

Daily changes of amino acids in soybean leaflets are modified by CO₂ enrichment

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Abstract

The effects of CO₂ enrichment on plant growth and on nitrogen partitioning were examined using soybean [*Glycine max* (L.) Merr. cv. Clark] leaflets. Plants were grown from single seeds in matching controlled environment chambers. Continuous ambient CO₂ partial pressures were from 38 to 40 Pa and elevated CO₂ treatments were 68 to 70 Pa. Total above ground biomass, total leaf area and specific leaf weight of soybean were increased 78%, 58% and 33%, respectively, in response to CO₂ enrichment when measured 25 days after sowing. Total chlorophyll (a+b) was 25% greater in third trifoliolate soybean leaflets in response to CO₂ enrichment but total soluble protein did not differ between treatments. These and other measurements indicated that soybean plants were nitrogen sufficient in this study. Variations of total soluble amino acids were observed in soybean leaflets and these were enhanced by CO₂ enrichment when measurements were performed mid-day. However, concentrations of total amino acids were similar in both CO₂ treatments by end of the photoperiod. Glycine was lower in the elevated compared to the ambient CO₂ treatment suggesting that rates of photorespiration were diminished by elevated CO₂. Alanine increased 20% in response to CO₂ enrichment. Overall, changes of soluble amino acids in response to CO₂ enrichment were smaller than in other crop species and a temporal shift occurred in the daily accumulation of amino acids in soybean leaflets.

Introduction

Photosynthetic capacity frequently decreases when terrestrial plants are grown continuously in CO₂ enriched atmospheres.^{1,2} For example, net CO₂ assimilation rates, photosynthetic proteins and Chl(a+b) of wheat, barley and tobacco leaves decreased in response to elevated CO₂ treatments both in season-long field studies and in controlled environments.³⁻¹⁰ In contrast to the above photosynthetic pro-

teins and *in vitro* Rubisco activity of soybean leaflets remained largely unaffected by plant growth in elevated CO₂.¹¹⁻¹⁵ However, Bernacchi *et al.*¹⁶ reported that the photosynthetic rates of soybean leaflets decreased slightly in response to long term CO₂ enrichment and Sicher *et al.*¹² observed that photosynthetic rates of CO₂ enriched soybean plants were approximately 35% lower than those of plants grown in ambient CO₂ when gas exchange measurements were performed at 70 Pa CO₂. Although photosynthetic protein levels were constant, the above evidence suggested that the photosynthetic capacity of soybean decreased in response to elevated CO₂ treatments. Therefore, we hypothesized that changes of net CO₂ fixation could alter the primary metabolism of soybean leaflets.

Basic biochemical processes that result in photosynthetic acclimation to elevated CO₂ are not fully understood, although Geiger *et al.*^{9,10} demonstrated that high N fertility partially or even fully reversed the negative effects of CO₂ enrichment on leaf proteins of tobacco. Photosynthetic proteins are synthesized from soluble amino acids and these leaf components are sensitive to N fertility, which in turn can be altered by CO₂ enrichment.^{9,17} Understanding the interaction of CO₂ enrichment and of soluble amino acid metabolism in soybean could provide valuable information about this crop. Rogers *et al.*¹³ and Ainsworth *et al.*¹⁴ published two years of data on soluble amino acid levels in soybean leaflets grown in the SoyFACE facility at the University of Illinois. Although CO₂ effects on soluble amino acids were noted in young leaflets, except for the Gly:Ser ratio, these metabolites were unaffected by CO₂ enrichment in mature trifoliolates. These experiments are consistent with what is known about changes of photosynthetic proteins in soybean and the results comprise a valuable dataset. However, the SoyFACE supplemented air with 150 μmol mol⁻¹ CO₂, which is less than a 50% increase over current ambient CO₂ levels. This is potentially important because effects of elevated CO₂ on plant growth and photosynthesis are usually proportional to the change in CO₂ concentration. Also, supplemental CO₂ was only provided during daylight hours and it is likely that nocturnal CO₂ levels alter dark respiration rates and plant growth rates of soybean.¹⁸ SoyFACE experiments also used ambient temperature and natural sunlight, both of which vary diurnally and during the growing season. Varying environmental conditions can obscure the responses of leaf metabolites to specific treatments.¹⁹ Therefore, the hypothesis of this study was that soluble amino acid metabolism in soybean leaflets would respond to CO₂ enrichment when using twice ambient CO₂ partial pressures over the entire light/dark cycle and

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constant temperature conditions provided by controlled environment chambers. We observed that a temporal shift in the accumulation of soluble amino acid occurred in soybean leaflets in response to CO₂ enrichment.

Materials and Methods

Plant materials

Soybean [*Glycine max* (L.) Merr. cv. Dixon] plants were grown in matching pairs of controlled environment chambers (model M-2, Environmental Growth Chamber Corp., Chagrin Falls, OH, USA) essentially as described previously.¹¹ Plants were seeded in 1.8 dm³ plastic pots filled with vermiculite, the air temperature was 27±1°C and the PPFD was 850±40 μmol m⁻² s⁻¹. The photoperiod used a 14 h day/10 h night cycle and pots were watered once daily with a complete mineral nutrient solution containing 12 mM nitrate and 2.5 mM ammonium. However, 19 d-old or older plants were watered with nutrient solution twice daily to ensure nutrient sufficiency and to avoid drought. Ambient and elevated chamber air CO₂ partial pressures were 38±10 Pa and to 70±10 Pa, respectively. Plant samples

were harvested 25 DAS. Terminal leaflets of fully-expanded third trifoliolate leaves were excised at indicated times, transferred to paper envelopes and quickly immersed in liquid N₂ to quench metabolism. Leaf samples could be stored at -80°C for up to 1 month prior to analysis without altering the results. At each time point five leaves were harvested from plants grown in each CO₂ treatment.

Leaf component analysis

Soluble amino acids were determined as described previously.¹⁷ Frozen leaf tissue was ground to a fine powder in a mortar and pestle with liquid N₂ and 50 mg of the leaf powder was extracted with 70% methanol in a ground glass tissue homogenizer. Extracts were heated to 45°C, centrifuged and the supernatants were evaporated to dryness at 37°C with a stream of N₂ gas. Samples were carefully resuspended in 20 mM HCL and were filtered by centrifugation using a 0.22 μm Ultrafree-MC membrane filter unit (Millipore Corp., Bedford, MA, USA). The filtered extracts could be stored at -20°C for up to 1 month prior to chromatography.

Soluble amino acids were determined by an HPLC procedure using the AccQTag pre-column derivatization method (Waters Corp., Milford, MA, USA). Samples and standards were derivatized using an AccQFluor kit from Waters according to the manufacturer's instructions. Separations were performed at 37°C on a Waters 600E Multisolute Delivery System equipped with a 3.9×150 mm AccQTag C₁₈ column. Detection was with a Shimadzu 535 fluorimeter (Shimadzu Corp., Columbia MD, USA) with excitation and emission wavelengths of 250 and 395 nm, respectively. The output of the detector was monitored using Empower2 software from Waters. Standard curves were prepared with a known mixture of 19 amino acids for each sample set. The amino acid standards, plus ammonia and the internal standard were separated by over 80% using the method described above. Plant samples were corrected to 100% recovery based on the detection of α-aminobutyric acid that was added to each extraction.

Other measurements. Various leaf measurements were performed using the same trifoliolate that was used for amino acid analysis and measurement details were essentially as described earlier.¹⁷ Soluble proteins were extracted in dilute buffer and were determined using Coomassie brilliant blue R-250. Total Chl(a+b) was extracted with 80% acetone and quantified spectrophotometrically. Inorganic NO₃⁻ was extracted in 80% methanol, microfiltered as above, diluted and analyzed by HPLC using a silica based strong anion exchange (SAX) column. For dry weight determinations, shoots were quickly separated into leaves and stems and these were oven-dried for 3 days at 70°C. Total leaf area was measured with an

Table 1. Effects of CO₂ enrichment on leaf N constituents and on plant growth parameters. Soybean plants grown for 25 days using ambient and elevated CO₂ treatments as described under Design and methods. Soluble protein and Chl (a+b) were analyzed using the central lobe of the third trifoliolate leaf of soybean or the most recently collared leaf of wheat. Leaf area and total above ground biomass are for whole plants. Values are means ± SE for 5 replicate samples. * and ** are for P≤0.05 and 0.01, respectively.

Parameter	36 Pa CO ₂	70 Pa CO ₂	P
Chl (a+b), mg g ⁻¹ FW	1.3±0.06	1.6±0.03	*
Soluble protein, mg g ⁻¹ FW	35.9±1.9	37.2±2.8	ns
Total leaf area, m ²	0.9±0.03	1.4±0.02	**
Total biomass, g	8.1±0.4	14.4±0.3	**
SLW, g m ⁻²	3.3±0.1	3.7±0.1	**

ns, no significant difference.

optical meter from Li-Cor Corp. (model 3100, Lincoln, NE, USA). Specific leaf weight (g m⁻²) was determined as the ratio of leaf mass per unit leaf area.

Statistical methods

Results of two completely replicated experiments were combined and significant differences were determined using a two-way Analysis of Variance procedure (StatView, Mountain View, CA, USA). Total soluble amino acid concentrations for a given treatment and time point were determined by summing the concentrations of 17 individual amino acids from each chromatogram.

Results

Effects of elevated CO₂ on soybean growth and N status

In the current study soybean plants grown at 70 Pa CO₂ were larger on average than the ambient controls (Table 1). When measured 25 DAS total above ground biomass, total leaf area and specific leaf weight (SLW) increased (P≤0.01) by 78%, 58% and 33%, respectively, in the elevated compared to the ambient CO₂ treatment. Similar to previous studies^{11,12} soluble protein did not differ among CO₂ treatments but Chl(a+b) in the 3rd trifoliolate leaflets increased 23% in response to CO₂ enrichment.

Effects of elevated CO₂ on nitrate and on total soluble amino acids in soybean leaflets

Inorganic nitrate concentrations in soybean trifoliolates were between 25 and 40 μmol g⁻¹ FW and nitrate was 22% lower on average (P<0.05) in the elevated compared to the ambient CO₂ treatment (Figure 1A). This difference was observed at all time points during the photoperiod. Total soluble amino acids in the ambient and elevated CO₂ treatments were

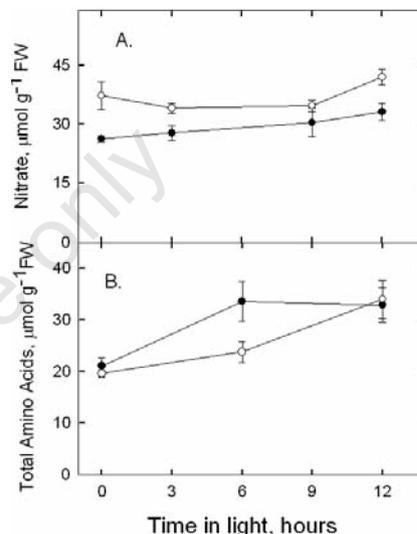


Figure 1. Effects of CO₂ enrichment on components of soybean trifoliolates. Inorganic nitrate (panel A) and total soluble amino acids (panel B) were determined at indicated times using the central lobe of the third trifoliolate leaf of 25 day old plants from the ambient (○) and the elevated (●) CO₂ treatments. Results are means ± SE for two replicate experiments with five leaflets per time point from each treatment.

similar at 0-h light (Figure 1B). Over the next 12 h total soluble amino acids in soybean leaflets increased about 50% on average in both CO₂ treatments. However, almost all of the total soluble amino acid accumulation in the elevated CO₂ treatment occurred during the first 6 h of the photoperiod. In comparison, total soluble amino acids in the ambient CO₂ treatment accumulated in nearly linear manner throughout the measurement period

Effects of elevated CO₂ on individual soluble amino acids

Changes of major soluble amino acids in soybean leaflets are shown in Figure 2. The four most abundant soluble amino acids in

third trifoliolate leaves were Glu, Asn, Asp, and Ser (Figures 2B, 2C, 2D and 2F, respectively) and levels of Asn and Ser accumulated during the light period. In fact, just less than 90% of the daily increase that was observed for total soluble amino acids from soybean leaflets in the ambient CO₂ treatment was attributed to these two individual amino acids. Unlike other species,^{9,17} Gln was present in very low concentrations in soybean leaflets (Figure 2A) when compared to Asn. Glu and Asp were essentially unchanged during the photoperiod when comparing samples from the ambient CO₂ treatment. In the elevated CO₂ treatment, Glu and Asp levels were low initially, increased about 30% at mid day and then this was reversed during the second half of the photoperiod. Therefore, changes of Glu and Asp in response to photoperiod and to CO₂ enrichment were qualitatively similar in soybean leaflets. Glu and Asn were significantly greater in the elevated compared to the ambient CO₂ treatment when comparing measurements for the 6 h sampling ($P \leq 0.05$). Gly was the only major soluble amino acid in soybean leaflets that was lower ($P \leq 0.05$) in the elevated than in the ambient CO₂ treatment (Figure 1E). Unlike Gly, Ser was unaffected by CO₂ enrichment in soybean leaflets ($P > 0.05$) by. The Gly/Ser ratio was initially about 0.19 in the ambient and elevated CO₂ treatments and this ratio decreased with time in the light (Figure 2G). However, the Gly/Ser ratio was not significantly ($P > 0.05$) affected by CO₂ enrichment. The Asp/Asn ratio also decreased in both CO₂ environments between 0 and 12 h of light. The Asp/Asn ratio was similar in both CO₂ treatments except at 0 h of the photoperiod.

Minor amino acid levels in soybean leaflets are shown in Figure 3. Ala, Arg, Thr and Phe increased in soybean leaflets during the photoperiod, whereas Pro, Val, Ile and Leu did not. In contrast to the other minor amino acids Ala levels were 29% greater in leaflets from the elevated compared to the ambient CO₂ treatment ($P \leq 0.05$) in response to CO₂ enrichment.

Discussion

Plants grown in atmospheres enriched with CO₂ often display symptoms of N deficiency. Various leaf properties, including levels of photosynthetic proteins, Rubisco activity, Chl (a+b) and photosynthetic capacity typically decrease and transient starch, the C/N ratio and SLW increase in response to CO₂ enrichment.²⁰ Geiger *et al.*^{9,10} demonstrated that the effects of CO₂ enrichment on tobacco were essentially eliminated when plants were fertilized with saturating N. Consequently, one likely reason for the acclimation responses of

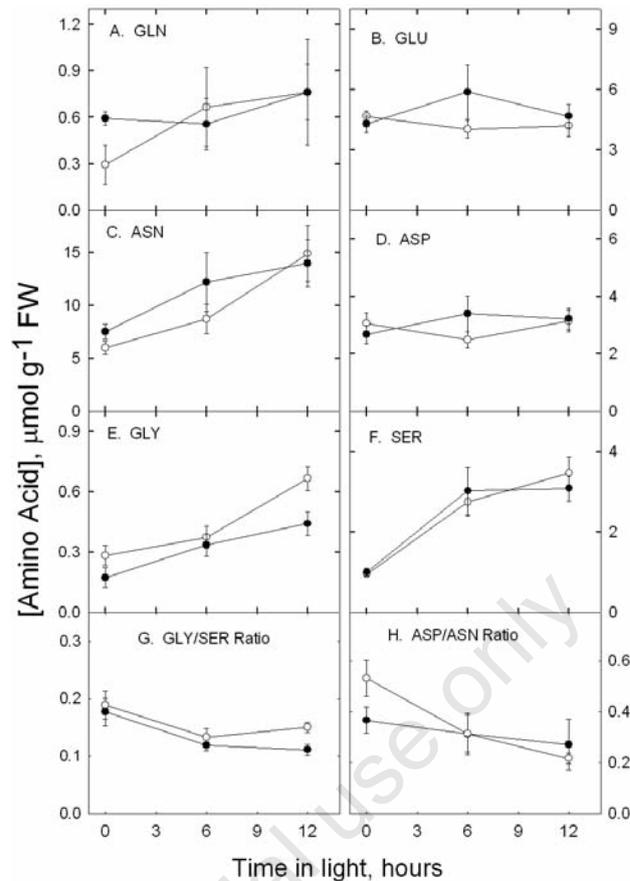


Figure 2. Effects of CO₂ enrichment on the accumulation of major soluble amino acids in soybean trifoliolates. Soluble amino acid levels were determined using the central lobe of the third trifoliolate leaf of 25 day old plants from the ambient (○) and the elevated (●) CO₂ treatments. Results are means \pm SE for two replicate experiments with five leaflets per time point from each treatment.

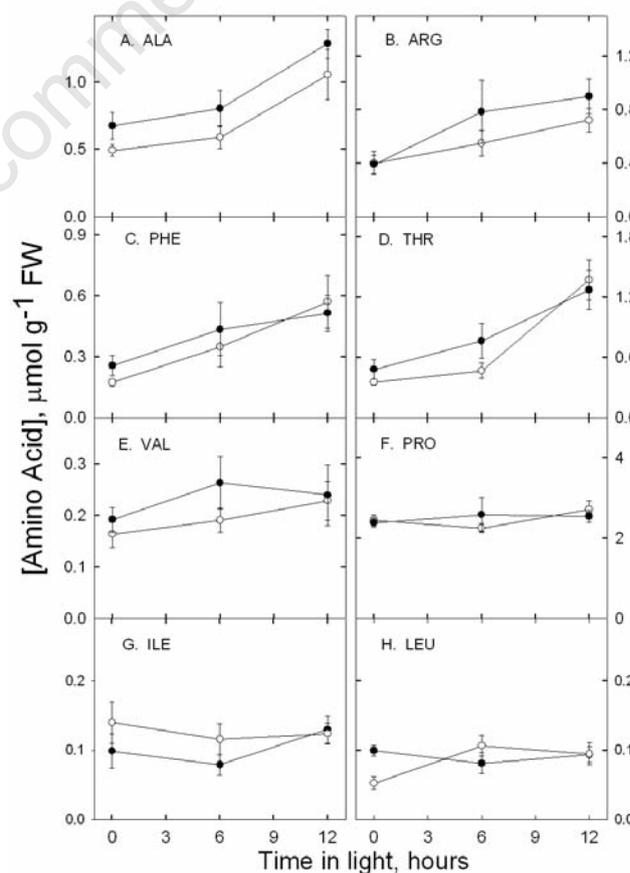


Figure 3. Effects of CO₂ enrichment on the accumulation of minor soluble amino acids in wheat leaves. Individual amino acid levels were determined using the most recently expanded leaf of 25 day old plants from the ambient (○) and the elevated (●) CO₂ treatments. Results are means \pm SE for two replicate experiments with five leaflets per time point from each treatment.

plants to CO₂ enrichment is an acquired N insufficiency. Effects of CO₂ enrichment on leaf properties and on the N status of soybean are usually much less than that reported for other C₃ crop species (i.e., 6, 10). Measurements of soluble protein and Chl(a+b) in the present study confirmed these prior observations. It is not obvious why the N status of soybean was less sensitive to the effects of CO₂ enrichment than other species. However, total soluble amino acids in soybean only increased about 50% during the light period, whereas changes of soluble amino acids in barley, tobacco and wheat increased from 2 to 4 fold.¹⁶ This implies that amino acid pools in soybean are closer to a metabolic stasis than in other species. Second, the most abundant soluble amino acid in soybean leaflets was Asn and, in the other species mentioned above, Glu was the predominant amino acid.

The finding that soluble protein and Chl(a+b) were not decreased by CO₂ enrichment suggested that the plants used in this study were N sufficient. This conclusion was supported by the inorganic nitrate measurements. Inorganic nitrate levels are typically lower in plants grown in elevated compared to ambient CO₂ and this may be due to reduced evapotranspiration rates that occur in response to CO₂ enrichment.²¹ Under N insufficient conditions foliar inorganic nitrate levels decreased during the photoperiod.⁹ Because inorganic nitrate concentrations in the present study were constant throughout the day it is unlikely the plants used in this study were N insufficient. Consequently, N fertility was not a major factor in determining soluble amino acid concentrations in this study.

In agreement with prior studies^{13,14} the effects of CO₂ enrichment on soluble amino acids in soybean leaflets were small. However, in the current study total soluble amino acid levels differed significantly between the elevated and the ambient CO₂ treatment when measured at mid-day. This was mostly attributed to Glu and Asn which were greater in the elevated than in the ambient CO₂ treatment when comparing measurements performed at mid-day. Note that differences between total soluble amino acids in the ambient and elevated CO₂ treatments were not observed at the beginning and end of the photoperiod and the major amino acids Glu, Asp and Ser either showed no net accumulation or a decrease in leaflet concentration during the latter half of the photoperiod. Therefore, a temporal shift in total soluble amino acid accumulation occurred in soybean leaflets in response to CO₂ enrichment and the effects of CO₂ enrichment on total amino acid levels in soybean leaflets were only observed during the first half of the light period.

Only two individual soluble amino acids, Gly

and Ala, differed significantly between CO₂ treatments. Gly was 28% lower in the elevated compared to the ambient CO₂ treatment and diminished levels of Gly indicated that CO₂ enrichment decreased flux through the photorespiratory pathway.⁹ In contrast to Gly, Ser levels and the Gly/Ser ratio were unaffected by CO₂ enrichment in this study. Rogers *et al.*¹³ reported that Gly and Ser were decreased by CO₂ enrichment, although this was only observed on three or four of the six harvest dates performed during the growing season. Along similar lines Ainsworth *et al.*¹⁴ reported that the Gly:Ser ratio was the only amino acid parameter affected by CO₂ enrichment in soybean leaflets. Taken together the above findings indicated that soybean leaflet amino acids involved in photorespiration were altered by CO₂ enrichment. The Asp/Asn ratio decreased in the light phase largely because Asn showed the largest daily variation of any amino acid in soybean leaflets. Ala was increased by CO₂ enrichment in soybean leaflets and Ala was one of the more abundant minor amino acids in soybean leaflets. Wallace *et al.*²² suggested that Ala in soybean leaflets was synthesized via the transamination of pyruvate and that Glu was the most likely amino donor. As noted above Glu was elevated by CO₂ enrichment when measured at mid-day and this potentially contributed to increased Ala concentrations in soybean leaflets. Ala is transported from soybean leaves in the phloem. Therefore, Ala may contribute additional C and N to sink tissues in soybean plants exposed to elevated CO₂.

In summary, small but significant changes in soluble amino acid concentrations were identified in N sufficient soybean leaflets in response to CO₂ enrichment. Most importantly total soluble amino acids were greater in the elevated than in the ambient CO₂ treatment when measured at mid-day. This was due to a temporal shift in soluble amino acid accumulation in soybean leaflets in response to CO₂ enrichment. Mid-day changes in total soluble amino acids resulted primarily from enhanced levels of Asn and Glu in response to CO₂ enrichment. In addition Gly was decreased due to a CO₂ dependent reduction in photorespiration and Ala increased, possibly because of elevated CO₂ effects on the putative amino donor, Glu.

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