RESEARCH PAPER

Does elevated atmospheric [CO₂] alter diurnal C uptake and the balance of C and N metabolites in growing and fully expanded soybean leaves?

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Abstract

Increases in growth at elevated [CO₂] 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 [CO₂] 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 [CO₂] in a free air CO₂ 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 [CO₂] 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 [CO₂] had smaller pools of ureides compared with developing leaves at ambient [CO₂], which suggests N assimilation in young leaves was improved by elevated [CO₂]. This work shows that elevated [CO₂] 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 [CO₂], FACE, Glycine max, hexose, starch, sucrose, ureide.

Introduction

Atmospheric CO₂ concentration ([CO₂]) 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 [CO₂] commonly leads to increased plant growth. However, maximum exploitation of a CO₂-rich atmosphere can only be achieved when a plant has sufficient capacity to use the increased supply of carbon (C) available at elevated [CO₂], and this is often limited by the availability of nitrogen (N) (Stitt and Krapp, 1999; Oren et al., 2001;...
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 \([\text{CO}_2]\) will depend on the metabolic status of growing tissue. Growing leaves are particularly important since greater growth at elevated \([\text{CO}_2]\) will lead to a larger leaf area and possibly compound gains in productivity. In addition to its agronomic importance, soybean (\textit{Glycine max} Merr.) is an interesting species to investigate in elevated \([\text{CO}_2]\) because it has an association with N-fixing bacteria (\textit{Rhizobiales}) 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 \([\text{CO}_2]\) (Rogers et al., 2006). Therefore, indeterminate soybeans are expected to escape the limitation of sink capacity that other species experience at elevated \([\text{CO}_2]\).

Determining the presence of an N limitation at elevated \([\text{CO}_2]\) 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 \([\text{CO}_2]\) 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 \([\text{CO}_2]\) 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 \([\text{CO}_2]\) (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 \(\text{CO}_2\) 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 \([\text{CO}_2]\), particularly under field conditions. Data are emerging that correlate changes in leaf growth at elevated \([\text{CO}_2]\) with altered metabolite pool sizes. Taylor et al. (2003) elegantly showed that the pattern of leaf expansion was altered in \textit{Populus×euvamericana} leaves exposed to elevated \([\text{CO}_2]\) 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 \([\text{CO}_2]\) in \textit{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 \([\text{CO}_2]\) 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 \([\text{CO}_2]\) were investigated using free air \(\text{CO}_2\) 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 \([\text{CO}_2]\) in the field. Diurnal courses of gas exchange of young and mature leaves were measured to investigate changes in C flux under elevated \([\text{CO}_2]\). Leaf carbohydrate and amino acid pools were measured throughout the diurnal course to investigate if elevated \([\text{CO}_2]\) 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 \([\text{CO}_2]\) 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 225) 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 \([\text{CO}_2}\) (c. 375 \(\mu\)mol mol\(^{-1}\)) and one fumigated from sunrise to sunset to an elevated target \([\text{CO}_2]\) of 550 \(\mu\)mol mol\(^{-1}\), using the FACE design of Miglietta et al. (2001). In 2004, the actual elevated \([\text{CO}_2]\) averaged across the growing season was 550 \(\mu\)mol mol\(^{-1}\) and 1 mi averages of \([\text{CO}_2]\) in the fumigated plots were within \(\pm 20\%\) 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 gs between trifoliate 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 [CO2] 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 CO2 uptake (A) was calculated by integrating under the area of the diurnal curve of photosynthesis.

Leaf CO2 assimilation rate (A) was determined in response to changes in the intercellular [CO2] (ci) 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 [CO2], then the [CO2] 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 cm²) 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 [CO2] considered as fixed effects and block as a random effect.

Results

Elevated [CO2] 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 [CO2] 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 [CO2], and intercellular [CO2] was 12–15% higher in developing leaves compared with fully expanded leaves in both ambient and elevated [CO2] (Fig. 1e, f). The A/c1 response curves (Fig. 3) showed that fully expanded leaves had higher photosynthetic capacity than developing leaves, but there was no effect of [CO2] treatment on the shape of the A/c1 response curve.

Elevated [CO2] 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 [CO2] 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 [CO2]-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 [CO2] (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 [CO2]. As expected from the photosynthesis measurements, total chlorophyll content was significantly
lower in developing leaves (Fig. 5g, h). Total protein content was not affected by [CO2] 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 [CO2] 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 [CO2] on ureide levels in fully expanded leaves but, in developing leaves, elevated [CO2] 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 [CO2] 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 acids...
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 [CO2], 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 [CO2] compared with ambient [CO2] (Fig. 8c).

Discussion

An increase in atmospheric [CO2] to levels predicted for 2050 caused changes in C flux and C and N metabolites in fully expanded and developing soybean leaves. Elevated [CO2] 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 [CO2], while in developing leaves carbohydrate content was low and unaffected by [CO2] (Fig. 3). By contrast,
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

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**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: \(*P < 0.05; **P < 0.01; ***P < 0.001.\)**
**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 [CO2] (open columns) and elevated [CO2] (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.
Responses of developing leaves to elevated [CO2]
How developing leaves respond to elevated [CO2] has received much less attention than mature leaves. Osborne et al. (1998) and Adam et al. (2000) reported that...
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 [CO2] in developing soybean leaves (Figs 1, 2); however, developing leaves had significantly higher intercellular [CO2] compared with fully expanded leaves (Fig. 1e, f). From the A/ci 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 [CO2], 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 [CO2] affect on chlorophyll content (Fig. 4g, h). There was a striking decrease in stomatal conductance in young leaves grown at elevated [CO2] (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 [CO2] 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 [CO2] 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 [CO2], there was no change in LMA of developing leaves.

**Implications for whole plant C and N status at elevated [CO2]**

When averaged across leaf ages and three time points, the Gly:Ser ratio was 15% lower in plants grown under elevated [CO2]. A decreased Gly:Ser ratio is frequently seen in elevated [CO2] (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 [CO2]; both observations, along with higher intercellular [CO2], provide strong evidence that there was a decrease in photorespiratory flux in soybeans growing at elevated [CO2]. 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 [CO2] than at ambient [CO2]. 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 [CO2] compared with those grown at current [CO2]. Further experiments would be needed to determine if more C were in fact exported from fully expanded leaves to developing leaves under elevated [CO2].

Another possibility is that long-distance signals related to higher carbohydrate status in elevated [CO2] drive
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 [CO₂] (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 [CO₂] surrounding the plant, not the [CO₂] surrounding the leaf. Controlled experiments where a mature leaf is maintained at one [CO₂] and a developing leaf at a different [CO₂] 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. 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.](image)

![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.](image)
reproductive phase compared with the vegetative phase in both ambient and elevated [CO2] (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 [CO2] 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 [CO2] increased C available for N2 fixation and/or enabled plants at elevated [CO2] 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 [CO2], 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.

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