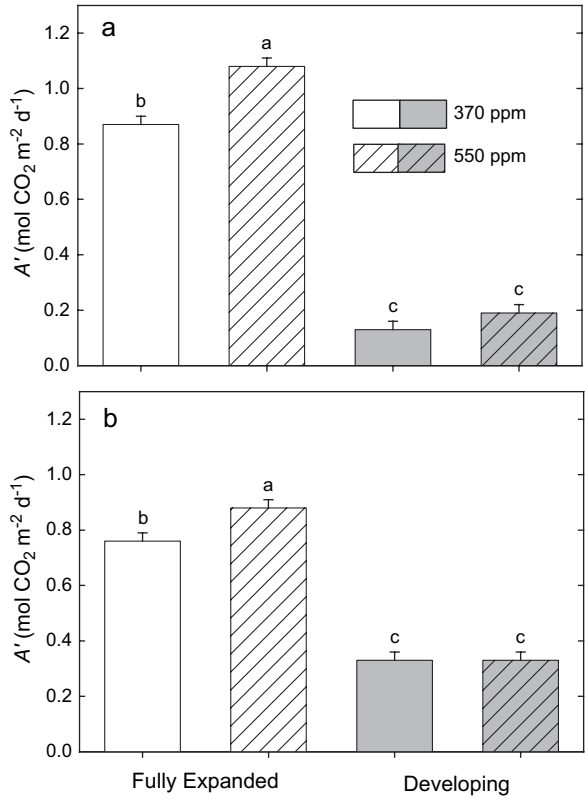

Fig. 2. Daily integral of net CO_2 assimilation on (a) DOY 189 and (b) DOY 223. On both days, there was a significant interaction between CO_2 and leaf age (P <0.05). Significant differences between elevated and ambient means in fully expanded leaves within time points are marked with letters (P <0.05).

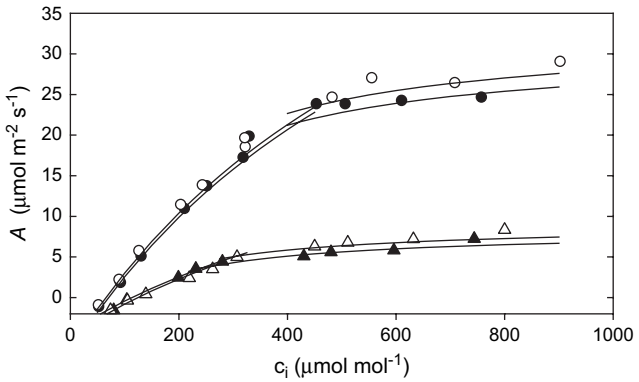

Fig. 3. Representative A/c_i responses of fully expanded (circles) and developing (triangles) soybeans exposed to ambient [CO_2] (open symbols) and elevated [CO_2] (closed symbols). $V_{c,max}$ was estimated from points below the inflexion and J_{max} was estimated from points above the inflexion.

acids also varied over the course of the day, with midday content highest for Glu (Fig. 7a), Gln (Fig. 7b), Ser (Fig. 8b), and Ala (Fig. 9c). The ratio of Glu:Gln was highest for fully expanded leaves exposed to elevated [CO_2], and more than three times higher in fully expanded leaves compared with young leaves (Fig. 7c). When averaged across leaf ages, the ratio of Gly:Ser was lower for plants grown in elevated [CO_2] compared with ambient [CO_2] (Fig. 8c).

Discussion

An increase in atmospheric [CO_2] to levels predicted for 2050 caused changes in C flux and C and N metabolites in fully expanded and developing soybean leaves. Elevated [CO_2] increased photosynthesis in fully expanded soybean leaves, and decreased stomatal conductance in leaves of both ages (Fig. 1). Carbohydrate content was high in fully expanded leaves and significantly increased by elevated [CO_2], while in developing leaves carbohydrate content was low and unaffected by [CO_2] (Fig. 3). By contrast,

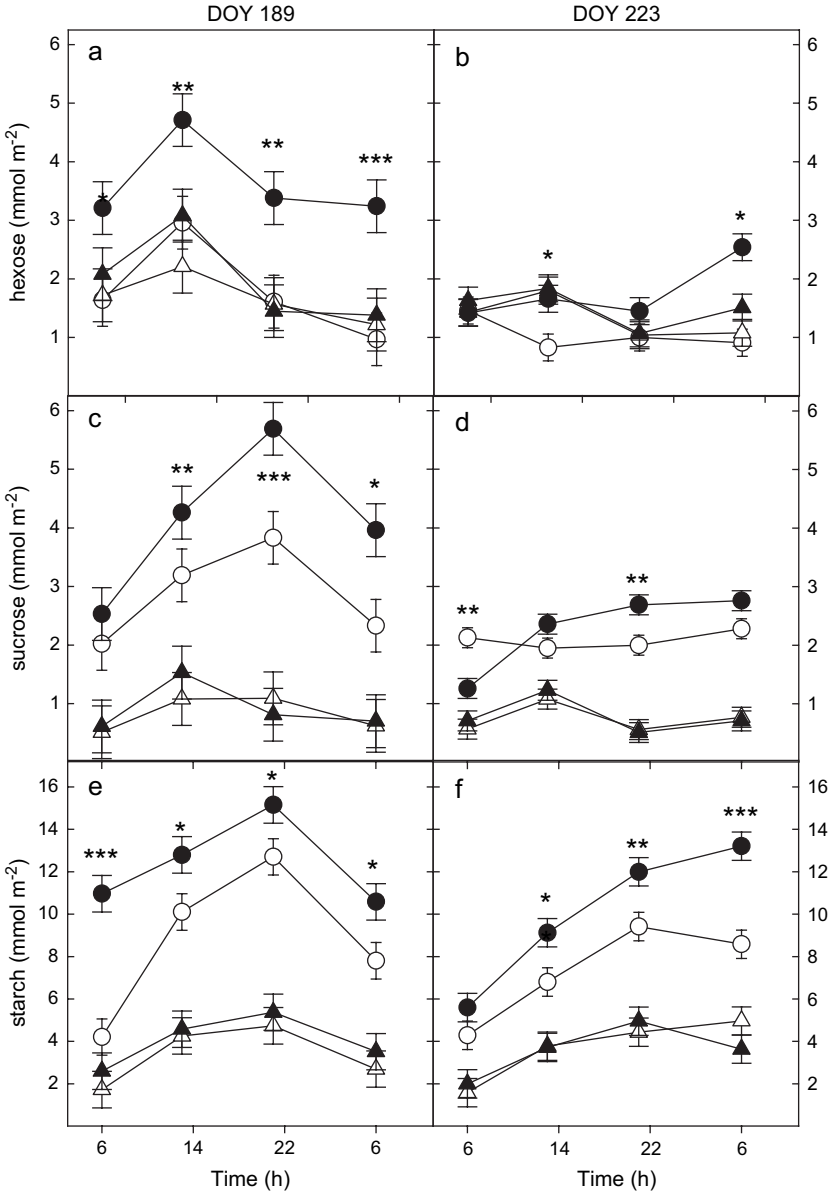

Fig. 4. Hexose, sucrose, and starch content of developing and fully expanded soybean leaves exposed to ambient and elevated [CO_2]. Results of the statistical analysis of hexose, sucrose, and starch content are shown in Table 1. Symbols and error bars are as described for Fig. 1. Significant differences between elevated and ambient means in fully expanded leaves within time points are marked with asterisks: *P <0.05; **P <0.01; ***P <0.001.

amino acid content was low and unaffected by [CO_2] in fully expanded leaves, while amino acid content in developing leaves was high (Figs 4, 6–8). Ureide content was also lower in fully expanded leaves than in developing leaves, and was significantly reduced by elevated [CO_2] in developing leaves (Fig. 5). These general changes were not affected by the developmental stage of the crop, nor was leaf area index significantly increased at elevated [CO_2] at any point during the 2004 growing season (O Dermody, personal communication). The variation that did occur between the dates of sampling was consistent with previously observed developmental patterns (e.g. ureide content from Rogers *et al*., 2006) and the effects of general variability in growth conditions (e.g. photosynthesis in Bernacchi *et al*., 2006).

Responses of mature leaves to elevated [CO_2]

The response of fully expanded soybean leaves to elevated [CO_2] resembled that observed in earlier studies (Rogers *et al*., 2004, 2006). Photosynthesis in mature leaves was very similar at both time points, despite different rates of stomatal conductance (Fig. 1). This can be explained by variation in carboxylation efficiency, which has been demonstrated to vary by up to 20% over the

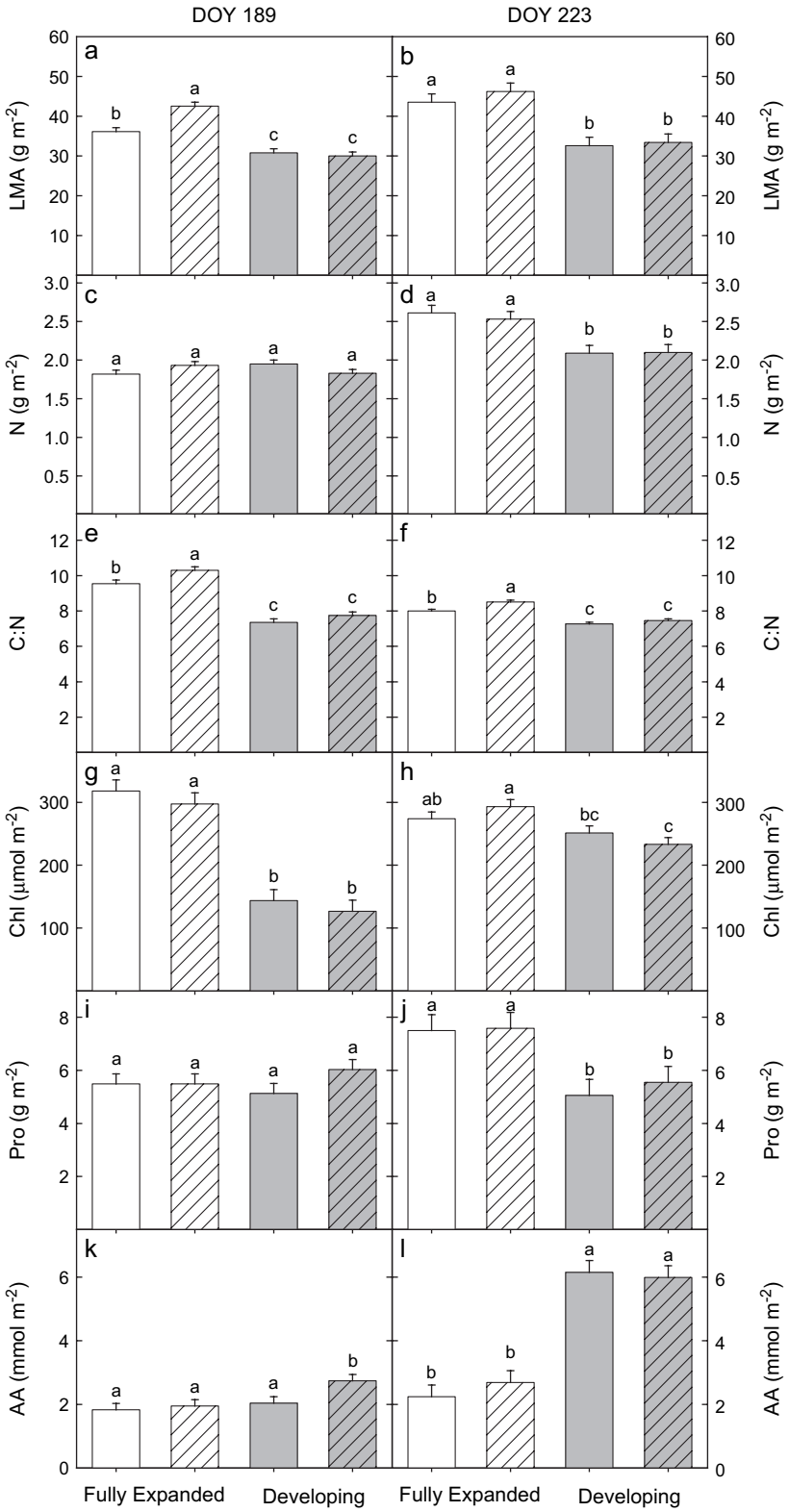

Fig. 5. Mean specific leaf weight (SLW) leaf N, leaf C:N, total chlorophyll (Chl), protein (Pro), and amino acid (AA) content of growing (grey columns) and fully expanded (white columns) soybeans exposed to ambient [CO_2] (open columns) and elevated [CO_2] (hatched columns). Error bars show the standard error of the least squared mean. Significant differences between means (P <0.05) within a time point are indicated with letters.

Table 2. *Statistical analysis (F, P) of leaf N, C, the C:N ratio, total leaf chlorophyll content, protein, amino acid content, and allantoin content shown in Figs 4 and 5*

A mixed model was fitted for overall comparisons, with [CO_2] treatment ([CO_2]) and leaf developmental age (Age) considered fixed effects. Each day was analysed independently. Significant results from the ANOVA (P <0.05) are shown in bold.

	DOY 189			DOY 223		
	[CO_2]	Age	[CO_2]×Age	[CO_2]	Age	[CO_2]×Age
SLW	**6.00**, **0.05**	**124.6**, **<0.001**	**20.06**, **0.004**	0.44, 0.53	**80.02**, **<0.001**	0.55, 0.49
N	0.02, 0.90	0.09, 0.77	**6.01**, **0.03**	0.11, 0.76	**57.28**, **<0.001**	0.58, 0.48
C:N	7.62, 0.06	**454.0**, **<0.001**	2.87, 0.14	**16.51**, **0.003**	**88.75**, **<0.001**	3.79, 0.08
Chlorophyll	1.80, 0.21	**154.4**, **<0.001**	0.02, 0.90	0.00, 0.96	**14.11**, **0.003**	2.95, 0.11
Protein	0.94, 0.37	0.09, 0.77	2.52, 0.16	0.32, 0.59	**19.12**, **0.002**	0.17, 0.69
Amino acid	4.42, 0.13	**9.56**, **0.02**	3.32, 0.12	0.17, 0.69	**100.7**, **<0.001**	0.72, 0.42
Allantoin	**6.27**, **0.03**	**19.01**, **<0.001**	**4.20**, **0.06**	**6.10**, **0.04**	**45.31**, **<0.001**	**9.38**, **0.01**

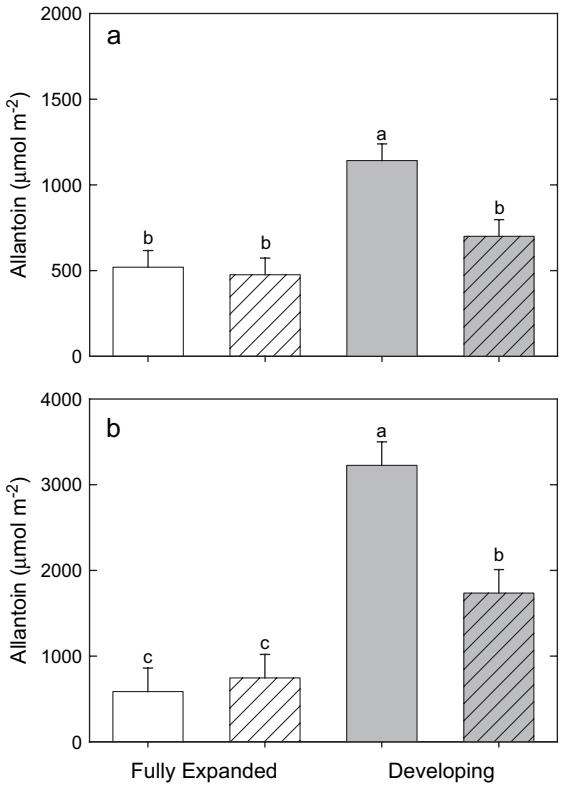

Fig. 6. Midday content of allantoin measured on (a) DOY 189 and (b) DOY 223 for plants grown at ambient [CO_2] (open columns) and elevated [CO_2] (hatched columns), as in Fig. 2. On both days, there was a significant interaction between CO_2 and leaf age (P <0.1). Allantoin content was not affected by elevated [CO_2] in fully expanded leaves, but was significantly lower in developing leaves exposed to elevated [CO_2]. Significant differences between elevated and ambient means in developing leaves within time points are marked with letters (P <0.05).

course of the growing season in field-grown soybean (Bernacchi *et al*., 2005). Based on the instantaneous increase in [CO_2] from 380 to 550 µmol mol^{-1}, an approximate 29% stimulation would be anticipated in photosynthesis in mature leaves (Fig. 3), which is consistent with the measured 22.5% and 24.9% differences in midday photosynthesis measured under field conditions (Fig. 1). Total leaf C, hexoses, sucrose, and starch were significantly higher in fully expanded leaves grown at elevated [CO_2], compared with fully expanded leaves grown at ambient [CO_2] (Fig. 4), as has been commonly reported in crops exposed to elevated [CO_2] (reviewed by Drake *et al*., 1997; Ainsworth *et al*., 2002; Ainsworth and Long, 2005). These physiological markers of leaf photosynthesis and carbohydrate status corresponded with increased above-ground productivity at elevated [CO_2] in multiple years at this field site (Rogers *et al*., 2004, 2006; Morgan *et al*., 2005). The greater starch content and LMA at elevated [CO_2] is also consistent with many previous studies in which greater starch content at elevated [CO_2] led to significantly greater LMA (Peterson *et al*., 1999; Ainsworth and Long, 2005). All metabolite data are therefore presented here on a leaf area basis.

By contrast to leaf C pools, leaf N pools were largely unaffected by elevated [CO_2], causing an increase in the C:N ratio of mature leaves. Ureide content was the same in fully expanded leaves grown at ambient and elevated [CO_2] (Fig. 6), and pools of amino acids were also largely unaffected (Figs 7–9). These results agree with prior results from SoyFACE, where ureide content in fully expanded leaves was significantly affected by growth stage, but not by [CO_2] (Rogers *et al*., 2006). Other studies have shown that ureide concentration in soybean leaves decreases at elevated [CO_2], and that elevated [CO_2] changes the response of N_2 fixation to soil water content (Serraj *et al*., 1998; Serraj and Sinclair, 2003). Crops at SoyFACE did not experience water stress at any time during the growing season in 2004 when the measurements were taken (Leakey *et al*., 2006); therefore, any potential changes in the response of N metabolites to drought at elevated [CO_2] were unlikely to occur during this study.

Responses of developing leaves to elevated [CO_2]

How developing leaves respond to elevated [CO_2] has received much less attention than mature leaves. Osborne *et al*. (1998) and Adam *et al*. (2000) reported that

Table 3. *Statistical analysis (*F, P*) of amino acid content measured on DOY 223*

A mixed model was fitted to repeated measures in time for overall comparisons, with [CO_2] treatment ([CO_2]) and leaf developmental age (Age) considered fixed effects, and time a repeated measure. Significant results (P <0.05) from the ANOVA are shown in bold.

	[CO_2]	Age	[CO_2]×Age	Time	[CO_2]×Time	[CO_2]×Age×Time
Glu	1.34, 0.33	**5.70**, **0.02**	0.58, 0.45	**16.3**, **<0.001**	0.39, 0.68	**3.20**, **0.03**
Gln	0.32, 0.59	**74.7**, **<0.001**	1.75, 0.20	**19.6**, **<0.001**	0.43, 0.66	1.50, 0.23
Glu:Gln	3.08, 0.09	**45.5**, **<0.001**	3.91, 0.06	**9.85**, **<0.001**	0.18, 0.84	**6.27**, **<0.001**
Gly	1.45, 0.24	**11.9**, **0.002**	1.48, 0.23	**13.2**, **<0.001**	0.87, 0.43	0.46, 0.77
Ser	0.49, 0.54	1.05, 0.31	0.12, 0.73	**38.4**, **<0.001**	0.03, 0.97	1.14, 0.36
Gly:Ser	3.11, 0.13	**27.9**, **<0.001**	3.0, 0.09	**13.3**, **<0.001**	0.51, 0.61	**3.38**, **0.02**
Asn	2.60, 0.21	**8.13**, **0.008**	0.92, 0.35	**15.5**, **<0.001**	0.54, 0.59	**3.74**, **0.01**
Asp	1.08, 0.34	**48.1**, **<0.001**	0.01, 0.94	**11.5**, **<0.001**	0.11, 0.90	**3.67**, **0.002**
Ala	0.78, 0.58	**6.45**, **0.02**	1.87, 0.18	**17.2**, **<0.001**	0.26, 0.77	**3.87**, **0.01**

acclimation of photosynthetic capacity in wheat occurred in older, shaded leaves, but not young, photosynthetically mature flag leaves. An increase in diurnal photosynthesis could not be detected at elevated [CO_2] in developing soybean leaves (Figs 1, 2); however, developing leaves had significantly higher intercellular [CO_2] compared with fully expanded leaves (Fig. 1e, f). From the A/c_i response curve, an 18.6% increase in light-saturated photosynthesis would be anticipated in developing leaves (Fig. 3) at 25 °C. In fact, on DOY 189, midday photosynthesis was *c*. 20% higher at elevated [CO_2], but this was outside of the range of statistical detection in the gas exchange measurements. On DOY 223, photosynthesis was not higher in developing leaves at any time during the day (Fig. 1). Developing leaves had low levels of chlorophyll compared with fully expanded leaves, but there was no [CO_2] affect on chlorophyll content (Fig. 4g, h). There was a striking decrease in stomatal conductance in young leaves grown at elevated [CO_2] (Fig. 1c, d), which may be a mechanism by which developing leaves prioritize water for expansion over transpiration. Ureides are delivered to the leaf in the transpiration stream and the reduced stomatal conductance at elevated [CO_2] may explain the decreased ureide content. However, it is felt that this is unlikely because stomatal conductance was markedly higher in fully expanded leaves where ureide levels were low and also where there was no effect of the reduced stomatal conductance on ureide content. Developing leaves had unique metabolite contents, including low levels of carbohydrate and high levels of ureides and amino acids, namely Glu, Gln, and Asn. These three amino acids were previously identified as markers of young leaves in quaking aspen (Jeong *et al*., 2004). The low LMA of developing leaves versus mature leaves means that these markers of juvenile status reflect an even greater change in ureide and amino acid concentration per unit dry mass with developmental stage. Elevated [CO_2] decreased ureide content (Fig. 6), transiently increased amino acid content (Fig. 4k), and had no obvious effect on leaf carbohydrates (Fig. 4). Without an increase in carbohydrate content at elevated [CO_2], there was no change in LMA of developing leaves.

Implications for whole plant C and N status at elevated [CO_2]

When averaged across leaf ages and three time points, the Gly:Ser ratio was 15% lower in plants grown under elevated [CO_2]. A decreased Gly:Ser ratio is frequently seen in elevated [CO_2] (Stitt and Krapp, 1999; Matt *et al*., 2001; Rogers *et al*., 2006). Novitskaya *et al*. (2002) demonstrated that with increased photorespiratory flux, C flooded into glycolate, leading to Gly accumulation and an increase in the Gly:Ser ratio. Novitskaya *et al*. (2002) also reported a negative correlation between Asp and photorespiration. The present data also show a higher Asp level in the fully expanded leaves grown at elevated [CO_2]; both observations, along with higher intercellular [CO_2], provide strong evidence that there was a decrease in photorespiratory flux in soybeans growing at elevated [CO_2]. Calculation of C export by mass balance (Rogers *et al*., 2004) provides a crude estimate of C available to developing sink tissue. Consistent with decreased photorespiration and increased photosynthesis, there was 15–29% more carbon exported from fully expanded source leaves at elevated [CO_2] than at ambient [CO_2]. The adjacent developing trifoliate leaves are likely to be strong proximal sinks for this additional photosynthate (Farrar and Williams, 1991; Farrar, 1996), particularly during vegetative growth (DOY 189), but also during the reproductive phase (DOY 223) in this indeterminate cultivar. Fixed N requires C skeletons for assimilation and further biosynthesis (Todd *et al*., 2006). Therefore, imported C could be used to fuel biosynthesis in the developing leaves, possibly explaining why ureide levels were lower in developing leaves grown at elevated [CO_2] compared with those grown at current [CO_2]. Further experiments would be needed to determine if more C were in fact exported from fully expanded leaves to developing leaves under elevated [CO_2].

Another possibility is that long-distance signals related to higher carbohydrate status in elevated [CO_2] drive

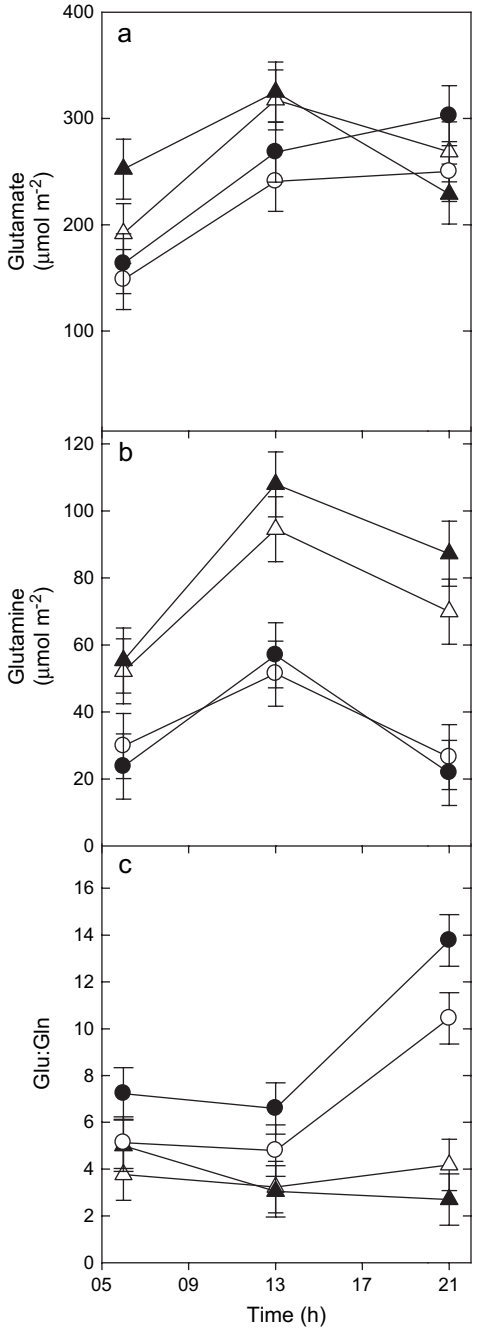

Fig. 7. Diurnal measurements of glutamate (a), glutamine (b), and the ratio of glutamate to glutamine [Glu:Gln (c)] measured on 11 August 2004. Symbols and error bars are as described for Fig. 1.

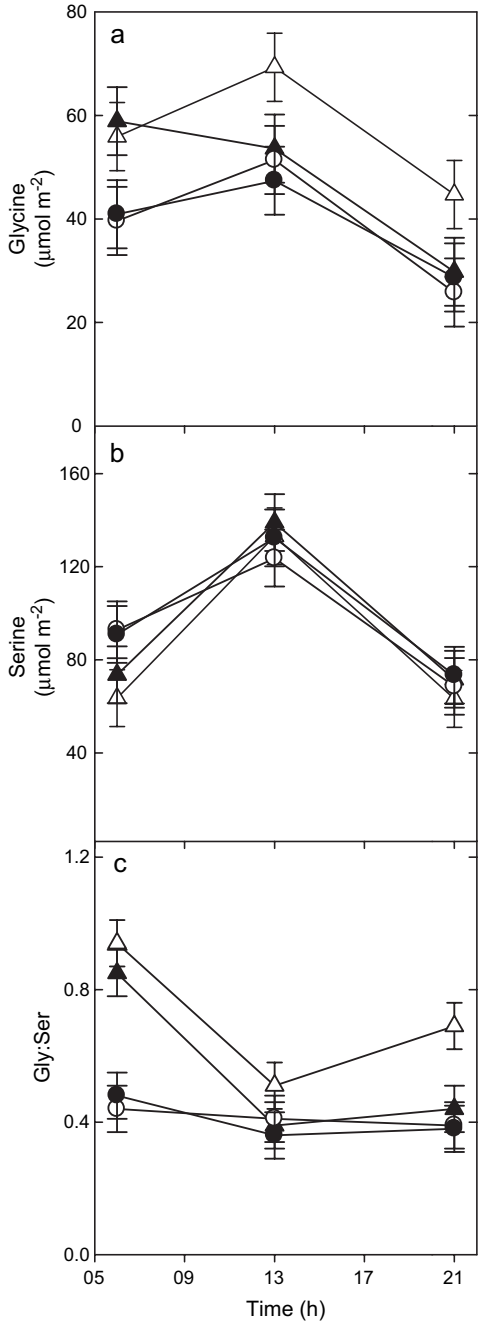

Fig. 8. Diurnal measurements of glycine (a), serine (b), and the Gly:Ser ratio (c) measured on 11 August 2004. Symbols and error bars are as described for Fig. 1.

increased growth in developing leaves; however, if growth were increased, it was not apparent by measurements of leaf area index (O Dermody, personal communication). Long-distance signalling from mature to developing leaves is one mechanism by which stomatal development responds to elevated [CO_2] (Lake *et al*., 2002), so it is possible that a similar signal stimulates growth. Sims *et al*. (1998) provided further evidence for the role of long-distance signals. They found that the photosynthetic capacity of a soybean leaf depended on the [CO_2] surrounding the plant, not the [CO_2] surrounding the leaf. Controlled experiments where a mature leaf is maintained at one [CO_2] and a developing leaf at a different [CO_2] would be needed to further investigate how growth is altered by long-distance signalling.

What do the changes in C and N metabolites suggest for the C and N balance of the soybean plants? N content of the soybeans was significantly higher during the early

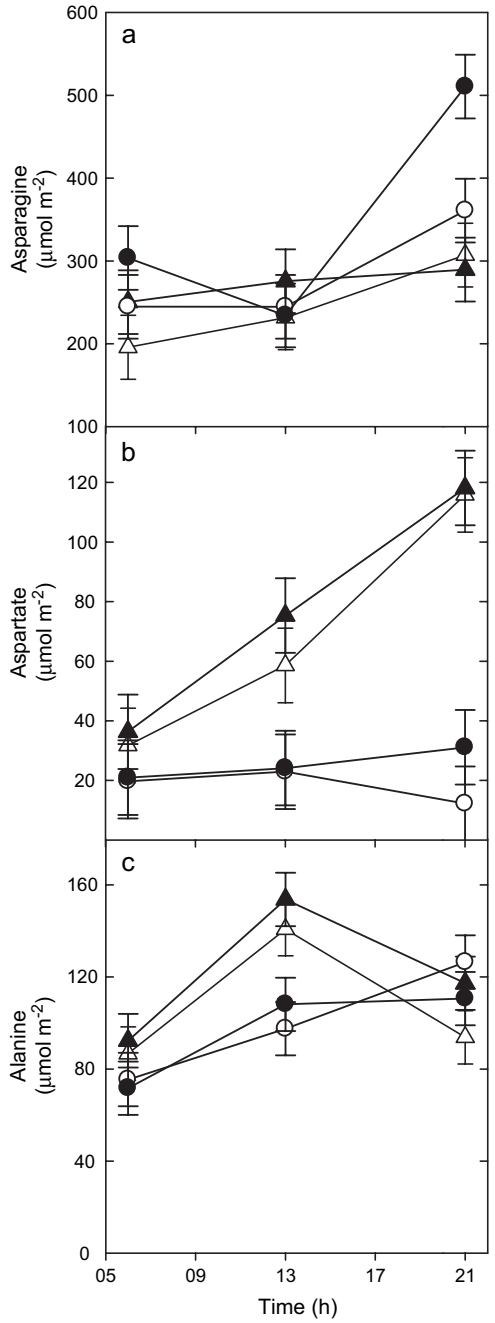

Fig. 9. Diurnal measurements of asparagine (a), aspartate (b), and alanine (c) measured on 11 August 2004. Symbols and error bars are as described for Fig. 1.

reproductive phase compared with the vegetative phase in both ambient and elevated [CO_2] (Fig. 4). Maximum N content of many crops is reached at the early reproductive phase and, with further development, available N is depleted and nodules senesce in annual legumes (Peoples and Gifford, 1997). Ureide content in young leaves harvested during early reproductive growth was nearly double that in young leaves harvested during vegetative growth (Fig. 5), consistent with the well-characterized changes in N fixation known to occur during crop development (Ritchie *et al*., 1997). Since there was no evidence of a reduction in N content, and soybean above-ground biomass was 32% and 24% greater in elevated [CO_2] at the approximate times when the sampling for the present study was made (K McConnaughay, personal communication), it suggests that N fixation per plant increased proportionally (Rogers *et al*., 2006), i.e. elevated [CO_2] increased C available for N_2 fixation and/or enabled plants at elevated [CO_2] to take advantage of fixed N pools by making more C skeletons available for N assimilation (Rogers *et al*., 2006). Interestingly, despite the increase in above-ground biomass at elevated [CO_2], leaf area index was not increased. Adapting soybeans to allocate resources towards increased leaf area may be an important strategy to improve plant performance in future environmental conditions.

Acknowledgements

We thank Steve Long, Orla Dermody, Kelly McConnaughay, Tim Mies, Joe Castro, Kelly Gillespie, Maja Christ, Katie Ciccodicola, and Regina Feil. EAA was supported by an Alexander von Humboldt post-doctoral research fellowship. MS acknowledges support from the Max Planck Society and from the European Commission (Integrated Project 'Grain legumes'). AR and LEH were supported by the US Department of Energy Office of Science contract No. DE-AC02-98CH10886 to Brookhaven National Laboratory and a Science Undergraduate Laboratory Internship to LEH. SoyFACE was supported by the Illinois Council for Food and Agricultural Research, Archer Daniels Midland Company, the US Department of Agricultural, and the Illinois Agricultural Experiment Station.

References

Adam NR, Wall GW, Kimball BA, *et al.* 2000. Acclimation response of spring wheat in a free-air CO_2 enrichment (FACE) atmosphere with variable soil nitrogen regimes. 1. Leaf position and phenology determine acclimation response. *Photosynthesis Research* **66,** 65–77.

Ainsworth EA, Davey PA, Bernacchi CJ, *et al.* 2002. A meta-analysis of elevated [CO_2] effects on soybean (*Glycine max*) physiology, growth and yield. *Global Change Biology* **8,** 695–709.

Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytologist* **165,** 351–372.

Bantan-Polak T, Kassai M, Grant KB. 2001. A comparison of fluorescamine and naphthalene-2,3-dicarboxaldehyde fluorogenic reagents for microplate-based detection of amino acids. *Analytical Biochemistry* **297,** 128–136.

Bernacchi CJ, Leakey ADB, Heady LE, *et al.* 2006. Hourly and seasonal variation in photosynthesis and stomatal conductance of soybean grown at future CO_2 and ozone concentrations for 3 years under fully open-air field conditions. *Plant, Cell and Environment* **29,** 2077–2090.

Bernacchi CJ, Morgan PB, Ort DR, Long SP. 2005. The growth of soybean under free air [CO_2] enrichment (FACE) stimulates photosynthesis while decreasing *in vivo* Rubisco capacity. *Planta* **220,** 434–446.

Drake BG, GonzalezMeler MA, Long SP. 1997. More efficient plants: a consequence of rising atmospheric CO_2? *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 609–639.

Ellsworth DS, Reich PB, Naumberg ES, Koch GW, Kubiske ME, Smith SD. 2004. Photosynthesis, carboxylation and leaf nitrogen responses of 16 species to elevated pCO_2 across four free-air CO_2 enrichment experiments in forest, grassland and desert. *Global Change Biology* **10,** 2121–2138.

Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO_2 fixation in C_3 species. *Planta* **149,** 178–190.

Farrar JF. 1996. Sinks-integral parts of a whole plant. *Journal of Experimental Botany* **47,** 1273–1279.

Farrar JF, Williams ML. 1991. The effects of increased atmospheric carbon dioxide and temperature on carbon partitioning, source-sink relations and respiration. *Plant, Cell and Environment* **14,** 819–830.

Foyer CH, Noctor G. 2002. Photosynthetic nitrogen assimilation: inter-pathway control and signaling. In: Foyer CH, Noctor G, eds. *Photosynthetic nitrogen assimilation and associated carbon and respiratory metabolism*. The Netherlands: Kluwer Academic Publishers, 1–22.

Foyer CH, Parry M, Noctor G. 2003. Markers and signals associated with nitrogen assimilation in higher plants. *Journal of Experimental Botany* **54,** 585–593.

Geigenberger P, Lerchl J, Stitt M, Sonnewald U. 1996. Phloem-specific expression of pyrophosphatase inhibits long-distance transport of carbohydrates and amino acids in tobacco plants. *Plant, Cell and Environment* **19,** 43–55.

Geiger M, Walch-Liu P, Engels C, Harnecker J, Schulze ED, Ludewig F, Sonnewald U, Scheible WR, Stitt M. 1998. Enhanced carbon dioxide leads to a modified diurnal rhythm of nitrate reductase activity in older plants, and a large stimulation of nitrate reductase activity and higher levels of amino acids in young tobacco plants. *Plant, Cell and Environment* **21,** 253–268.

Hungate BA, Dukes JS, Shaw MR, Luo Y, Field CB. 2003. Nitrogen and climate change. *Science* **320,** 1512–1513.

Jeong ML, Jiang HY, Chen HS, Tsai CJ, Harding SA. 2004. Metabolic profiling of the sink-to-source transition in developing leaves of quaking aspen. *Plant Physiology* **136,** 3364–3375.

Jones MGK, Outlaw WH, Lowry OH. 1977. Enzymic assay of 10^{-7} to 10^{-14} moles of sucrose in plant tissues. *Plant Physiology* **60,** 379–383.

Kruse J, Hetzger I, Hansch RH, Mendel RR, Rennenberg H. 2003. Elevated pCO_2 affects C and N metabolism in wild type and transgenic tobacco exhibiting altered C/N balance in metabolite analysis. *Plant Biology* **5,** 540–549.

Lake JA, Woodward FI, Quick WP. 2002. Long-distance CO_2 signalling in plants. *Journal of Experimental Botany* **53,** 183–193.

Leakey ADB, Bernacchi CJ, Dohleman FG, Ort DR, Long SP. 2004. Will photosynthesis of maize (*Zea mays*) in the US Corn Belt increase in future $[CO_2]$ rich atmospheres? An analysis of diurnal courses of CO_2 uptake under free-air concentration enrichment (FACE). *Global Change Biology* **10,** 951–962.

Leakey ADB, Uribelarrea M, Ainsworth EA, Naidu SL, Rogers A, Ort DR, Long SP. 2006. Photosynthesis, productivity and yield of *Zea mays* are not affected by open-air elevation of CO_2 concentration in the absence of drought. *Plant Physiology* **140,** 779–790.

Long SP, Ainsworth EA, Rogers A, Ort DR. 2004. Rising atmospheric carbon dioxide: plants FACE the future. *Annual Review of Plant Biology* **55,** 591–628.

Luo Y, Su B, Currie WS, *et al.* 2004. Progressive nitrogen limitation of ecosystem responses to rising atmospheric carbon dioxide. *Bioscience* **54,** 731–739.

Masclaux C, Valadier M-H, Brugiere N, Morot-Gaudry J-F, Hirel B. 2000. Characterization in the source/sink in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. *Planta* **211,** 510–518.

Matt P, Geiger M, Walch-Liu P, Engels C, Krapp A, Stitt M. 2001. The diurnal changes of nitrogen metabolism in leaves of nitrate-replete tobacco are driven by an imbalance between nitrate reductase activity and the rate of nitrate uptake and ammonium and glutamine metabolism during the first part of the light period. *Plant, Cell and Environment* **24,** 177–190.

Miglietta F, Peressotti A, Vaccari FP, Zaldei A, de Angelis P, Scarascia-Mugnozza G. 2001. Free-air CO_2 enrichment (FACE) of a poplar plantation: the POPFACE fumigation system. *New Phytologist* **150,** 465–476.

Morgan PB, Bollero GA, Nelson RL, Dohleman FG, Long SP. 2005. Smaller than predicted increase in aboveground net primary production and yield of field-grown soybean under fully open-air $[CO_2]$ elevation. *Global Change Biology* **11,** 1856–1865.

Nielsen TH, Stitt M. 2001. Tobacco transformants with strongly decreased expression of pyrophosphate:fructose-6-phosphate expression in the base of their sink leaves contain much higher levels of fructose-2,6-bisphosphate but exhibit no major changes in fluxes. *Planta* **214,** 106–116.

Novitskaya L, Trevanion SJ, Driscoll S, Foyer CH, Noctor G. 2002. How does photorespiration modulate leaf amino acid contents? A dual approach through modelling and metabolite analysis. *Plant, Cell and Environment* **25,** 821–835.

Oren R, Ellsworth DS, Johnsen KH, *et al.* 2001. Soil fertility limits carbon sequestration by forest ecosystems in a CO_2-enriched atmosphere. *Nature* **411,** 469–472.

Ort DR, Ainsworth EA, Aldea M, *et al.* 2006. SoyFACE: the effects and interactions of elevated $[CO_2]$ and $[O_3]$ on soybean. In: Nösberger J, ed. *Managed ecosystems and CO2: case studies, processes and perspectives*. Berlin: Springer Verlag, 71–86.

Osborne CP, LaRoche J, Garcia RL, Kimball BA, Wall GW, Pinter PJ, LaMorte RL, Hendrey GR, Long SP. 1998. Does leaf position within a canopy affect acclimation of photosynthesis to elevated CO_2? Analysis of a wheat crop under free-air CO_2 enrichment. *Plant Physiology* **117,** 1037–1045.

Peoples MB, Gifford RM. 1997. Regulation of the transport of nitrogen and carbon in higher plants. In: Dennis DT, Turpin DH, Lefebvre DD, Layzell DB, eds. *Plant metabolism*. Harlow: Addison Wesley Longman, 525–538.

Peterson AG, Ball JT, Luo Y, *et al.* 1999. Quantifying the response of photosynthesis to changes in leaf nitrogen content and leaf mass per area in plants grown under atmospheric CO_2 enrichment. *Plant, Cell and Environment* **22,** 1109–1119.

Prentice IC, Farquhar GD, Fasham MJR, Goulden ML, Heimann M, Jaramillo VJ, Kheshgi HS, LeQuere C, Scholes RJ, Wallace DWR. 2001. The carbon cycle and atmospheric carbon dioxide. In: Houghton JT, Ding Y, Griggs DJ, Noguer M, Van der Linder PJ, Dai X, Maskell K, Johnson CA, eds. *Climate change 2001: the scientific basis. Contributions of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge: Cambridge University Press, 183–238.

Ritchie SW, Hanaway JJ, Thompson HE, Benson GO. 1997. *How a soybean plant develops*. Special Report No. 53, Iowa State University, Ames, IA.

Rogers A, Ainsworth EA. 2006. The response of foliar carbohydrates to elevated carbon dioxide concentration. In: Nösberger J, ed. *Managed ecosystems and CO2: case studies, processes and perspectives*. Berlin: Springer Verlag, 293–308.

Rogers A, Allen DJ, Davey PA, *et al*. 2004. Leaf photosynthesis and carbohydrate dynamics of soybeans grown throughout their life-cycle under free-air carbon dioxide enrichment. *Plant, Cell and Environment* **27,** 449–458.

Rogers A, Gibon Y, Stitt M, Morgan PB, Bernacchi CJ, Ort DR, Long SP. 2006. Increased carbon availability at elevated carbon dioxide concentration improves N assimilation in a legume. *Plant, Cell and Environment* **29,** 1651–1658.

Serraj R, Sinclair TR. 2003. Evidence that carbon dioxide enrichment alleviates ureide-induced decline of nodule nitrogenase activity. *Annals of Botany* **91,** 85–89.

Serraj R, Sinclair TR, Allen LH. 1998. Soybean nodulation and N-2 fixation response to drought under carbon dioxide enrichment. *Plant, Cell and Environment* **21,** 491–500.

Sims DA, Luo Y, Seemann JR. 1998. Importance of leaf versus whole plant CO_2 environment for photosynthetic acclimation. *Plant, Cell and Environment* **21,** 1189–1196.

Stitt M, Krapp A. 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. *Plant, Cell and Environment* **22,** 583–621.

Taylor G, Tricker PJ, Zhang FZ, Alston VJ, Miglietta F, Kuzminsky E. 2003. Spatial and temporal effects of free-air CO_2 enrichment (POPFACE) on leaf growth, cell expansion, and cell production in a closed canopy of poplar. *Plant Physiology* **131,** 177–185.

Todd CD, Tipton PA, Blevins DG, Piedras P, Pineda M, Polacco JC. 2006. Update on ureide degradation in legumes. *Journal of Experimental Botany* **57,** 5–12.

Vadez V, Sinclair TR. 2000. Ureide degradation pathways in intact soybean leaves. *Journal of Experimental Botany* **51,** 1459–1465.

Walsh KB, Vessey JK, Layzell DB. 1987. Carbohydrate supply and N-2 fixation in soybean: the effect of varied daylength and stem girdling. *Plant Physiology* **85,** 137–144.

Walter A, Christ MM, Barron-Gafford GA, Grieve KA, Murthy R, Rascher U. 2005. The effect of elevated CO_2 on diel leaf growth cycle, leaf carbohydrate content and canopy growth performance of *Populus deltoides*. *Global Change Biology* **11,** 1207–1219.