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Effects of CO_2 enrichment on soluble amino acids and organic acids in barley primary leaves as a function of age, photoperiod and chlorosis

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ABSTRACT

Responses of soluble amino acids and organic acids to either ambient (36 Pa) or elevated (100 Pa) CO_2 treatments were determined using barley primary leaves (*Hordeum vulgare* L. cv. Brant). Total soluble amino acids were increased 33% by CO_2 enrichment 9 days after sowing (DAS), but a decrease relative to the ambient CO_2 treatment was observed with increasing leaf age. Marked declines of glutamine and asparagine were observed under CO_2 enrichment, both diurnally and with advancing leaf age. Consequently, total soluble amino acids were 59% lower in the elevated compared to the ambient CO_2 treatment 17 DAS. It was likely that chlorosis in response to CO_2 enrichment negatively impacted soluble amino acid levels in older barley primary leaves. In contrast to the ambient CO_2 treatment, glutamine and most other soluble amino acids decreased as much as 60% during the latter half of a 12 h photoperiod in primary leaves of 13-day-old seedlings grown under enhanced CO_2. Malate was decreased about 9 percent by CO_2 enrichment and citrate and succinate were increased by similar amounts when measured 9 and 13 DAS. Malate accumulation was also decreased about 20% by CO_2 enrichment on a diurnal basis. The onset of CO_2-dependent leaf yellowing had much less of an effect on organic acids than on soluble amino acids. This above results emphasized the sensitivity of N metabolism to CO_2 enrichment in barley. Increased levels of citrate and succinate in response to CO_2 enrichment suggested that the tricarboxylic acid cycle was upregulated in barley by CO_2 enrichment. In summary, organic and amino acid levels in barley primary leaves were dynamic and were altered by age, diurnally and in response to CO_2 enrichment.

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1. Introduction

Growth rates usually accelerate when terrestrial plants are grown in CO_2 enriched atmospheres. However, this growth stimulation typically subsides within a few days or weeks as plants acclimate to the elevated CO_2 treatment [1]. Transient effects of elevated CO_2 on plant growth were correlated with changes of net photosynthesis and were attributed to altered levels of chlorophyll (a + b), ribulose-1,5-bisphosphate carboxylase/oxygenase and various other photosynthetic proteins [2,3]. The acclimation of growth and photosynthesis to enhanced CO_2 was usually less pronounced in seedlings than in larger, older plants [3,4]. Therefore, seedlings and developing tissues are an important tool for studying plant responses to CO_2 enrichment.

Several plant species developed foliar injuries, such as chlorosis, necrosis, leaf thickening and brittleness, when exposed to elevated CO_2 (i.e., ref. [5]). Leaf yellowing was more pronounced on older than younger leaves and was attributed to photobleaching, photoinhibition, nutrient deficiency, premature senescence and other causes [6,7]. Barley primary leaves are often utilized in physiological investigations [8,9] and large numbers of genetically uniform leaves can be produced quickly in a confined growing area with minimal nutritional or developmental problems. In previous studies [10–12] leaf yellowing was observed on primary leaves of 2–3-week-old barley plants grown at moderate to high irradiance in controlled environment chambers. The yellowing of barley primary leaves increased with age, was accelerated by elevated CO_2, and was associated with an accumulation of nonstructural carbohydrates and decreased rates of net CO_2 assimilation. However, total soluble protein and Rubisco protein in barley primary leaves were little changed in response to CO_2-dependent chlorosis. Therefore, it is plausible that yellowing of barley leaves in response to CO_2 enrichment resulted from photochemical damage within the chloroplasts.

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Abbreviations: Gln, glutamine; Glu, glutamate; Asn, asparagine; Asp, aspartate; Gly, glycine; Ser, serine; Ala, alanine; Mal, malate; Cit, citrate; Scc, succinate.

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Other than changes to nonstructural carbohydrates little is known about the effects of CO_2-dependent chlorosis on leaf metabolites. Unlike older leaves, soluble amino acids were increased in young soybean and tobacco leaves exposed to atmospheric CO_2 enrichment [4,14]. However, it is not clear if these findings apply to monocot species and the impact of leaf yellowing on foliar amino acids has not been examined in detail. The hypothesis that organic acids would be altered by chlorosis in response to CO_2 enrichment was also examined here. The synthesis of organic acids is dependent on nonstructural carbohydrates and in some instances uses reductant manufactured in chloroplasts. Consequently, factors that alter photosynthetic rates, such as CO_2 enrichment, should impact foliar organic acids. Other than a handful of reports (i.e., refs. [4,12]), responses of organic acids to CO_2 enrichment have received little prior attention. Therefore, a concurrent study of the effects of CO_2 enrichment on both organic and amino acids could provide insights into the regulation of assimilate partitioning in response to atmospheric change.

2. Materials and methods

2.1. Plant materials

Barley (Hordeum vulgare L. cv. Brant) plants were grown from single seeds in plastic pots filled with 2 dm^3 of vermiculite. Experiments were performed in matching pairs of controlled environment chambers (model M-2, Environmental Growth Chambers Corp., Chagrin Falls, OH) using a 14 h light and a 10 h dark period with a constant air temperature of 22 ± 1 °C. Irradiance was 850 ± 30 $\mu mol\ m^{-2}\ s^{-1}$ and chamber air CO_2 partial pressures were controlled at 36 ± 1 Pa (ambient CO_2) and 100 ± 2 Pa (elevated CO_2). Plants were watered once daily and received 15 mM total N and 1 mM P from a complete mineral nutrient solution. Further details regarding plant growth conditions have been reported previously [13].

2.2. Leaf sampling

Individual barley primary leaves were harvested between 4 and 5 h of light 9, 13 and 17 DAS. On these sampling dates leaf yellowing affected an average of about 0, 15 and 80% of the total primary leaf area, respectively, in the elevated CO_2 treatment [10,11]. Diurnal measurements were performed using primary leaves of 13-day-old plants harvested at indicated times during the photoperiod. Sampled primary leaves were quickly placed in envelopes and frozen in liquid N_2 to quench metabolism. Frozen leaves were extracted immediately or were stored at −80 °C for up to 2 months prior to use. Datasets comprised 5 primary leaves each from plants grown in the ambient and elevated CO_2 treatments. Experiments were replicated at least once and the results were combined.

2.3. Leaf components

Leaf tissue was ground to a fine powder in a mortar and pestle using liquid N_2. For amino acid analysis approximately 50 mg frozen tissue was extracted with 2 ml 70% aqueous methanol in a ground glass tissue homogenizer. An internal standard containing 50 nmol of α-aminobutyric acid, α-ABA, was injected into each extract. The homogenates were incubated in a H_2O bath at 45 °C for 15 min and then centrifuged for 15 min at 5800 × g in a Beckman Coulter centrifuge (model Avanti J-20 XP, Palo Alto, CA, USA). The resultant supernatants were evaporated to dryness under a stream of N_2 at 37 °C and the dried samples were resuspended in 0.5 ml of 20 mM HCl. The resuspended samples were then filtered by centrifugation using a 0.22 μm Ultrafree-MC membrane filter unit (Millipore Corp., Bedford, MA) and stored at −20 °C for up to 1 month.

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 on a Waters 600E Multisolvent Delivery System equipped with a 3.9 mm × 150 mm AccQTag C_{18} column using a temperature of 37 °C. The column was equilibrated with 140 mM sodium acetate buffer, pH 5.60 (adjusted with 50% H_3PO_4), 2.6 mM EDTA and 6.9 mM triethylamine. Amino acids were eluted using a series of linear gradients prepared by mixing acetonitrile with the sodium acetate buffer described above. Acetonitrile was 0% initially and was increased linearly in steps to 3% at 16 min, 6% at 25 min and to 14% at 35 min. The acetonitrile component was held constant at 14% for 5 min and was then increased linearly to 18% at 50 min. The column was washed with 60% acetonitrile and 40% H_2O for 5 min prior to reconditioning. Detection was with a Gilson 121 fluorimeter using optical filters to obtain 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 17 amino acids obtained from Waters (WAT088122). Two individual amino acids, Gln and Asn, were added to this mixture prior to derivatization. All of the 19 amino acid standards, plus ammonia and the internal standard, α-ABA, were separated by over 90% using the method described above. Quantitation was based on standard curves using 0, 25, 50 and 100 pmol of each standard amino acid, except for Cys which was present in one-half the usual amount. Each individual sample set was quantified using a separate standard curve. Plant samples were corrected to 100% recovery based on the quantity of internal standard that was detected.

Tissue for organic acid analysis was extracted using methods developed for metabolite profiling [15]. Samples were extracted as described above except that the solvent was 100% methanol and the initial extraction volume was 1.4 ml. Prior to extraction each sample was spiked with 0.1 mg of adonitol (ribitol) which served as an internal standard. The homogenates were incubated in a H_2O bath at 70 °C for 15 min and allowed to cool before dilution with an equal volume of deionized H_2O. After thorough mixing 20 μl of each extract was transferred to a 1 ml reactivial and dried overnight in a desiccator under vacuum. Dried samples and appropriate standards were resuspended in 50 μl of pyridine containing 1 mg of methoxyamine and were then incubated in a H_2O bath at 30 °C for 90 min with continuous shaking. Subsequently, 50 μl of MSTFA [*N*-methyl-*N*-(trimethylsilyl)fluoracetamide] was added to each vial which was then incubated as above for 30 min at 37 °C. Derivatized samples were separated by gas chromatography (model 6890A, Hewlett Packard) and organic acids were detected with a mass selective detector (model 7125, Agilent Technologies, Wilmington, DE) coupled to Agilent MSD Chemstation Software. Separations were performed with a 30 m x 0.25 mm Supelco SPB-50 column (Sigma–Aldrich, St. Louis, MO, USA) using high-purity helium as a carrier gas at 1.2 ml min^{-1}. The oven temperature was increased at 5 °C min^{-1} from 70 to 310 °C and a solvent delay of 8.5 min was used. The detector was operated in full scan mode at 50 scans min^{-1} with a range of 0–550 *m*/*z*. Total ion chromatograms were quantified using peak identification and calibration parameters within the Chemstation program. Standard curves were prepared with a mixture of known concentrations of specific organic acids.

3. Results

3.1. Changes of soluble amino acids in barley primary leaves in response to CO_2 enrichment

The combined effects of leaf age and of CO_2 enrichment on six major soluble amino acids in barley primary leaves are shown in Fig. 1. The most abundant soluble amino acids were Gln, Glu and Asn (Fig. 1A–C). These three amino acids represented about 70% of the total soluble amino acids in this tissue when measured 9 DAS under both CO_2 treatments. Other relatively abundant soluble amino acids were Asp (Fig. 1D), Gly (Fig. 1E), Ser (Fig. 1F) and Ala (not shown). Concentrations of total soluble amino acids in 9-day-old barley primary leaves in the ambient CO_2 treatment were about 73 μmol g^{-1} FW and this was increased 33% by CO_2 enrichment. These values were obtained by summing the individual concentrations of 18 soluble amino acids including that of γ-aminobutyric acid (GABA). Maximal concentrations of Asn and Gly were observed 9 DAS in both CO_2 treatments and levels of these two compounds decreased progressively with leaf age. Asp and Asn displayed an apparent reciprocal relationship with increasing leaf age in the ambient CO_2 treatment. In contrast to Asn and Gly, foliar concentrations Gln, Glu, Asp and Ser were maximal 13 DAS. The only soluble amino acid that was decreased by CO_2 enrichment when determined 9 DAS was Gly. With the exception of Asn, all of the amino acids shown here were lower in the elevated than in the ambient CO_2 treatment when measured 17 DAS. For the final harvest total soluble amino acid levels in barley primary leaves were about 69 and 29 μmol g^{-1} FW in the ambient and elevated CO_2 treatments, respectively.

Soluble amino acids were also measured after 0, 6 and 12 h of light using primary leaves of 13-day-old barley plants grown in the ambient and elevated CO_2 treatments (Fig. 2). Diurnal variations of Gln and Glu were small but discernible under ambient CO_2 (Fig. 2A and B, respectively). At the beginning of the photoperiod Gln was 14% lower in the elevated than in the ambient CO_2 treatment and this difference increased proportionally with time in the light. As a result Gln levels were 75% lower in the elevated compared to the ambient CO_2 treatment when determined after 12 h of light. In contrast to Gln, Glu was 34% lower on average in the elevated compared to the ambient CO_2 treatment and this difference persisted throughout the first 12 h of the photoperiod. Diurnal changes of Asn, Asp (Fig. 1C and D) and Glu were similar in the ambient CO_2 treatment. Levels of all three amino acids were low initially, increased to maxima about midday and then decreased toward the end of the light period. Levels of Asn in the elevated CO_2 treatment also declined markedly during the light period. Levels of Asn were greater in the elevated than in the ambient CO_2 treatment at the earliest time point but Asn decreased over 80% after 12 h of light in response to enhanced CO_2. Additional amino acid measurements performed here showed that diurnal changes and the effects of CO_2 enrichment on Val, Phe, Leu, Ile, and Tyr, although present at lower overall concentrations, were similar to that of Asn (data not shown). Foliar concentrations of Gly (Fig. 2E) were about 0.1 μmol g^{-1} FW in dark-adapted barley primary leaves from both CO_2 treatments. When measured during the middle of the light period, Gly levels increased about 60- and 15-fold in the ambient and elevated CO_2 treatments, respectively. Foliar concentrations of Ser (Fig. 2F) also were strongly decreased by CO_2 enrichment in the light. Similar to Gly, this was not

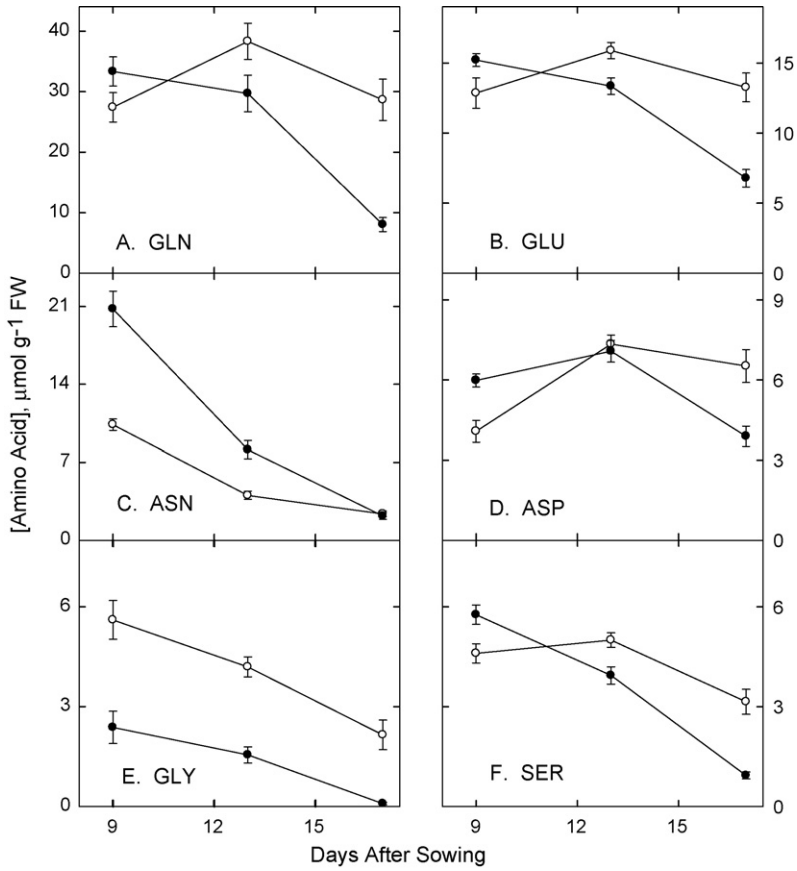

Fig. 1. Effects of CO_2 enrichment on age-dependent changes of soluble amino acids in barley primary leaves. Plants were grown in controlled environment chambers for 9, 13 and17 days using either ambient (36 Pa; ○) or elevated (98 Pa; ●) CO_2. Major soluble amino acids in the leaf were quantified using the AccuTag pre-column derivitization procedure. Values are means ± S.E. for n = 10.

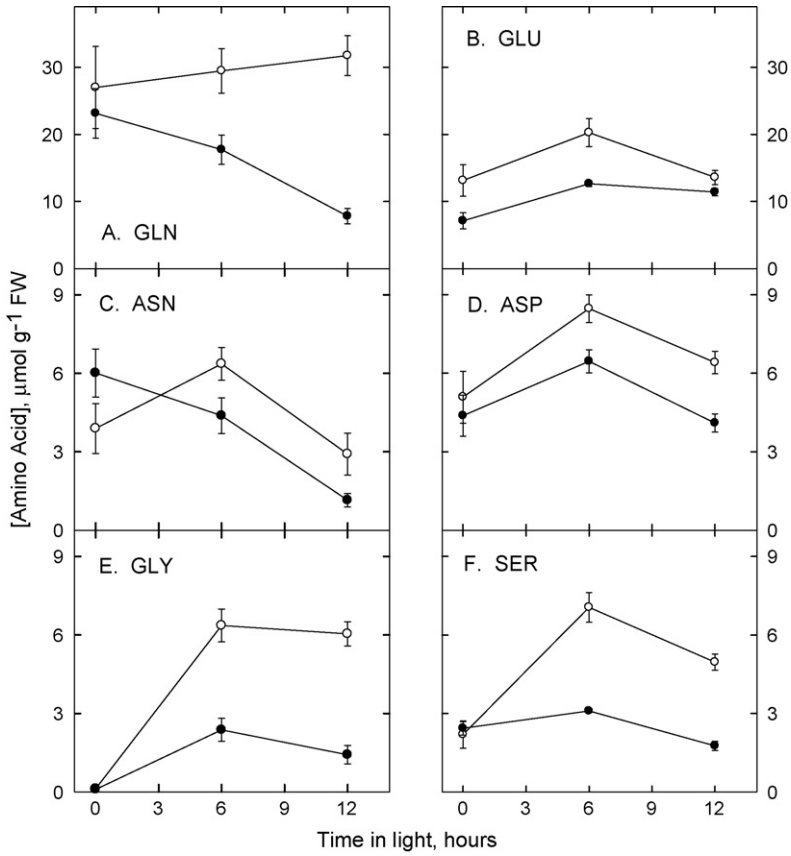

Fig. 2. Effects of CO_2 enrichment on diurnal changes of soluble amino acids in barley primary leaves. Treatments, symbols and other details were as in Fig. 1.

observed at the end of the dark period when levels of Ser were about 3 μmol g^{-1} FW in either CO_2 treatment.

3.2. Changes of organic acids in barley primary leaves in response to CO_2 enrichment

Concentrations of Mal, Cit and Scc were determined 9, 13 and 17 DAS using primary leaves of barley plants grown in the ambient and elevated CO_2 treatments (Fig. 3). These were the three most abundant organic acids detected in tissue samples examined here. Foliar concentrations of Mal and Cit increased but Scc decreased with leaf age. Levels of Mal in the ambient and elevated CO_2 treatments increased 54 and 36%, respectively, between 9 and 17 DAS. Mal was decreased 14% by CO_2 enrichment ($P \leq 0.01$) when averaged over all the three harvest dates. In contrast to Mal, Cit was increased by CO_2 enrichment when determined 9 and 13 DAS but this difference was not observed 17 DAS. Levels of Scc in barley primary leaves were 37% greater ($P \leq 0.01$) in the elevated than in the ambient CO_2 treatment when results for all three harvests were combined.

Diurnal changes of organic acids in barley primary leaves also were measured using 13-day-old plants (Fig. 4). Concentrations of Mal in barley primary leaves were about 2.9 mg g^{-1} FW at the beginning of the light period in both the ambient and elevated CO_2 treatments. During the next 12 h of light the concentration of Mal increased by 100% and by 66% in the ambient and elevated CO_2 treatments, respectively. Levels of Cit and Scc in barley primary leaves were greater in the dark than in the light under the ambient CO_2 treatment. Moreover, Cit and Scc were greater in the elevated than in the ambient CO_2 treatment when measured after 6 and 12 h of light. Therefore, diurnal variations of these two organic acids were smaller in the elevated than in the ambient CO_2 treatment.

4. Discussion

The initial stable products of photosynthetic carbon assimilation can be separated into neutral, acidic and basic fractions [16]. These alcohol-soluble fractions principally contain carbohydrates, organic acids and amino acids, respectively. It is widely agreed that plant growth in CO_2 enriched atmospheres enhances the accumulation of both leaf starch and soluble carbohydrates [3]. Since the metabolism of carbohydrates is essential for the synthesis of amino acids and organic acids, it is reasonable to assume that the effects of CO_2 enrichment should be similar for all three classes of compounds. However, published results and present findings concerning the effects of CO_2 enrichment on amino acids and organic acids have not supported this conclusion.

Experiments with mature tobacco leaves showed that soluble amino acids were largely unaffected by CO_2 enrichment when the plants were N sufficient [4,17] but decreased foliar amino acid levels occurred in response to CO_2 enrichment when the N supply was limited. Furthermore, CO_2 enrichment increased soluble amino acid concentrations in young, developing tobacco leaves. Rogers et al. [18] reported that foliar amino acid levels for soybean plants grown in the field were decreased by CO_2 enrichment early in the growing season but that no treatment differences were observed later in the year when the plants were fully established. Ainsworth et al. [14], in an extension of the above soybean study, reported that soluble amino acids were increased by CO_2 enrichment in young, developing soybean leaves but again treatment differences were not observed in fully expanded leaves. The above findings generally agreed that young leaves have the capacity to augment amino acid synthesis in response to CO_2 enrichment whereas older, mature leaves do not.

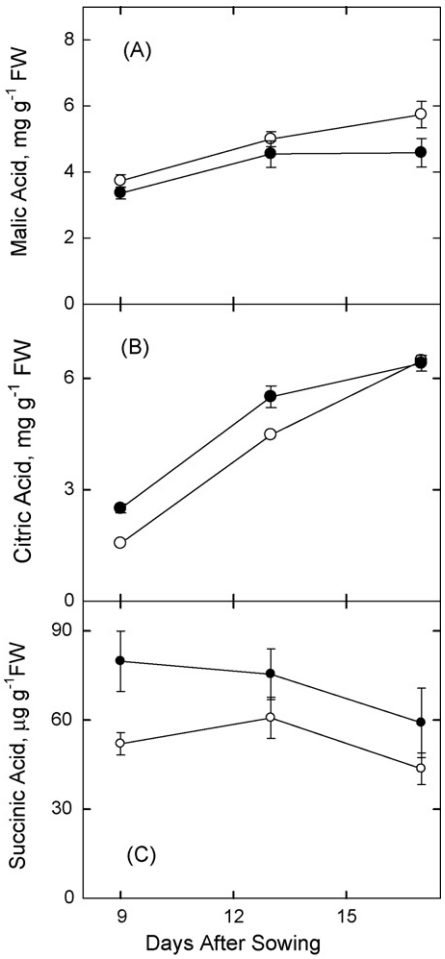

Fig. 3. Effects of CO_2 enrichment on age-dependent changes of organic acids in barley primary leaves. Plants were grown in controlled environment chambers with treatments, symbols and other details as in Fig. 1. The most abundant organic acids in the leaf were quantified by gas chromatography coupled to a mass selective detector. Values are means ± S.E. for $n = 10$.

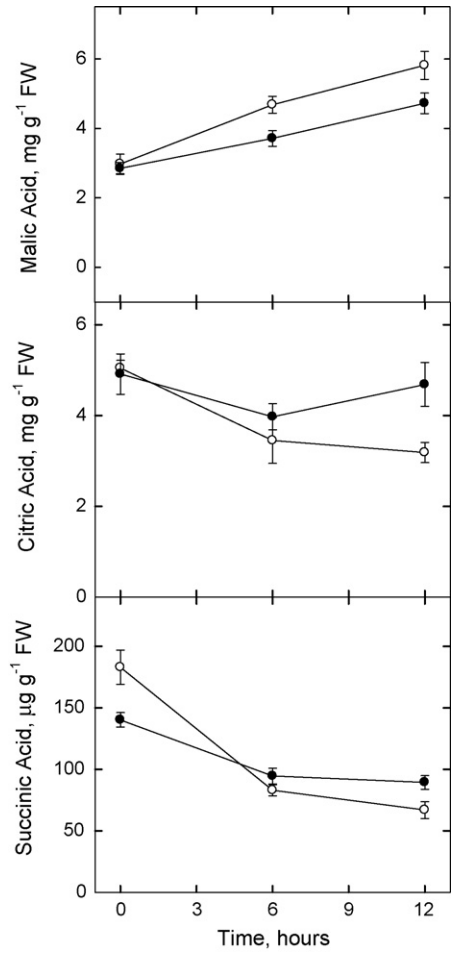

Fig. 4. Effects of CO_2 enrichment on diurnal changes of organic acids in barley primary leaves. Plants were grown in controlled environment chambers with treatments, symbols and other details as in Figs. 1 and 3.

In the current study total soluble amino acids were increased 33% by CO_2 enrichment when measured 9 DAS. Increased soluble amino acids in barley in response to enhanced CO_2 only occurred briefly and 4–8 days after the initial measurements amino acid levels were up to 70% lower in the elevated than in the ambient CO_2 treatment. The finding that soluble amino acids were initially increased by CO_2 enrichment in developing leaves was in agreement with prior reports by Geiger et al. [4] in tobacco and by Ainsworth et al. [14] in soybean. Geiger et al. [4] argued that the accumulation of soluble amino acids in young leaves in response to CO_2 enrichment was necessary to support subsequent protein synthesis. It is also possible that soluble amino acids accumulated in young seedlings in response to CO_2 enrichment because of an inadequate sink capacity.

All of the amino acids reported here decreased markedly with increasing leaf age in the elevated CO_2 treatment. Therefore, it is clear that CO_2-dependent leaf yellowing had a large negative impact on the levels of major amino acids in older barley primary leaves. Soluble carbohydrates and starch were increased by CO_2 enrichment in prior experiments using conditions similar to those of the present study [10,12]. Consequently, ample C was available to support amino acid synthesis in this tissue and reasons for the decline in soluble amino acids under CO_2 enrichment were likely due to inadequate rates of N acquisition and assimilation. Prior findings that both nitrate and ammonia were decreased by CO_2 enrichment in barley primary leaves supported this conclusion [13].

Concentrations of Asn and Gly were strongly influenced by age in barley primary leaves under both CO_2 treatments. This was in contrast to Glu, Gln and Asp which exhibited only small changes throughout development in the ambient CO_2 treatment. Results for Glu and Gln reported here during leaf development and in response to CO_2 enrichment were broadly in agreement with earlier results for barley primary leaves based on enzymatic measurements [13]. Note that leaf concentrations for Glu and Gln in the current study were corrected to 100% recovery, whereas the prior findings were not.

Jeong et al. [19] previously observed that Asn was elevated in developing quaking aspen leaves and suggested that Asn was a metabolic marker for young, leaf tissue. These authors further suggested that high levels of Asn may be a reserve source of N under stress conditions and that excess Asn in young leaves was dissipated via photorespiration later in development. An apparent reciprocal relationship was noted in the current study between Asn and Asp during leaf development in the ambient CO_2 treatment. This is consistent with the fact that Asp is transaminated to form Asn [19,20]. Note that Asn levels were not consistently increased by CO_2 enrichment in Figs. 1 and 2 when measured 13 DAS. This may be attributed to the fact that these results were from different plantings and that the overall concentration differences for Asn

between the ambient and elevated CO_2 treatments were small on this date.

Large diurnal changes of soluble amino acids also were observed when using primary leaves from 13-day-old plants. Note that CO_2-dependent differences for Glu, Asp, Gly, Ser and Asn were relatively constant during the day except for the first sampling when light/dark effects were observed. The exception to this rule was Gln which decreased progressively throughout the photoperiod in the elevated compared to the ambient CO_2 treatment. Since soluble carbohydrates and starch accumulated in primary leaves throughout the light period [3], this finding again indicated that N uptake and assimilation, rather than C availability, were negatively impacted by CO_2 enrichment in this tissue during the light. Therefore, understanding the effects of CO_2 enrichment on Gln synthesis in barley leaves will likely to be an important research question in the future. The fact that Gln levels were similar in both treatments at the 7 a.m. harvest suggested that during the dark period Gln synthesis was not impaired by CO_2 enrichment. Alternately, Gln may have been imported into leaf tissue from roots during the dark [19]. Effects of CO_2 enrichment on Gln levels throughout the day were not likely the result of chlorosis or related factors because 13-day-old leaves displayed only limited amounts of leaf yellowing. Also, Gln levels in older, vegetative barley leaves were similarly affected by elevated CO_2 on a diurnal basis when using a different cultivar that did not develop yellowing (data not shown).

Geiger et al. [4] measured Mal and Cit in young tobacco leaves and showed that these organic acids were increased significantly by CO_2 enrichment. Also, these authors reported that Mal increased throughout the light period but only in response to elevated CO_2 and that Cit decreased by about one-half in the light under both CO_2 treatments. In contrast to the above findings, Backhausen and Scheibe [21] reported that Mal levels in tobacco decreased by about one-half in response to a doubling of growth CO_2 regardless of leaf age. In the latter study leaf samples were analyzed at the end of the dark period. Robinson and Sicher [12] further observed that total Asc levels in barley primary leaves were 22% lower on average in the elevated than in the ambient CO_2 treatment on the same three dates used in the present experiments.

Responses of organic acids to CO_2 enrichment in barley primary leaves throughout development were small in comparison to that of soluble amino acids (Fig. 3). Consequently, CO_2-dependent leaf yellowing did not have large effects on organic acid levels in this study. Concentrations of both Mal and Cit increased in barley primary leaves between 9 and 17 DAS, whereas Scc was little changed or decreased slightly. Concentrations of Mal and Cit were each over 30-fold greater than that of Scc, which is consistent with prior findings that Mal and Cit are normally stored in vacuoles [22]. The finding that Cit and Scc were enhanced by CO_2 enrichment in younger barley primary leaves is a possible indication that C flux through the tricarboxylic acid cycle was increased in association with elevated carbohydrate levels.

Changes in organic acid levels also were measured throughout the light period using primary leaves of 13-day-old plants. Although differences were small in the present study, Mal was lower in the elevated than the ambient CO_2 treatment after 6 and 12 h of the light period. Note that Mal and Cit were similar in the ambient and elevated CO_2 treatments at the end of the dark period (i.e., at 0 h light). Therefore, current findings for Mal were not in close agreement with prior results by Geiger et al. [4] or Backhausen and Scheibe [21]. However, effects of CO_2 enrichment on Cit in barley primary leaves were generally in agreement with the earlier findings of Geiger et al. [4]. Scc was maximal at the 0 h measurement and subsequently decreased by over one-half during the light period. Decreases of Scc levels in the light were less for the elevated than for the ambient CO_2 treatment, again suggesting that CO_2 enrichment increased C flux through the tricarboxylic acid cycle.

In summary, organic and amino acid levels in barley primary leaves were dynamic and changed markedly with age, CO_2 treatment and in response to light. Initially, CO_2 enrichment increased total soluble amino acids in barley primary leaves. However, large decreases of Asn and Gln and other soluble amino acids occurred with advancing leaf age in the elevated compared to the ambient CO_2 treatment. These findings suggested that soluble amino acids were negatively impacted by CO_2-dependent leaf yellowing in older barley primary leaves. Also, Gln levels decreased progressively on a diurnal basis in the elevated compared to the ambient CO_2 treatment using 13-day-old primary leaves. Since carbohydrates were abundant [10] this was an indication that rates of N uptake and assimilation in the enhanced CO_2 treatment were limiting for Gln synthesis. Since Gln can be used in transamination reactions to synthesize several other amino acids, the negative impact of CO_2 enrichment on Gln synthesis also could explain the low levels of soluble amino acids observed in older primary leaves of the elevated compared to the ambient CO_2 treatment. Effects of CO_2 enrichment on organic acids were small in comparison to the above results for soluble amino acids. This was an indication that CO_2-dependent leaf yellowing and low rates of N assimilation in the light did not affect organic acids to the same extent as amino acids.

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