Nitrogen assimilation and growth of wheat under elevated carbon dioxide

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Simultaneous measurements of CO_2 and O_2 fluxes from wheat (*Triticum aestivum*) shoots indicated that short-term exposures to elevated CO_2 concentrations diverted photosynthetic reductant from NO_3^- or NO_2^- reduction to CO_2 fixation. With longer exposures to elevated CO_2, wheat leaves showed a diminished capacity for NO_3^- photoassimilation at any CO_2 concentration. Moreover, high bicarbonate levels impeded NO_2^- translocation into chloroplasts isolated from wheat or pea leaves. These results support the hypothesis that elevated CO_2 inhibits NO_3^- photoassimilation. Accordingly, when wheat plants received NO_3^- rather than NH_4^+ as a nitrogen source, CO_2 enhancement of shoot growth halved and CO_2 inhibition of shoot protein doubled. This result will likely have major implications for the ability of wheat to use NO_3^- as a nitrogen source under elevated CO_2.

Atmospheric CO_2 concentrations have increased from about 280 to 370 μmol mol^{-1} since 1800 (1, 2) and may reach 500–900 μmol mol^{-1} by the end of the century (3). Several responses of higher plants to such changes were not anticipated (4). For example, a doubling of CO_2 level initially accelerates carbon fixation in C_3 plants by about 30%, yet after days to weeks of exposure to high CO_2 concentrations, depending on species, carbon fixation declines until it stabilizes at a rate that averages 12% above ambient controls (5). This general phenomenon, known as CO_2 acclimation, is correlated with a decline in the activity of Rubisco and other enzymes in the Calvin cycle (6, 7). The change in Calvin cycle enzyme activities is not necessarily selective; rather, it often follows a decline in overall shoot protein and N contents (8). Shoot N contents diminish by an average of 14% with a doubling of CO_2 (9), a difference that exceeds what would be expected if a given amount of N were diluted by additional biomass (8).

In wheat, CO_2 acclimation varies with N supply (10, 11). Wheat shoots accumulate free NO_3^- under elevated CO_2 (12), and shoot protein declines (13) despite little change in total N (12, 14). Here, we present several lines of evidence that elevated CO_2 concentrations inhibit NO_3^- assimilation in wheat shoots and suggest two physiological mechanisms for this phenomenon.

Materials and Methods

We surface-sterilized wheat (*Triticum aestivum* cv. Veery 10) seeds for 1 min in 2.6% NaClO, washed them thoroughly with water, and germinated them for several days on thick paper toweling (germination paper) saturated with 1 mM $CaSO_4$. Twenty seedlings were transplanted to 19-liter opaque plastic containers filled with an aerated nutrient solution containing 0.2 mM NH_4NO_3, 1 mM $CaSO_4$, 0.5 mM K_2HPO_4, 0.5 mM KH_2PO_4, 1 mM $MgSO_4$, 0.2 g liter^{-1} Fe-NaEDTA, and micronutrients (15). The nutrient solution was replenished every 3 days.

The containers were placed in a controlled environment chamber [Conviron (Winnipeg, MB, Canada) PGV-36] equipped with a nondispersive infrared CO_2 analyzer (Horiba, Kyoto, no. APBA-250E) and a Conviron process controller that added CO_2 to maintain the chamber concentration at 360 μmol mol^{-1} for the photon flux density (PFD) response experiments (i.e., shoot photosynthesis as a function of photosynthetic PFD at plant height) and either 360 or 700 μmol mol^{-1} for the A–C_i response (i.e., shoot photosynthesis as a function of internal CO_2 concentration) experiments. The growth/N relations experiments were also conducted at 360 or 700 μmol CO_2 mol^{-1}. The CO_2 added was filtered through a $KMnO_4$ column to remove contaminating hydrocarbons such as ethylene. A combination of high-pressure sodium, metal halide, and incandescent lamps provided a photosynthetic PFD of 700 μmol m^{-2} s^{-1} at plant height. The light/dark period was 16 h/8 h at 25°C and 15°C, respectively.

Gas-Exchange Measurements. We transferred a seedling about 14 days old into a measurement system in which a split rubber stopper was fitted around the stem, sealing the root into an acrylic plastic and stainless steel cuvette (16) and the shoot into a gold-plated cuvette with a glass top (17). The leaves in the shoot cuvette were at their normal orientation; thus, the angle of incidence was 70–80°. Shoot gas fluxes were monitored with a commercial nondispersive infrared CO_2 analyzer (Horiba VIA-500R), a custom O_2 analyzer, and relative humidity sensors (Vaisala, Helsinki) (17). The custom O_2 analyzer contains two cells of calcia-stabilized zirconium oxide ceramic similar to those found in an Applied Electrochemistry model N-37 M. When heated to 752.00 ± 0.01°C, these cells become selectively permeable to O_2 and generate a 106-nV Nernst potential per μmol mol^{-1} difference in O_2 partial pressures between the cells; in practice, this analyzer can resolve O_2 concentration differences to within 2 μmol mol^{-1} on the normal background of 209,700 μmol mol^{-1} (17). Mass flow controllers (Tylan, Torrance, CA) prepared the various gas mixtures, and a pressure transducer (Validyne, North Ridge, CA) monitored gas flows through the cuvette. In the photosynthetic PFD experiments, a 1,000-W metal halide lamp with an adjustable ballast (Wide-Lite, San Marcos, TX) and neutral density filters controlled PFD at plant height between 0–1900 μmol m^{-2} s^{-1}. Plants exhibited photoinhibition if the PFD was increased to levels where the response became light-saturated (3000 μmol m^{-2} s^{-1}). In the A–C_i response experiments, the PFD was 1200 μmol m^{-2} s^{-1}.

In the PFD experiments, a plant was exposed to 360 μmol mol^{-1} CO_2 and received an aerated nutrient solution of 1 mM $CaSO_4$, 0.5 μM K_2HPO_4, and either 0.2 mM KNO_3 or 0.2 mM NH_4Cl for 36 h before taking any measurements. In the A–C_i response experiments, a plant received an aerated nutrient solution of 0.2 mM NH_4Cl, 1 mM $CaSO_4$, and 0.5 μM K_2HPO_4 for 16 h before taking any measurements. A plant after such a pretreatment contained no detectable NO_3^- in its tissues (data not shown). This plant then received a nutrient solution containing 0.2 mM KNO_3, 1 mM $CaSO_4$, and 0.5 μM K_2HPO_4 for 16 h before measurements of the A–C_i response were repeated.

Abbreviations: PFD, photon flux density; AQ, assimilatory quotient.

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We followed standard protocols to assess the response of photosynthesis to PFD or intercellular CO_2 (18). The calculation of fluxes included an adjustment for changes in leaf area during the measurements.

Chloroplast Isolation. Wheat and pea (*Pisum sativum* cv. Progress 9) were grown for about 2 weeks at ambient CO_2. The wheat received the nutrient solution described above, whereas the pea was in vermiculite that was watered daily. The following procedures were conducted at 0–4°C. The wheat (30–50 g) or pea (100–130 g) shoots were blended for 10 seconds in 0.2 liters of a grinding buffer (0.05 M K-Hepes, pH 7.3/0.33 M sorbitol/1 mM $MgCl_2$/1 mM $MnCl_2$/2 mM Na_2EDTA/0.1% BSA). The homogenate was squeezed through two layers of miracloth and centrifuged at 2,900 × *g* for 5 min. The pellet was resuspended in 3 ml of a homogenization buffer (0.05 M K-Tricine, pH 8.0/0.33 M sorbitol) and layered onto a 30-ml Percoll gradient that was generated by centrifugation of 50% (vol/vol) Percoll in an equal volume of the grinding buffer at 37,000 × *g* for 30 min. After centrifugation of the overlayered gradients at 8,000 × *g* for 10 min, the intact chloroplasts formed a band near the bottom of the Percoll gradient. These were washed twice with 45 ml of the homogenization buffer and pelleted at 1,500 × *g* for 5 min. To test for the intactness of the chloroplasts, 15 μl of chloroplast material were layered on top of 100 μl of silicone oil that floated above 100 μl of a buffer solution (0.1 M K-Tricine, pH 8.0/0.66 M sorbitol) in a 4-ml Eppendorf tube. After centrifugation at maximum speed for 15 seconds (about 50,000 × *g*), broken chloroplasts floated on top of silicone oil, whereas intact chloroplasts passed through the silicone oil phase and formed a pellet at the bottom of the tube. To determine the amount of chlorophyll in the isolated chloroplasts, 10 μl of the chloroplast suspension were diluted into 5 ml of 80% acetone and filtered through Whatman no. 1 paper; the absorbance was read at 720, 663, and 645 nm, where Chl (mg liter^{-1}) = $4.02 \times (A_{663} - A_{720}) + 10.14 \times (A_{645} - A_{720})$ (19).

Chloroplast Nitrite Absorption. We incubated intact chloroplasts containing about 1 g liter^{-1} chlorophyll in 50 mM K-Tricine (pH 8.0), 330 mM sorbitol, 0.3 mM KNO_2, and 0, 0.3, 1.0, or 3.0 mM $KHCO_3$ at about 22°C and at a PFD of 650 μmol quanta m^{-2} s^{-1}. A concentration of 0.3 mM HCO_3^- is probably higher than is present *in vivo*, but at lower concentrations, the relative depletion of HCO_3^- by carbon fixation during the incubation period was prohibitive. We took samples of the incubation mixture between 15 and 60 min, placed them immediately in the dark at 0–4°C, and centrifuged them at 3,000 × *g* for 3 min. To analyze NO_2^- in the supernatant, we pipetted a 0.1-ml aliquot into 0.5 ml of a sulfanilamide solution [0.5 g sulfanilamide in 150 ml of 15% (vol/vol) CH_3COOH], added after 5 min 0.5 ml of a NED solution [0.2 g of *N*-(1-naphthyl)ethylenediamide·2HCl in 150 ml of 15% (vol/vol) CH_3COOH], allowed color to develop for 15 min, and measured absorbance at 540 nm (20). We fitted a cubic spline curve to the data for NO_2^- concentration as a function of time and derived net NO_2^- uptake from the slope of the cubic spline (MATHCAD, MathSoft, Cambridge, MA).

Growth and Nitrogen Parameters Under NH_4^+ or NO_3^-. We germinated seeds as described above and, when they were 6 days old, transferred them to two controlled environmental chambers, one maintained at 360 μmol mol^{-1} CO_2 and the other at 700 μmol mol^{-1} CO_2. Each chamber had a continuous-flow nutrient solution system that supplied NH_4^+ as the sole N source and one that supplied NO_3^- as the sole source. A solution system consisted of a 100-liter main reservoir, a centrifugal chemical pump, a distribution manifold with 6 4-liter hour^{-1} drip irrigation emitters, 6 opaque 4-liter polyethylene containers, and a manifold that returned the overflow from the 6 containers to the main reservoir. Each container held two plants. The nutrient solutions were composed of 0.1 mM $(NH_4)_2SO_4$ or 0.2 mM KNO_3, 2 mM $CaCl_2$, 1 mM K_2HPO_4, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 g liter^{-1} Fe-NaEDTA, and micronutrients (15). To minimize nitrification and denitrification, we added ampicillin (20 mg liter^{-1}) and cefotaxime (10 mg liter^{-1}) (21). Every 2 days, we measured and adjusted the concentration of NH_4^+ (22) or NO_3^- (23) in the solution systems. In another study, increasing the NO_3^- supply from 0.1 to 1.0 mM had little effect on wheat biomass production at 360 or 1000 μmol mol^{-1} CO_2 (12). Here, the plants received 0.2 mM NH_4^+ or NO_3^-, and shoot N contents under all treatments were high at around 5%, indicating that N availability was not limiting growth. We conducted four replicate experiments in which we switched the ambient and elevated chambers and rotated the positions of the NH_4^+ and NO_3^- treatments in a chamber.

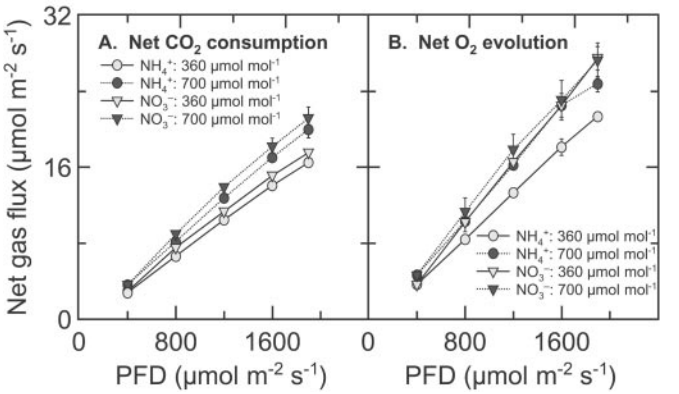

Fig. 1. Net CO_2 consumption (*A*) and O_2 evolution (*B*) by the shoot of a wheat seedling as a function of photosynthetic PFD at plant height. The plants had been grown in controlled environment chambers at 360 μmol mol^{-1} CO_2 and measured at 360 (light symbols) or 700 (dark symbols) μmol mol^{-1} CO_2. They received either NH_4^+ (circles) or NO_3^- (triangles) as a sole N source during measurements. The leaves in the gas-exchange cuvette were at their natural orientation. Shown are mean ± SE for 5–8 replicate plants.

After 2 weeks, we evaluated plant growth and nitrogen parameters. Eight to 10 plants were dried in a forced-air oven at 70°C, weighed, and ground in a ball mill. We measured total N in one subsample via a carbon, hydrogen, and nitrogen (CHN) elemental analyzer (PDZ Europa, Cheshire, England, ANCA-SL), extracted another subsample with 1 mM KCl adjusted to pH 2 with H_2SO_4, and analyzed the extract for free NO_3^- by means of HPLC (23). We analyzed two plants for total protein by using the Coomassie dye binding protein assay (Bio-Rad Bradford Protein Assay) and for *in vitro* activities of NO_3^- and NO_2^- reductases based on the appearance or disappearance, respectively, of NO_2^- using a colorimetric assay (24). We used a general linear model (GLM procedure, SAS Institute, Cary, NC) to perform Tukey's Studentized Range and Bonferroni *t* tests on the ambient vs. elevated CO_2 treatments under NH_4^+ or NO_3^- nutrition and designated probabilities of less than 5% as significant.

Results and Discussion

Gas-Exchange Measurements. For wheat shoots grown at ambient CO_2, net CO_2 consumption at any given PFD was higher at elevated CO_2 than ambient CO_2 (Fig. 1*A*). Net O_2 evolution was also higher at elevated CO_2 than ambient CO_2 under NH_4^+, but was insensitive to CO_2 concentration under NO_3^- (Fig. 1*B*). The response of net CO_2 consumption vs. C_i (shoot internal CO_2 concentration) was similar among all treatments (Fig. 2), as is usually observed for C_3 plants (25). Net O_2 evolution, by

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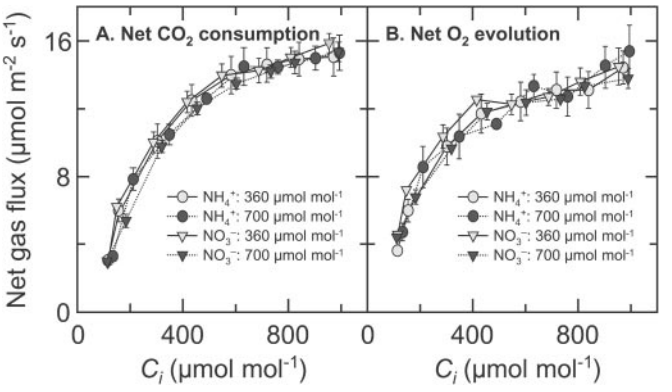

Fig. 2. Net CO_2 consumption (*A*) and O_2 evolution (*B*) by the shoot of a wheat seedling as a function of internal CO_2 concentration (C_i), estimated from changes in CO_2 and water vapor concentrations. The plants had been grown in controlled environment chambers at 360 (light symbols) or 700 (dark symbols) μmol mol^{-1} CO_2. During measurements, the plants were exposed to NH_4^+ (circles) and then to NO_3^- (triangles). The PFD at plant height was 1200 μmol quanta m^{-2} s^{-1}. Shown are mean ± SE for 6 replicate plants.

contrast, was lower under NH_4^+ than NO_3^- for the wheat grown under ambient CO_2 and measured at the two lowest C_is (Fig. 2).

The assimilatory quotient (AQ), the ratio of net CO_2 consumption to net O_2 evolution, highlights these differences (Fig. 3). The AQ was verified as a nondestructive measure of *in planta* NO_3^- assimilation over 50 years ago for algae (26) and over a decade ago for higher plants by using barley mutants deficient in NO_3^- reductase activity (17). Transfer of electrons to NO_3^- and NO_2^- during photoassimilation increases O_2 evolution from the light-dependent reactions of photosynthesis, while CO_2 consumption by the light-independent reactions continues at similar rates. Therefore, plants that are photoassimilating NO_3^- exhibit a lower AQ and the difference in the AQ with a shift from NO_3^- to NH_4^+ nutrition (ΔAQ) is proportional to NO_3^- photoassimilation. The AQ may respond to other shoot processes—principally, photorespiration and the Mehler-peroxidase reaction—but these processes probably would not differ with N form in the root medium and, thus, would not influence ΔAQ.

Here, the ΔAQs measured at elevated CO_2 concentrations did not differ significantly from zero over a range of light levels, indicating little NO_3^- photoassimilation (Fig. 3*A*). This finding is consistent with a tight coupling between the light-dependent and light-independent reactions of photosynthesis. Net O_2 evolution under NO_3^- remained high at both CO_2 levels (Fig. 1*B*), suggesting that the rate of photosynthetic electron transport and the amount of photosynthetic reductant generated were independent of CO_2 level. In contrast, the ΔAQs measured at ambient CO_2 increased with PFD (Fig. 3*A*), indicating that NO_3^- photoassimilation increased with light intensity. Thus, the shoots seemed to conduct NO_3^- photoassimilation only to the extent that carbon fixation was CO_2-limited and surplus photosynthetic reductant became available. Giving priority to carbon fixation seems an appropriate strategy in that plants can store moderate levels of NO_3^- with little difficulty until reductant becomes available, but cannot directly store significant amounts of CO_2.

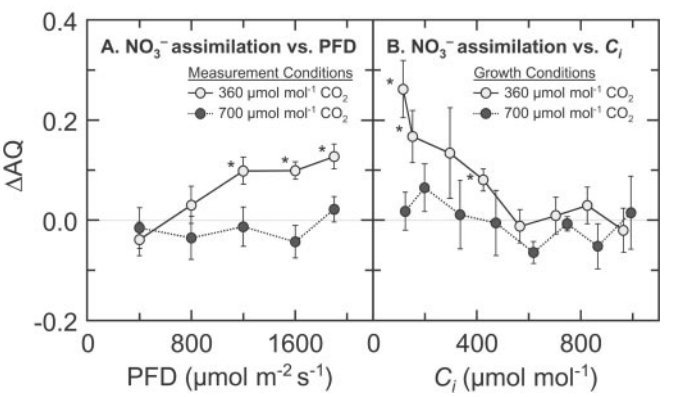

Fig. 3. The change in AQ (AQ = CO_2 consumed/O_2 evolved) with a shift in N source from NO_3^- to NH_4^+ as a function of either photosynthetic flux density (*A*) or internal CO_2 concentration (*B*), based on the data presented in Figs. 1 and 2. The plants had been measured (*A*) or grown (*B*) in controlled environment chambers at 360 (light symbols) or 700 (dark symbols) μmol mol^{-1} CO_2. Shown are means ± SE for 5–8 replicate wheat plants. Asterisks mark the means that were significantly different from zero ($P < 0.05$, a Student's *t* test).

The response of ΔAQ as a function of internal CO_2 concentration (C_i) supports this interpretation. Wheat grown under ambient CO_2 and measured at the lower C_is exhibited ΔAQs greater than zero (Fig. 3*B*). These results indicate that exposure to elevated CO_2 concentrations, either in the short term (hours) or long term (weeks), diminished NO_3^- photoassimilation. The same mechanism could account for both these responses: short-term inhibition of NO_3^- assimilation caused a specific down-regulation of shoot NO_3^- and NO_2^- reductase activities (Fig. 6) and, therefore, a long-term decline in the capacity of the shoot to assimilate NO_3^- even under ambient CO_2 conditions.

Our ability to monitor shoot O_2 fluxes simultaneously with CO_2 fluxes under normal physiological conditions provided a unique perspective. Previous studies of photosynthetic responses to PFD and C_i have monitored primarily CO_2 exchange, which is relatively insensitive to N source (Figs. 1*A* and 2*A*). Measurements of photosynthetic O_2 exchange are generally conducted by using polarographic O_2 electrodes at saturating CO_2 concentrations. Under these conditions, measurements of CO_2 exchange are not feasible, and N source would have little effect on O_2 evolution (Figs. 1*B* and 2*B*). For example (27), oxygen evolution monitored with a polarographic O_2 electrode at saturating CO_2 did not differ between detached barley leaves given NH_4^+ and those given NO_3^- at levels similar to the xylem NO_3^- concentrations measured here (15.4 ± 0.7 mM; mean ± SE, $n = 55$). Another technique, chlorophyll fluorescence, in contrast with O_2 fluxes, does not provide an accurate measure for electron transport rates of an entire wheat shoot (28).

Carbon fixation may interfere with NO_3^- photoassimilation at several junctures. First, reduction of NO_3^- to NO_2^- occurs in the cytosol (29, 30) and requires NADH generated from malate that is shuttled from the chloroplast (31). The demands of carbon fixation for reductant might limit this malate shuttle. Second, the reduction of NO_2^- to NH_4^+, the incorporation of NH_4^+ into amino acids, and the Calvin cycle all occur in the stroma of a chloroplast (32) and require ferredoxin that is reduced via photosynthetic electron transport (33). Elevated CO_2 stimulates the Calvin cycle and, under light-limited conditions, can diminish the amount of reduced ferredoxin available for NO_2^- reduction or NH_4^+ assimilation (34, 35). Our finding that ΔAQ declined to zero in low light or elevated CO_2 (Fig. 3) is consistent with a diminished availability of NADH or reduced ferredoxin for NO_3^- assimilation.

Chloroplast Nitrite Absorption. NO_2^- transport from the cytosol into the chloroplast involves the diffusion of HNO_2 across chloroplast membranes and, therefore, requires the stroma to be more alkaline than the cytosol (36). Carbon dioxide at elevated concentrations can dissipate this pH gradient because additional CO_2 movement into the chloroplast acidifies the stroma (37) and because enhanced carbon fixation hydrolyzes ATP faster and requires supplementary proton exchange across the thylakoid membrane to regenerate this ATP. The addition of 0.3, 1.0, or 3.0 mM HCO_3^- decreased chloroplast NO_2^- absorption by an average of 38, 45, or 61% in wheat and 32, 48, or 60% in pea (Fig.

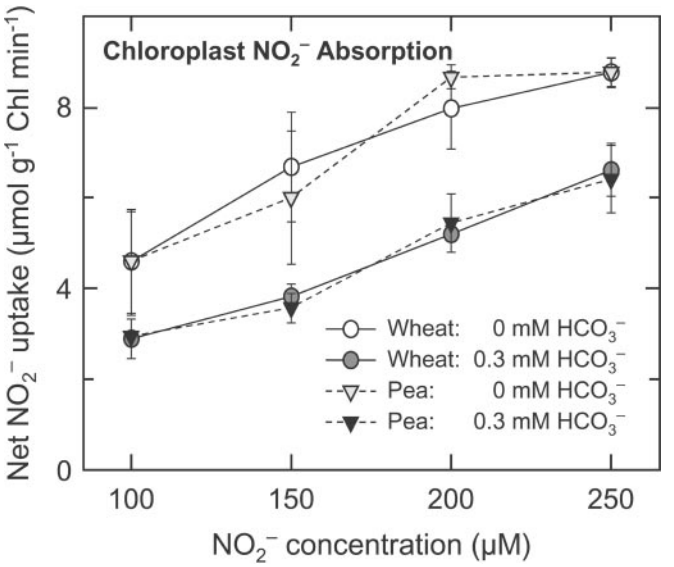

Fig. 4. Net NO_2^- uptake (μmol mg^{-1} chlorophyll min^{-1}) by isolated chloroplasts as a function of NO_2^- concentration when the medium contained 0 (light symbols) or 0.3 (dark symbols) mM HCO_3^-. Shown are the mean ± SE ($n = 3$) for wheat (circles) and pea (inverted triangles).

4 shows the 0 and 0.3 mM data). These results confirm that high CO_2 levels can interfere with NO_2^- transport into the chloroplast and thereby provide another mechanism through which elevated CO_2 might inhibit shoot NO_3^- assimilation.

Growth and Nitrogen Parameters under NH_4^+ and NO_3^-. If CO_2 at elevated concentrations inhibits NO_3^- photoassimilation, then plants receiving NH_4^+ as a N source should prove more responsive to CO_2 enrichment. To test this prediction, we grew wheat seedlings in controlled environment chambers where CO_2 was controlled at ambient or elevated levels (360 or 700 μmol mol^{-1}) and the plants received either 0.2 mM NH_4^+ or 0.2 mM NO_3^- as the sole N source. The form in which N was supplied did not influence plant growth at 360 μmol mol^{-1} (ambient) CO_2, but had a dramatic effect at 700 μmol mol^{-1} (elevated) CO_2 (Fig. 5).

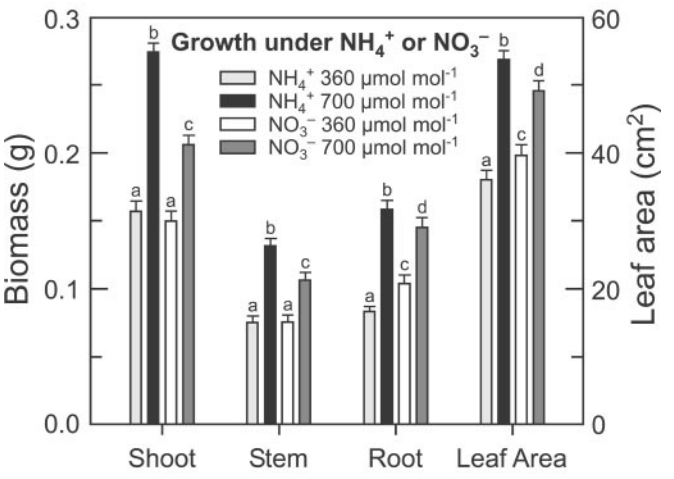

Fig. 5. Biomass (g of dry mass) and leaf area (cm^2) per plant of wheat seedlings grown for 14 days in controlled environment chambers at 360 or 700 μmol mol^{-1} CO_2 and under NH_4^+ or NO_3^- nutrition. Shown are means ± SE for four replicate experiments, each with 8–10 plants per treatment. Treatments labeled with different letters differ significantly ($P < 0.05$).

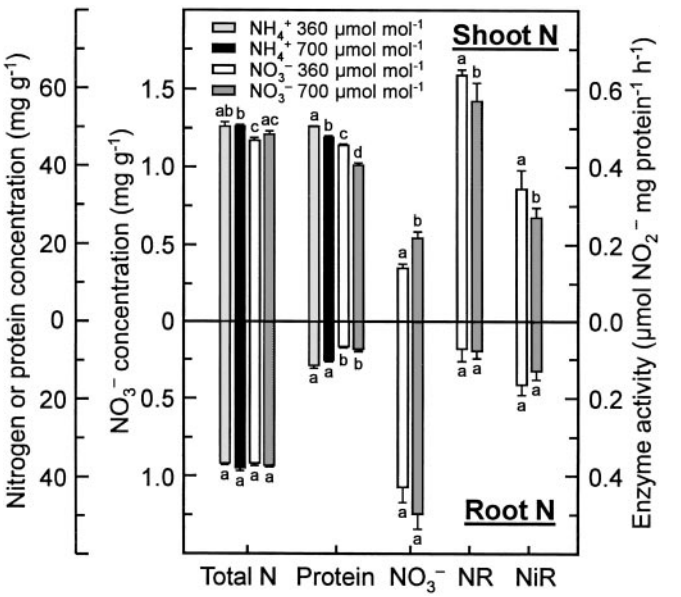

Fig. 6. Total N concentration (mg g^{-1} dry mass), protein concentration (mg g^{-1} fresh mass), NO_3^- concentration (mg g^{-1} fresh mass), NO_3^- reductase activity (μmol NO_2^- generated mg^{-1} protein h^{-1}), and NO_2^- reductase activity (μmol NO_2^- consumed mg^{-1} protein h^{-1}) in the shoot (*Top*) or root (*Bottom*) of wheat grown for 14 days in controlled environment chambers at 360 or 700 μmol mol^{-1} CO_2 and under NH_4^+ or NO_3^- nutrition. Shown are means ± SE for four replicate experiments, each with 8–10 plants per treatment. Treatments labeled with different letters differ significantly ($P < 0.05$). Chlorophyll concentrations were 0.32 ± 0.02 and 0.34 ± 0.07 g $liter^{-1}$ (mean ± SE, $n = 2$) for the NH_4^+ treatment at 360 and 700 μmol mol^{-1}, respectively, and 0.30 ± 0.01 g $liter^{-1}$ and 0.26 ± 0.04 g $liter^{-1}$ (mean ± SE, $n = 2$) for the NO_3^- treatment at 360 and 700 μmol mol^{-1}, respectively.

Leaf area in the elevated CO_2 treatment relative to the ambient CO_2 treatment increased 49% under NH_4^+ nutrition but only 24% under NO_3^- nutrition (Fig. 5). Total plant biomass increased 78% under NH_4^+ nutrition but only 44% under NO_3^- nutrition (Fig. 5). Thus, the plants receiving NH_4^+ were more responsive to CO_2 enrichment than those receiving NO_3^-.

Shoot and root N concentrations were similar under the two CO_2 regimes, indicating that N absorption per unit plant mass remained unchanged (Fig. 6). The fate of N after it was absorbed, however, differed under ambient and elevated CO_2 as demonstrated by the balance between inorganic and organic N (Fig. 6). In the elevated CO_2 treatment relative to the ambient CO_2 treatment, shoot protein concentrations decreased 6% under NH_4^+ nutrition, as might be expected given the dilution by additional biomass, but decreased 13% under NO_3^- nutrition despite less additional biomass (Fig. 6). Thus, shoot protein per plant increased 73% and 32% under NH_4^+ and NO_3^-, respectively. Shoot NO_3^- concentrations were undetectable in plants receiving NH_4^+, but increased 62% at elevated CO_2 in those receiving NO_3^- (Fig. 6). *In vitro* shoot activities of NO_3^- reductase and NO_2^- reductase decreased 12% and 27% from ambient to elevated CO_2, respectively, on a total protein basis (Fig. 6) and decreased 33% and 30%, respectively, on a fresh mass basis. Root protein, NO_3^-, and enzyme activities were similar under both CO_2 treatments (Fig. 6).

These results support a hypothesis that elevated CO_2 inhibits NO_3^- photoassimilation (12). Although the plants received the various CO_2 and N treatments only from day 6 through day 20, the differences were substantial. Elevated CO_2 concentrations stimulated shoot growth of the plants receiving NO_3^- to only half the extent of those receiving NH_4^+. Shoot protein concentrations at elevated CO_2 concentrations declined more than twice as

much under NO_3^- than under NH_4^+ (Fig. 6). Shoot activities of NO_3^- assimilatory enzymes declined even more than the overall protein concentrations (Fig. 6), suggesting that they were selectively inhibited. Studies on *Plantago major* (38), *Nicotiana tabacum* (39), *Nicotiana plumbaginifolia* (40), and spinach (41) have also found that longer exposures (4 h to over 2 weeks) to elevated CO_2 inhibited shoot NO_3^- reductase activity. Selective inhibition of NO_3^- assimilation led to the accumulation of NO_3^- in the shoots of *N. plumbaginifolia* (40) and wheat (12).

Nitrogen parameters from the growth analysis were consistent with the gas-exchange measurements. Changes in O_2 evolution after exposure to NO_3^- indicated that the NO_3^- photoassimilation rate for plants grown and measured under ambient CO_2 concentrations was about 0.3 μmol NO_3^- m^{-2} s^{-1}.[§] This rate was sufficient to account for the organic N that accumulated in the plants receiving NO_3^- nutrition under ambient CO_2 during the current experiment.[¶] Moreover, maximum NO_3^- reductase activity in the shoots of these plants was 1.9 μmol NO_3^- m^{-2} s^{-1}, a value consistent with a NO_3^- photoassimilation rate of 0.3 μmol NO_3^- m^{-2} s^{-1} considering that maximum NO_3^- reductase activity may exceed the actual rate of NO_3^- assimilation by a factor of 6 (41).

Despite extensive evidence on the importance of N availability for determining plant responses to CO_2 enrichment (4, 14, 25, 39, 42–45), few other studies have considered the form of N. The two major N forms, NH_4^+ and NO_3^-, have distinct physiological effects on plant growth and development (46), yet this may be the first study to examine CO_2 responses under controlled levels of NH_4^+ vs. NO_3^- as sole N sources. Periodic watering of pots with solutions containing various N forms may not provide adequate control because N transformations are both rapid in nonsterile cultures (47) and sensitive to atmospheric CO_2 (48). In the present study, we compared NH_4^+ and NO_3^- as sole N sources by using a continuous flow system to maintain constant nonlimiting levels of nutrients and a mixture of antibiotics to minimize conversion among N forms (21).

Our results may explain several responses of wheat to elevated CO_2. In a multiyear Free Air CO_2 Enrichment (FACE) experiment conducted at Maricopa, Arizona, wheat received either moderate (about 200 kg N ha^{-1}) or high (about 500 kg N ha^{-1}) N as a mix of NH_4^+ and NO_3^- and was exposed to 360 or 550 μmol mol^{-1} CO_2 (14). Grain yields did not vary with CO_2 level in the moderate N treatment, but were 15% higher at elevated vs. ambient CO_2 in the high N treatment (13). Leaf N concentrations and grain protein declined by more than 10% at elevated vs. ambient CO_2 in the moderate N treatment, whereas these parameters varied only slightly with CO_2 level in the high N treatment (13, 14). In the moderate N treatment, NO_3^- was the predominate N form (14); thus, CO_2 inhibition of NO_3^- photoassimilation might account for the decline in leaf N and grain quality at elevated CO_2 in this treatment. Plants in the high N treatment could compensate for CO_2 inhibition of shoot NO_3^- assimilation because they received additional NH_4^+. A treatment of about 500 kg N ha^{-1}, however, exceeds the average fertilizer recommendations for wheat by a factor of 3 or 4 (49, 50) and would exacerbate NO_3^- leaching, NH_4^+ volatilization, and N_2O release (51). Consequently, addition of such high N levels to compensate for CO_2 inhibition of shoot NO_3^- assimilation is unlikely for both economic and environmental reasons.

We feel that our laboratory results have implications for the real world of crop production. Wheat is grown on over 200 million hectares worldwide (50) and receives 18 million metric tons of N annually (49) or 20% of the world's production (50). In the well drained soils generally devoted to wheat cultivation, NO_3^- is a major N form. Were CO_2 inhibition of NO_3^- photoassimilation common among wheat cultivars, rising atmospheric CO_2 levels would probably require major changes in fertilizer practices associated with wheat production.

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[§]When plants grown under ambient CO_2 were exposed to an atmospheric concentration of 360 μmol mol^{-1}, their C_is averaged 295 μmol mol^{-1}. At this C_i, net CO_2 uptake did not differ with N source (Fig. 2*A*), but net O_2 evolution increased by 0.6 μmol O_2 m^{-2} s^{-1} with the shift from NH_4^+ to NO_3^- nutrition (Fig. 2*B*). Assuming that all of the extra O_2 evolution was associated with generating reductant for NO_3^- photoassimilation, (0.6 μmol O_2 m^{-2} s^{-1} × 4 electrons per O_2)/(8 electrons per NO_3^- converted to NH_4^+) = 0.3 μmol NO_3^- m^{-2} s^{-1} would be the potential NO_3^- photoassimilation rate.

[¶](7200 μg of organic N $plant^{-1}$)/(0.3 μmol m^{-2} s^{-1} × 0.0014 m^2 average leaf area $plant^{-1}$ × 14 μg of N μmol^{-1} × 3600 s h^{-1} × 16 h light d^{-1}) = 21 days, the age of the plant.

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