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Responses of nitrogen metabolism in N-sufficient barley primary leaves to plant growth in elevated atmospheric carbon dioxide

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Abstract

Effects of atmospheric carbon dioxide enrichment on nitrogen metabolism were studied in barley primary leaves (Hordeum vulgare L. cv. Brant). Seedlings were grown in chambers under ambient (36 Pa) and elevated (100 Pa) carbon dioxide and were fertilized daily with complete nutrient solution providing 12 millimolar nitrate and 2.5 millimolar ammonium. Foliar nitrate and ammonium were 27% and 42% lower ($P \leq 0.01$) in the elevated compared to ambient carbon dioxide treatments, respectively. Enhanced carbon dioxide affected leaf ammonium levels by inhibiting photorespiration. Diurnal variations of total nitrate were not observed in either treatment. Total and Mg^{2+}inhibited nitrate reductase activities per gram fresh weight were slightly lower ($P \leq 0.01$) in enhanced compared to ambient carbon dioxide between 8 and 15 DAS. Diurnal variations of total nitrate reductase activity in barley primary leaves were similar in either treatment except between 7 and 10 h of the photoperiod when enzyme activities were decreased ($P \leq 0.05$) by carbon dioxide enrichment. Glutamate was similar and glutamine levels were increased by carbon dioxide enrichment between 8 and 13 DAS. However, both glutamate and glutamine were negatively impacted by elevated carbon dioxide when leaf yellowing was observed 15 and 17 DAS. The above findings showed that carbon dioxide enrichment produced only slight modifications in leaf nitrogen metabolism and that the chlorosis of barley primary leaves observed under enhanced carbon dioxide was probably not attributable to a nutritionally induced nitrogen limitation.

Abbreviations: DAS – days after sowing; PAR – photosynthetically active radiation; NADH:NR – NADH-dependent nitrate reductase

Introduction

Terrestrial C_3 plants generally possess increased rates of net photosynthesis and greater biomass accumulation under atmospheric CO_2 enrichment. Increased rates of net photosynthesis occurred primarily because CO_2 assimilation rates were not saturated by the current atmospheric CO_2 concentration (Long 1991). Accelerated growth rates in response to elevated CO_2 altered the demand for mineral nutrients and this affected C/N ratios in the foliage and other plant parts (Conroy and Hocking 1993). The uptake and assimilation of N is coordinated with net rates of photosynthesis and interactions between these metabolic processes also were altered by CO_2 enrichment (Fonseca et al. 1997).

Numerous studies have shown that growth in elevated CO_2 can result in decreased foliar [NO_3^-](Purvis et al. 1974; Fonseca et al. 1997), [NH_4^+](Ferrario-Méry et al. 1997, Geiger et al. 1999), soluble protein (Wong 1979; Conroy 1992; Nie et al. 1995), and ribulose-1,5 bisphosphate carboxylase/oxygenase (Rubisco) protein (Rowland-Bamford et al. 1991; Sicher et al. 1997; Sims et al. 1998). Moreover, total

reduced N in the foliage was decreased, particularly with respect to dry matter accumulation, and N use efficiency was improved by CO_2 enrichment (Hocking and Meyer 1991; Poorter et al. 1997). Effects of elevated CO_2 on leaf N metabolism also were greater in N deficient compared to N sufficient plants (Hocking and Meyer 1991; Ferrario-Méry et al. 1997; Geiger et al. 1999). The above observations suggested that plant growth under CO_2 enrichment was potentially N limited (Conroy and Hocking 1993).

Several possible reasons have been advanced to explain the reduction of total N in the shoots of CO_2 enriched plants. One suggested possibility was that growth in elevated CO_2 accelerated plant growth and development with the result that leaf senescence occurred prematurely (Miller et al. 1997; Sicher and Bunce 1997). Diminished soluble protein, Rubisco protein and chlorophyll observed in numerous CO_2 enrichment studies were consistent with accelerated senescence. Carbohydrate regulated gene expression also could be a factor in accelerated leaf senescence and in the photosynthetic decline observed under elevated CO_2 (Sheen 1997). An alternative proposal was that premature senescence during growth in elevated CO_2 was the result of a growth induced N deficiency (Ferrario-Méry et al. 1997; Stitt and Krapp 1999). Altered nutritional status certainly could be an important factor in many of the observed adverse effects of CO_2 enrichment on plant growth and development. Understanding how a growth induced N-limitation develops at elevated CO_2 has potential implications for sustaining agricultural productivity and for improving the nutritional quality of foods in the near future.

Changes of leaf N metabolism in response to CO_2 enrichment are not thoroughly understood at the biochemical level. Fonseca et al. (1997) reported a transient increase of NADH:NR activity in shoots and roots of *Plantago major* within days after plants were transferred from ambient to twice ambient CO_2. Conversely, other investigators (Purvis et al. 1974; Hocking and Meyer 1991; Ferrario-Méry 1997) observed that for a variety of species NADH:NR was 25 to 50% lower when plants were grown at elevated compared to ambient CO_2. The activity of NADH:NR *in vivo* is controlled by several, complex regulatory mechanisms and the expression of mRNA encoding this enzyme and other steps in N assimilation is dependent on [NO_3^-] (Galangau et al. 1988; Huber et al. 1992; Geiger et al. 1998). The conflicting observations cited above and the complexity of interactions between photosynthesis and N metabolism suggested that additional research on this subject is needed.

Chlorosis and other forms of leaf injury have been reported in prior CO_2 enrichment studies performed with tomato (Tripp et al. 1991), cotton (Betsche 1994), tobacco (Miller et al. 1997) and eggplant (Nederhoff and Buitlear 1992). Extensive leaf yellowing also was observed on primary leaves of barley seedlings grown under high light and elevated CO_2 (Sicher 1997, 1999). Primary leaves from the elevated CO_2 treatment contained less than 0.1 g m^{-2} Chl between 16 and 18 DAS. These values were 78% below maximal Chl concentrations observed 12 DAS. By comparison, Chl concentrations of primary leaves in the ambient CO_2treatment decreased 36% between 12 and 18 DAS. Increased growth rates in response to elevated CO_2 occurred early in ontogeny and diminished with plant age (Fonseca et al. 1997). Consequently, understanding the effects of CO_2 enrichment on seedlings is of critical importance. The objective here was to ascertain the effects of elevated CO_2 on N metabolism of barley primary leaves and to determine if leaf yellowing was influenced by an induced N deficiency.

Materials and methods

Plant materials

Barley (*Hordeum vulgare* L. cv. Brant) plants were raised from single seeds sown in plastic pots filled with 1 dm^3 of vermiculite (Sicher 1997). Plants were grown at 22 °C in matching controlled environment chambers (model M-2, Environmental Growth Chambers Corp., Chagrin Falls, Ohio) using a 14-h light/10-h dark cycle. Irradiance was 950 ± 30 μmol m^{-2} s^{-1} PAR and was provided by twenty high-pressure Na^+ and metal halide lamps (Lucolux LU 400 and Multivapor MVR 400, General Electric Co., Cleveland Ohio) located in Sunbrella reflectors provided by the manufacturer. Relative humidity was not controlled but was about 80% in the light and never fell below 50% in the dark. Growth chambers were flushed continuously with CO_2-free air and chamber air CO_2 partial pressures were controlled at 36 ± 1 (ambient) and 100 ± 2 Pa (elevated) using infrared analyzers equipped with set-point controllers (model WMA-3, PP Systems, Haverhill, Massachusetts). Plants were watered once daily during the 1st h of the light period with a complete mineral nutrient solution containing 12 mm NO_3^- and 2.5 mm NH_4^+ (Robinson 1984).

Care was taken during watering to prevent the surface contamination of leaves.

Leaf sampling and data analysis

Experiments were started 8 or 9 days after sowing (DAS) and usually were terminated 17 DAS. Primary leaves were either harvested at indicated times or were collected between 4 and 5 h of light. Primary leaves are developed partly from seed reserves. This reduced the impact of treatment differences on leaf development in this study. Leaf segments (5–10 cm length) from the middle of each primary leaf were transferred rapidly to labeled envelopes and frozen in liquid N_2 to quench metabolism. Leaf area was determined using the width measured at the midpoint of each leaf segment. Samples were either extracted immediately or were frozen at –80 °C until use. Primary leaves from 5 ambient and 5 elevated CO_2-grown plants were sampled at five or six time-points per experiment and results from 2 or 3 experiments (n = 100–180) were combined for analysis. Ambient and elevated CO_2 treatments were randomly assigned to two chambers for each experiment. Significant differences were determined using a three-way Analysis of Variance procedure (Statview 5.0, SAS Inc., Raleigh, North Carolina) with date or time, experiment and CO_2 treatment as nominal factors.

Leaf component analysis

Frozen leaf segments were weighed and quickly transferred to 15 ml ground-glass tissue homogenizers on ice. Samples were extracted at 0 °C with 2 ml 80% ethanol and the homogenates were transferred to 2 ml micro-screw cap cryovials and stored on ice. Homogenates were centrifuged at full line voltage (Eppendorf model 5415 C, Brinkmann Instr., Westbury, New York) and 0.5 ml of the supernatant was transferred to a fresh, tightly sealed cryovial and stored at –20 °C. A 1 ml aliquot of the supernatant was partitioned with 1 ml $CHCl_3$ and 1 ml H_2O and the resultant aqueous fractions were evaporated to a minimum volume under a stream of N_2 at 45 °C. Concentrates were diluted to a final volume of 1 ml with deionized H_2O and stored at –20 °C as described above. All enzymes and reagents were from Sigma-Aldrich Co., St. Louis, Missouri. NH_4^+ was quantified enzymically with 0.1 ml of the 80% alcohol extracts using a diagnostic kit (Sigma No. 171-A). Tissue concentrations of NH_4^+ were calculated according to the manufacturer's instructions. Aqueous fractions of the partitioned 80% alcohol extracts were diluted from 1:20 or 1:50 and NO_3^- was quantified by high pressure liquid chromatography according to Thayer and Huffaker (1980). Samples were separated isocratically at 1 ml min^{-1} using a 0.5 by 25 cm Partisil-10-SAX column (Whatman Inc., Clifton, New Jersey) with 50 mm phosphate buffer (pH 3.O) in the mobile phase. Absorbance due to NO_3^- was measured at 210 nm using a Waters model 490E Multiwavelength Detector. Glutamate and glutamine were also measured enzymically using aqueous fractions derived from the 80% ethanol extracts (Brent and Bergmeyer 1974). Glutamate was detected at 30 °C in 1 ml reactions containing 300 mM glycine-250 mM hydrazine buffer (pH 9.0), 1.2 mM ADP, 1.75 mM NAD^+ and 0.02 – 0.1 ml sample. Reactions were initiated with 3–5 International Units of L-glutamate dehydrogenase (G-2626) and increased absorbance was determined after 5 min at 340 nm. Glutamine was deaminated enzymically and was detected using the glutamate assay described above. An aliquot of the aqueous fraction was incubated for 2 h at 37 °C with an equal volume of 0.5 M acetate buffer (pH 5.0) containing 0.1 International Units of L-glutaminase (G-5382). Recoveries of standard glutamate and glutamine were from 80 to 85%.

The activity of NADH:NR in barley primary leaf extracts was determined at 25 °C using an *in vitro* colorimetric procedure (Huber et al. 1992). Assays were performed both in the presence and absence of Mg^{2+} to differentiate the total and inhibited activities of NADH:NR, respectively. Reaction mixtures contained 50 mM Hepes-NaOH (pH 7.5), 5 mM KNO_3, 5 μM FAD, 0.2 mM NADH and either 6 mM $MgCl_2$ (plus Mg^{2+} assay) or 3 mM EDTA (minus Mg^{2+} assay). These are also referred to as total and Mg^{2+} inhibited NADH:NR activities, respectively. Assays were initiated with leaf extract and were terminated after O and 5 min in a boiling H_2O bath. The reaction product, NO_2^-, was detected as a diazo compound formed with sulphanilamide (Aslam et al. 1979). Blanks were also prepared using leaf extracts kept in a boiling H_2O bath for 2 min prior to assay.

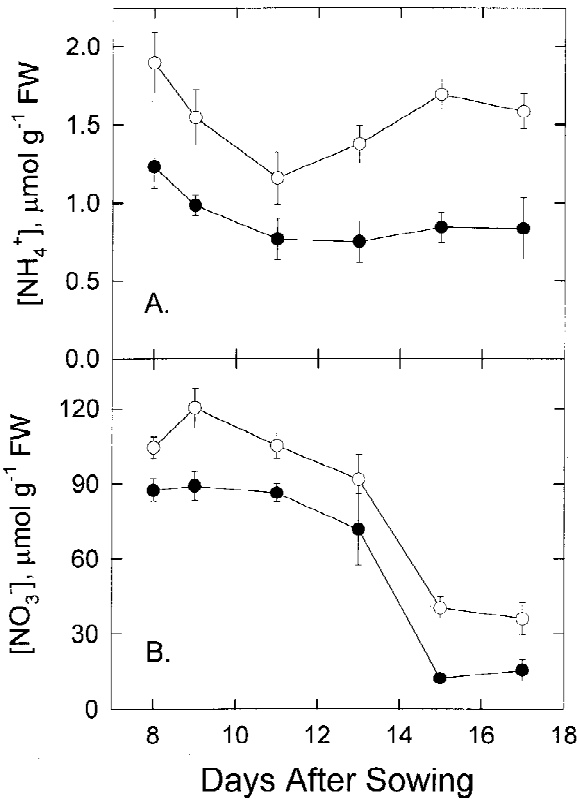

Figure 1. Changes of $[NH_4^+]$ and $[NO_3^-]$ in barley primary leaves in response to CO_2 enrichment and increasing leaf age. Barley primary leaf segments were sampled between 4 and 5 h of light on indicated dates during growth in ambient (○) or elevated (●) atmospheric CO_2. (A) NH_4^+ and (B) NO_3^-. Values are means ± SE.

Results

Effects of CO_2 enrichment on barley primary leaf $[NO_3^-]$ and $[NH_4^+]$

Both $[NH_4^+]$ and $[NO_3^-]$ were significantly lower ($P \leq 0.01$) in barley primary leaves in the elevated compared to the ambient CO_2 treatment (Figure 1). Mean $[NH_4^+]$ for all six dates between 8 and 17 DAS was 1.54 ± 0.07 and 0.90 ± 0.06 μmol g^{-1} FW in the ambient and elevated CO_2 treatments, respectively (Figure 1A). The treatment by date interaction was not significant ($P > 0.05$), indicating that effects of elevated CO_2 on $[NH_4^+]$ were detected each sampling date. The $[NH_4^+]$ in barley primary leaves from the ambient CO_2 treatment was maximal 8 DAS, decreased 40% to a minimum 11 DAS and then increased again in older leaves. Age dependent changes of $[NH_4^+]$ in primary leaves from the ambient and elevated CO_2 treatments were similar. However, the buildup of $[NH_4^+]$ observed in leaves 15–17 DAS was less evident in the elevated compared to the ambient CO_2 treatment.

Typical of N-sufficient plants grown with a complete mineral nutrient solution (Geiger et al. 1999), barley primary leaves accumulated substantial amounts of $[NO_3^-]$ under both CO_2 treatments between 8 and 13 DAS (Figure 1B). Estimated on a dry weight basis, $[NO_3^-]$ comprised approx. 5% of the mass of a barley primary leaf. Note also that for this time period, leaf $[NO_3^-]$ was 50–100-fold greater than values for $[NH_4^+]$. For both the ambient and elevated CO_2 treatments, leaf $[NO_3^-]$ decreased by more than two-thirds between 9 and 15 DAS. Mean leaf $[NO_3^-]$ averaged over all measurement dates was 80.6±4.2 μmol g^{-1} FW in the ambient CO_2 treatment. This value was 27% lower ($P \leq 0.01$) for leaves of seedlings in the elevated CO_2 treatment and a treatment by date interaction was not detected for this parameter ($P \geq 0.05$). Effects of CO_2 enrichment on NO_3^- accumulation were about half as great when expressed per unit area. This reflected the fact that primary leaves contained about 10% greater mass per unit leaf area in the elevated compared to ambient CO_2 growth treatments (data not shown). Nevertheless, both foliar $[NO_3^-]$ and $[NH_4^+]$ were significantly lower ($P \leq 0.01$) in the enhanced compared to the ambient CO_2 treatment when results were expressed per unit leaf area (data not shown).

Effects of growth in elevated CO_2 on leaf $[NH_4^+]$ and $[NO_3^-]$ were then determined for a single diurnal cycle 9 DAS (Figure 2). Foliar $[NH_4^+]$ was low in the dark and initially was similar in the ambient and elevated CO_2 treatments (Figure 2A). A 2-fold increase of $[NH_4^+]$ was observed in the light for leaves of barley seedlings under ambient CO_2. By comparison, $[NH_4^+]$ in primary leaves under CO_2 enrichment remained low and was essentially unchanged in light and dark. In contrast to the above results for $[NH_4^+]$, $[NO_3^-]$ remained high in barley primary leaves throughout the diurnal cycle and any observed changes were negligible (Figure 2B). Mean primary leaf $[NO_3^-]$ was 13% lower ($P \leq 0.01$) in the elevated compared to the ambient CO_2 treatment when all measurements taken 9 DAS were combined. A treatment by date interaction also was not detected for these measurements ($P > 0.05$).

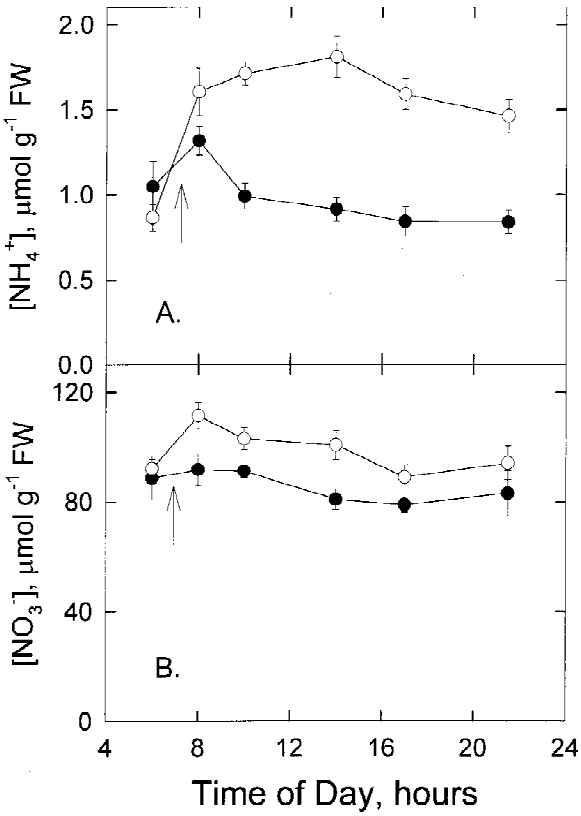

Figure 2. Diurnal variations of $[NH_4^+]$ and $[NO_3^-]$ in barley primary leaves in response to CO_2 enrichment. Barley primary leaf segments were sampled at indicated times 9 DAS under either ambient (○) or elevated (●) atmospheric CO_2. Other experimental details were as in Figure 1.

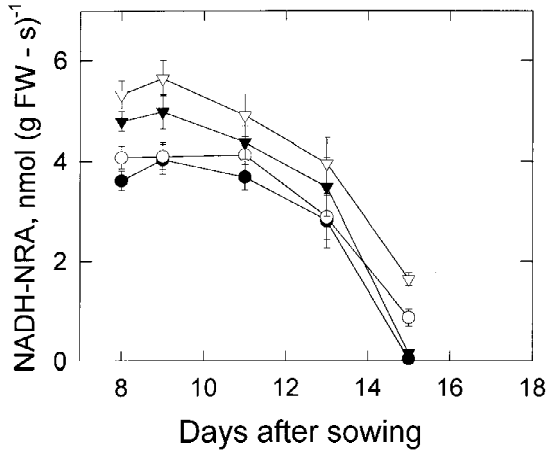

Figure 3. Changes of NADH-dependent nitrate reductase activity in barley primary leaves in response to CO_2 enrichment and increasing leaf age. Data were for total (Δ, ▲) and Mg^{2+} inhibited (○, ●) NADH:NR activity under ambient (○, Δ) and elevated (▲, ●) CO_2. Values are means ± SE.

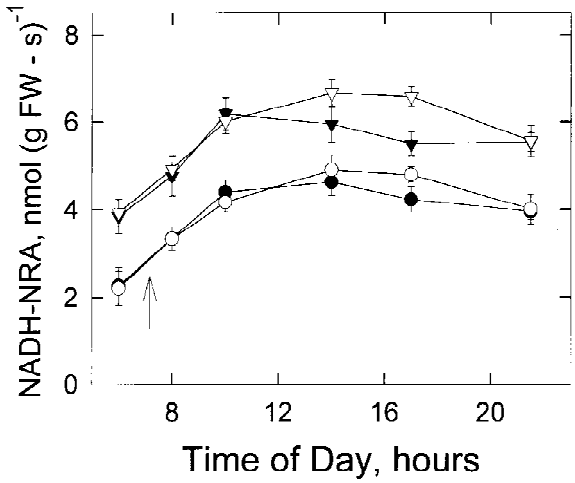

Figure 4. Diurnal variations of NADH-dependent nitrate reductase activity in barley primary leaves in response to CO_2 enrichment. Barley primary leaf segments were sampled at indicated times 9 DAS. Symbols and other experimental details were as in Figure 3.

Changes of barley primary leaf NADH:NR activity in response to CO_2 enrichment

The activity of NADH:NR in barley primary leaves was maximal immediately after full leaf expansion and then decreased with leaf age (Figure 3). Between 9 and 15 DAS, total primary leaf NADH:NR activity decreased 71% and 97%, respectively, in leaves of seedlings grown with ambient and elevated CO_2. NADH:NR activity measured in the presence of Mg^{2+} also dropped by more than 70% between 9 and 15 DAS. Both the plus and minus Mg^{2+} NADH:NR activities in barley primary leaves were negligible 15 DAS in elevated CO_2 and there was no detectable NADH:NR activity for leaves from either CO_2 treatment 17 DAS (data not shown). Mean NADH:NR activities averaged over all five dates in the ambient CO_2 treatment were 4.3 ± 0.3 and 3.2 ± 0.2 nmol g^{-1} FW s^{-1} for the total and Mg^{2+} inhibited assays, respectively. These mean enzyme activities were 17% and 12% lower, respectively ($P < 0.01$), when performed on leaves from the elevated CO_2 treatment. The treatment by date interaction was not significant for both the plus and minus Mg^{2+} NADH:NR assays.

Effects of CO_2 enrichment on the NADH:NR activity of barley primary leaves also were determined over one diurnal cycle 9 DAS (Figure 4). The total activity of NADH:NR from primary leaves in the ambient CO_2 treatment was minimal in the dark, increased 69% during the first 7 h of light and then

decreased gradually until the end of the photoperiod. Changes of NADH:NR activity throughout the diurnal cycle were qualitatively similar for the minus and plus Mg^{2+} assays. Most of the diurnal increase in enzyme activity occurred during the first 3 h of light. Total NADH:NR activities in the ambient and elevated CO_2 treatments were similar throughout the diurnal cycle, except between 7 and 10 h of light. During this part of the photoperiod, total NADH:NR activity was decreased by CO_2 enrichment. Averaged over all six measurements 9 DAS, total NADH:NR activities were 5.6 ± 0.2 and 5.3 ± 0.2 nmol g^{-1} FW s^{-1} for the ambient and elevated CO_2 treatments, respectively ($P \leq 0.05$). However, there were no CO_2 treatment effects on mean NADH:NR activities in the plus Mg^{2+} assay ($P \leq 0.05$). Ratios of the Mg^{2+}–inhibited total NADH:NR activity 9 DAS were 0.71 and 0.68 for the ambient and elevated CO_2 treatments, respectively ($P \leqslant 0.05$). Soluble protein concentrations for the above NADH:NR dataset 9 DAS were 23.3 ± 0.4 and 24.3 ± 0.5 mg g^{-1} FW ($P > 0.05$) for the ambient and elevated CO_2 treatments, respectively (data not shown).

Effects of elevated CO_2 on glutamate and glutamine levels in barley primary leaves

Changes of glutamate and glutamine in barley primary leaves were measured as a function of leaf age (Figure 5). Amounts of glutamate in leaves from the ambient CO_2 treatment were 8.5 ± 0.5 μmol g^{-1} FW when measured 8 DAS (Figure 5A). Glutamate levels were 50% greater 11 DAS but then returned to initial values by 17 DAS. Changes of glutamate in primary leaves in the ambient and elevated CO_2 treatment were similar, except 15 and 17 DAS when glutamate levels were decreased by CO_2 enrichment. In contrast to glutamate, glutamine levels in primary leaves from the ambient CO_2 treatment were high initially, decreased to a minimum value11 DAS and then returned to about original levels between 15 and 17 DAS (Figure 5B). In comparison to the ambient CO_2 grown plants, effects of CO_2 enrichment were to increase glutamine levels in barley primary leaves between 8 and 11 DAS and to decrease glutamine 15 and 17 DAS. Since treatment effects differed in younger and older leaves, mean glutamine concentrations summed over all measurement dates were unaffected ($P \leq 0.05$) by the elevated CO_2 treatment.

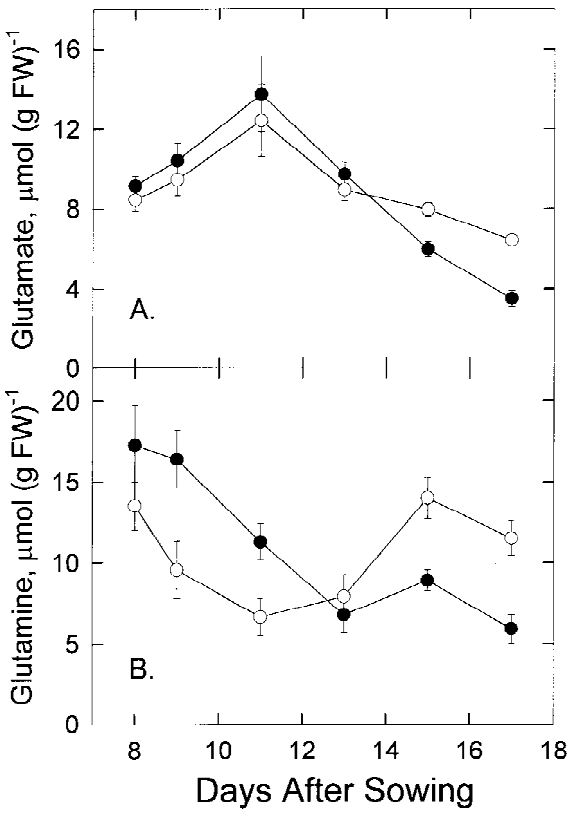

Figure 5. Changes of glutamate and glutamine levels in barley primary leaves in response to CO_2 enrichment and increasing leaf age. Data were for (A) glutamate and (B) glutamine, and symbols were as in Figure 1. Values are means ± SE.

Discussion

Growth in elevated CO_2 generally leads to pronounced increases of photosynthesis and growth of well-fertilized plants (Fonseca et al. 1997). However, these changes are usually transitory because plant growth under CO_2 enrichment is subject to a possible N-limitation (Conroy and Hocking 1993; Geiger et al. 1999). Enhanced photosynthetic rates under elevated CO_2 could interact with the N metabolism of leaves in a number of ways. First, NO_2^- and CO_2 reduction potentially compete for reducing equivalents produced by the electron transport chain of the chloroplast (Bloom 1997). Second, increased CO_2 partial pressures inhibit photorespiration and decrease both NH_4^+ release and the C flux through serine and glycine, as well as other amino acids (Ferrario-Méry 1997). A buildup of photosynthate in response to CO_2 enrichment provides extra C skeletons for amino acid synthesis and would be expected to enhance NO_3^-

assimilation via feed-forward regulatory mechanisms (Champingy and Foyer 1992). Both NADH:NR activity and enzymes involved in photosynthetic carbohydrate synthesis are coordinately regulated by post-translational modification (Huber et al. 1992; Hoff et al. 1994). There is also a growing body of evidence that NO_3^- and various soluble sugars are capable of altering the expression of specific genes via complex molecular signaling pathways (Stitt and Krapp 1999).

Decreased foliar [NO_3^-] has been observed in several prior CO_2 enrichment studies (Purvis et al. 1974; Hocking and Meyer 1991; Geiger et al. 1999). However, other investigators (Geiger et al. 1998, 1999) reported that CO_2 effects on leaf [NO_3^-] varied with N-nutrition, species and leaf age. In the present study, increasing the growth CO_2 partial pressure from 36 to 100 Pa decreased [NO_3^-] in barley primary leaves 13 – 27% on a FW basis. In contrast to earlier reports (Gebauer et al. 1984; Scheible et al. 1997), large diurnal variations of [NO_3^-] were not observed in barley primary leaves. This finding indicated that the ratio of NO_3^- supplied to NO_3^- assimilated was greater for seedlings than for older plants grown in pots. The lack of a diurnal variation of [NO_3^-] in barley primary leaves also indicated that for seedlings rates of NO_3^- uptake and accumulation were balanced with rates of NO_3^- utilization. The reason for decreased NO_3^- in leaves of CO_2 enriched plants is unknown. It has been postulated that a CO_2-dependent reduction in transpiration rates could decrease the flux of NO_3^- from roots to shoot (Conroy and Hocking 1993). It was also possible that leaf NO_3^- assimilation rates were greater in elevated compared to ambient CO_2-grown plants.

In the present study, [NH_4^+] was significantly lower in primary leaves of elevated compared to ambient CO_2-grown plants. Previous investigators (Ferrario-Méry et al. 1997; Geiger et al. 1999) attributed the effects of CO_2 enrichment on [NH_4^+] to a suppression of the photorespiratory pathway. The present finding that elevated CO_2 only affected [NH_4^+] in barley primary leaves in the light supported this conclusion. However, it is difficult to interpret the significance of decreased photorespiration rates under elevated CO_2 on NH_4^+ metabolism. Rates of NH_4^+ released via photorespiration were greater than rates of NH_4^+ assimilation and there is evidence that NH_4^+ released from the oxidation of glycine is reassimilated by glutamine synthetase in the chloroplast (Wallsgrove et al. 1980). It was interesting to note that the age dependent changes of foliar [NH_4^+] in this study compared favorably to results for leaves of varying ages on elongating tobacco shoots (Masclaux et al. 2000). Conversely, these same authors observed that [NO_3^-] was greatest in the oldest tobacco leaves.

Glutamine and glutamate levels in barley primary leaves responded differently to CO_2 enrichment. Glutamate did not differ in ambient and elevated CO_2-grown barley primary leaves except on the last two measurement dates. Decreased photosynthetic capacity due to extensive CO_2-dependent leaf yellowing impacted on glutamate concentrations in these older primary leaves. Glutamine initially was higher in elevated compared to ambient CO_2 when comparing young, recently expanded primary leaves. As noted for glutamate, effects of CO_2 enrichment were to reduce leaf glutamine levels in older primary leaves in association with leaf yellowing. The above results for glutamate and glutamine in younger barley primary leaves were in agreement with those of Ferrario-Méry et al. (1997) using N. plumbaginifolia grown in hydroponic culture. Total α-amino N levels in barley primary leaves also were greater under elevated CO_2 in younger but not older leaf samples (Sicher 1998). Geiger et al. (1998) reported a 200–300% increase of glutamine in young leaves of tobacco seedlings grown with elevated CO_2. The finding that glutamate and glutamine responded differently to growth in elevated CO_2 indicated that increased total α-amino N of CO_2 enriched barley primary leaves was due to altered levels of specific amino acids and not a general increase of foliar photoassimilates.

NADH:NR activity *in vivo* is controlled by several complex regulatory mechanisms. NADH:NR activity, mRNA transcript levels and protein levels vary diurnally (Galangau et al. 1988). Also, foliar NADH:NR activity is modulated by light, leaf [NO_3^-], carbohydrate levels, post-translational modification and by other possible mechanisms (Huber et al. 1992; Hoff et al. 1994). Because NO_3^- is the most common source of nutritional N for plant growth, it is important to understand the impact of CO_2 enrichment on NADH:NR activity. NADH:NR activity was greatest in barley primary leaves immediately after full leaf expansion but enzyme activity disappeared when leaves still possessed 50% or more of maximal photosynthetic capacity (Sicher 1998). There was a 12–17% decrease of total and mg^{2+} inhibited NADH:NR activity in primary leaves of elevated compared to ambient CO_2-grown seedlings in this study. These CO_2 enrichment effects on NADH:NR activity were smaller when results were expressed on a leaf area basis. CO_2-dependent changes of NADH:NR activity were magni-

fied for older leaves by leaf yellowing that was evident beginning 15 DAS. Large numbers of studies have purportedly examined the effects of CO_2 enrichment on foliar NADH:NR activity. These were almost invariably based on a limited number of time points, repetitions have not been carefully documented, sample sizes were usually very limited and statistical analysis has rarely been attempted. Unfortunately, due to the inherent variability of *in vitro* enzyme measurements, much of the earlier work on this subject is difficult to evaluate.

A diurnal variation of total and Mg^{2+} inhibited NADH:NR activity was observed for 9-day-old barley primary leaves in the present study. However, an effect of CO_2 enrichment on the diurnal variation of total NADH:NR activity was only evident during the latter half of the photoperiod. Geiger et al. (1998) also noted a modified diurnal rhythm of NADH:NR activity in tobacco source leaves, although in their experiments enzyme activity was increased by CO_2 enrichment but primarily during the latter half of the light period. Galangau et al. (1988) attributed the decline of NADH:NR activity towards the end of the photoperiod in ambient CO_2-grown tobacco and tomato to a feedback control mechanism resulting from foliar metabolite accumulation. Results of the present study were consistent with the suggestion that feedback control altered the diurnal rhythm of total NADH:NR in CO_2 enriched barley primary leaves. Clearly, the diurnal rhythm of NADH:NR in barley primary leaves and its modification by CO_2enrichment were not attributable to changes of total leaf $[NO_3^-]$.

Several lines of evidence indicated that the N metabolism of barley primary leaves underwent a transition beginning 11 DAS. Both $[NO_3^-]$ and NADH:NR activity in barley primary leaves were high in younger leaves and decreased after this point in time. Concomitantly in the ambient CO_2 treatment, foliar glutamate levels were maximal, and glutamine and $[NH_4^+]$ were minimal 11 DAS. Presumably, N assimilation rates were greatest in younger leaves and diminished beginning 11 DAS. Changes of nitrogenous metabolites in older primary leaves were likely the result of N mobilization.

Collectively, the above results showed that growth in elevated CO_2 altered the N metabolism of barley primary leaves. However, the responses of $[NO_3^-]$, NADH:NR activity and glutamate to CO_2 enrichment were small relative to previous reports (Fonseca et al. 1997; Ferrario-Méry et al. 1997; Geiger et al. 1998) and the observed changes of $[NH_4^+]$ were likely the result of a diminished photorespiratory pathway. It should be noted that greater effects of CO_2 enrichment on foliar N metabolism would be expected using N deficient growth conditions (Geiger et al. 1999). The above evidence suggested that leaf yellowing of barley primary leaves was not the result of an N limitation induced by growth in elevated CO_2. The most likely explanation for chlorosis of barley primary leaves under elevated CO_2 is oxidative damage to the photosystems (Sicher 1999). It is important to note that this photoxidation was potentially exacerbated by a reduction of the photorespiratory pathway under CO_2 enrichment.

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