

Nitrate assimilation is inhibited by elevated CO₂ in field-grown wheat

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Total protein and nitrogen concentrations in plants generally decline under elevated CO₂ atmospheres^{1,2}. Explanations for this decline include that plants under elevated CO₂ grow larger, diluting the protein within their tissues^{3,4}; that carbohydrates accumulate within leaves, downregulating the amount of the most prevalent protein Rubisco²; that carbon enrichment of the rhizosphere leads to progressively greater limitations of the nitrogen available to plants⁴; and that elevated CO₂ directly inhibits plant nitrogen metabolism, especially the assimilation of nitrate into proteins in leaves of C₃ plants⁵. Recently, several meta-analyses have indicated that CO₂ inhibition of nitrate assimilation is the explanation most consistent with observations^{6–8}. Here, we present the first direct field test of this explanation. We analysed wheat (*Triticum aestivum* L.) grown under elevated and ambient CO₂ concentrations in the free-air CO₂ enrichment experiment at Maricopa, Arizona. In leaf tissue, the ratio of nitrate to total nitrogen concentration and the stable isotope ratios of organic nitrogen and free nitrate showed that nitrate assimilation was slower under elevated than ambient CO₂. These findings imply that food quality will suffer under the CO₂ levels anticipated during this century unless more sophisticated approaches to nitrogen fertilization are employed.

Many lines of evidence from laboratory studies demonstrate that elevated CO₂ concentrations in the atmosphere inhibit leaf nitrate (NO₃[−]) assimilation in C₃ plants. These include: plants receiving NO₃[−] as their sole source of nitrogen (N) accumulate less organic N under elevated than ambient CO₂ (refs 7,9–11); plants subjected to a pulse of ¹⁵N–NO₃[−] incorporate less ¹⁵N into organic N compounds under elevated than ambient CO₂ (ref. 10); plant growth is slower under elevated than ambient CO₂ when NO₃[−] serves as the sole N source and faster when NH₄⁺ serves as the sole N source^{5,12}; ΔAQ (changes in the ratio of net CO₂ consumption to net O₂ evolution after shifting N nutrition from NH₄⁺ to NO₃[−]), a real-time measure of leaf NO₃[−] assimilation, decreases with increasing leaf internal CO₂ concentration^{9,12}; and maximum NO₃[−] reductase activity *in vitro* is usually less under elevated than ambient CO₂ (refs. 11–13). Verification of CO₂ inhibition of NO₃[−] assimilation in the field, however, is still lacking.

Here, we conducted chemical analyses of wheat (*Triticum aestivum* L.) grown in 1996 and 1997 under elevated or ambient atmospheric CO₂ concentrations in the free-air CO₂ enrichment (FACE) experiment at Maricopa, Arizona. This experiment originally assessed grain yield¹⁴, total N of green leaves¹⁵, grain protein¹⁶ and soil N dynamics¹⁷. Leaf material collected from this experiment was stored on ice, transported to the laboratory, oven dried at 70 °C, stored in evacuated plastic bags that were

sealed in paint cans, and kept in a storeroom at the US Water Conservation Laboratory in Phoenix, Arizona, USA until air freighted to UC Davis for the chemical analyses described below. This preparation and storage of samples minimized changes over time in total N, nitrate and nitrogen isotope ratios¹⁸. What prompted these additional analyses of the Maricopa leaf material was the development of a new technique to assess the N isotope signature of NO₃[−] (ref. 19) as well as a new perspective about the interactions between elevated CO₂ and NO₃[−] assimilation^{5,9,10,12}.

The values for leaf total N (Fig. 1) did not differ from those reported over a decade earlier¹⁵, supporting the assumption that the samples were well preserved. Plants in the low-N treatment of either CO₂ treatment contained no detectable leaf NO₃[−] on most sampling dates (data not shown). In contrast, a significant percentage of leaf N remained as unassimilated NO₃[−] (ratio of NO₃[−] to total N) in plants subjected to the high-N treatment (Fig. 2). The first fertilization, applied 4 weeks after plant emergence, increased leaf NO₃[−] concentration in the short term an average of fivefold and twofold in 1996 and 1997, respectively (Figs 1 and 2). Therefore, we focused our analysis on the high-N treatment from week 6 onwards.

Leaf total N in the high-N treatment did not differ significantly between the CO₂ treatments ($P = 0.12$; Fig. 1), but overall the ratio of NO₃[−] to total N was greater under elevated than ambient CO₂ from week 6 onwards ($P < 0.0001$; Fig. 2). Total N and the ratio of NO₃[−] to total N were lower in 1996 than in 1997 ($P < 0.003$; Figs 1 and 2). The analysis of variance tables are available in Supplementary Tables 2–5.

In leaves, both organic N and unassimilated NO₃[−] in 1996 were less enriched in ¹⁵N under elevated than ambient CO₂ from week 6 onwards ($P < 0.0001$; Figs 3 and 4). The δ¹⁵N of leaf organic N and NO₃[−] declined as the plants matured under both CO₂ treatments ($P < 0.0001$; Figs 3 and 4).

All three measures of NO₃[−] assimilation assessed in this study confirm that elevated CO₂ inhibited leaf NO₃[−] assimilation in field-grown wheat. The first measure was the proportion of leaf N that remained as free NO₃[−]. Leaf total N in the high-N treatment did not differ significantly between the CO₂ treatments (Fig. 1), as reported earlier¹⁵. The percentage of leaf total N that remained as unassimilated NO₃[−] was higher under elevated than ambient CO₂ from week 6 onwards in both years (Fig. 2 and Supplementary Tables 3–5). Higher free NO₃[−] relative to total N suggests that NO₃[−] assimilation was slower under elevated CO₂.

The second measure was the δ¹⁵N of leaf organic N. It was more depleted in ¹⁵N under elevated than ambient CO₂ from 6 weeks onwards (Fig. 3). If NO₃[−] availability does not limit assimilation, leaves preferentially assimilate ¹⁴N–NO₃[−] (ref. 20). Therefore, the lower leaf δ¹⁵N_{organic} signatures under elevated than ambient CO₂

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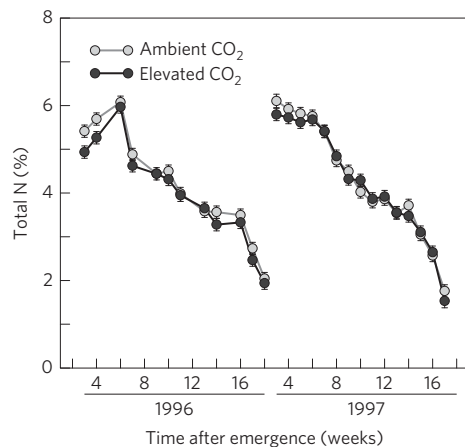


Figure 1 | Total nitrogen (percentage of dry matter) in wheat leaves as a function of time after emergence (weeks). Shown are data from the 1996 and 1997 field seasons for plants grown under ambient ($363 \mu\text{mol mol}^{-1}$ in 1996 and $370 \mu\text{mol mol}^{-1}$ in 1997) or elevated ($548 \mu\text{mol mol}^{-1}$ in 1996 and $559 \mu\text{mol mol}^{-1}$ in 1997) CO_2 atmospheres in the high-N fertilization treatment at Maricopa, Arizona. Plotted are the estimated means and standard errors (SAS Proc Mixed version 9.3).

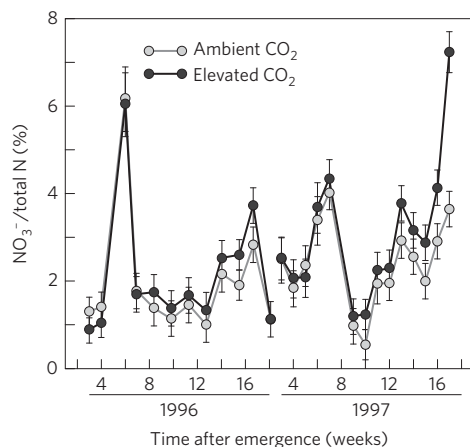


Figure 2 | Nitrate as a percentage of total N in wheat leaves as a function of time after emergence (weeks). Shown are data from the 1996 and 1997 field seasons for plants grown under ambient ($363 \mu\text{mol mol}^{-1}$ in 1996 and $370 \mu\text{mol mol}^{-1}$ in 1997) or elevated ($548 \mu\text{mol mol}^{-1}$ in 1996 and $559 \mu\text{mol mol}^{-1}$ in 1997) CO_2 atmospheric conditions in the high-N fertilization treatment at Maricopa, Arizona. Plotted are the estimated means and standard errors (SAS Proc Mixed version 9.3).

(Fig. 3) indicate that leaf NO_3^- assimilation was slower relative to replenishment of leaf NO_3^- from roots under elevated than ambient CO_2 .

The third measure was $\delta^{15}\text{N}$ of free NO_3^- in the leaves. If leaf NO_3^- assimilation is slower relative to replenishment of leaf NO_3^- from roots under elevated than ambient CO_2 , NO_3^- assimilation more slowly depletes leaf tissues of ^{14}N - NO_3^- and $\delta^{15}\text{N}_{\text{nitrate}}$ becomes less enriched in ^{15}N . Here, unassimilated NO_3^- in wheat leaves was less enriched in ^{15}N under elevated than ambient CO_2 from 6 weeks onwards (Fig. 4), indicating that leaf NO_3^- assimilation was slower under elevated than ambient CO_2 .

The isotopic signature of free NO_3^- in leaves also depends on the $\delta^{15}\text{N}$ of NO_3^- translocated from the roots. For example, if NO_3^- assimilation rates in the roots are faster under elevated than ambient CO_2 (ref. 21), isotope discrimination by nitrate reductase will enrich the root NO_3^- pool in ^{15}N , and so NO_3^- translocated to the leaves

will be more ^{15}N enriched. The $\delta^{15}\text{N}$ of leaf NO_3^- , however, was lower under elevated than ambient CO_2 (Fig. 4), indicating that the isotopic signature of NO_3^- derived primarily from leaf NO_3^- assimilation being slower under elevated than ambient CO_2 .

These field results are consistent with those of previous laboratory studies showing that several physiological mechanisms are responsible for CO_2 inhibition of leaf NO_3^- assimilation in C_3 plants^{5,9,10,12}. One mechanism involves the first biochemical step of NO_3^- assimilation, the conversion of NO_3^- to NO_2^- in the cytoplasm of leaf mesophyll cells. Photorespiration stimulates the export of malic acid from chloroplasts²² and increases the availability of NADH in the cytoplasm²³ that powers this first step^{24,25}. Elevated CO_2 decreases photorespiration and thereby decreases the amount of reductant available for NO_3^- reduction. Another physiological mechanism is that elevated CO_2 inhibits NO_2^- influx into chloroplasts, and this decreases NO_3^- assimilation¹². A third physiological mechanism is that processes in the chloroplast stroma compete for reduced ferredoxin: because ferredoxin-NADP reductase has a higher affinity for reduced ferredoxin than nitrite reductase²⁶, NO_3^- assimilation proceeds only if the availability of reduced ferredoxin exceeds that needed for NADPH formation^{24,27}. For most plants, this occurs when CO_2 availability limits C_3 carbon fixation¹⁰.

Several earlier studies at the Maricopa FACE site examined soil N in wheat plots that received irrigation, fertilizer and CO_2 treatments similar to the high-N treatment here. Total inorganic N through the soil profile was similar in the ambient and elevated CO_2 treatments from 6 weeks onwards²⁸. Nitrogen mineralization was unaffected by CO_2 treatment¹⁷, and soil NO_3^- constituted between 90 and 98% of inorganic N extractable by 2M KCl at harvest (S. A. Prior and H. A. Torbert personal communication, 2013). Therefore, these soil N data support the conclusion that the leaf N differences that we observed between the elevated and ambient CO_2 treatments derived from altered plant responses and not altered soil N availability.

Several recent meta-analyses of the literature on plant responses to elevated CO_2 support that CO_2 inhibits leaf NO_3^- assimilation. One⁷ based on 43 studies of wheat protein and grain yield under ambient and elevated CO_2 concluded that 'elevated CO_2 has a direct negative effect on grain protein accumulation independent of the yield effect, supporting recent evidence of CO_2 -induced impairment of nitrate uptake/assimilation'. Another meta-analysis⁶ based on 38 studies of soil NH_4^+ and NO_3^- concentrations and plant NH_4^+ and NO_3^- uptake in 58 species concluded that 'differential CO_2 effects on soil NH_4^+ and NO_3^- ... were consistent qualitatively with recent discoveries of eCO_2 effects on plant N utilization', citing our laboratory studies on CO_2 inhibition of leaf NO_3^- assimilation.

Under elevated CO_2 , protein concentrations in wheat grain^{16,29}, rice grain⁸, potato tuber⁸ and barley grain^{29,30} decline an average of around 8%. Wheat, rice, potato and barley, respectively, provide 21, 13, 2 and 0.3% of the protein in the human diet³¹. Consequently, protein available for human consumption may diminish by about 3% as atmospheric CO_2 reaches the levels anticipated during the next few decades.

Increased yields under CO_2 enrichment and heavy N fertilization may partially compensate for the decrease in food quality resulting from elevated CO_2 . In the low-N treatment at Maricopa, elevated CO_2 increased grain yields by 9% (ref. 32), but decreased grain protein concentrations by 11% (ref. 16), and so grain protein yields decreased by about 2%. In the high-N treatment, elevated CO_2 increased grain yields about 16% (ref. 32), but had an insignificant effect on grain protein concentrations¹⁶, and so grain protein yields increased about 16%. In the high-N treatment, however, the fertilizer and soil supplied 430 and 490 kg N ha^{-1} during 1996 and 1997, respectively, but crop biomass N under elevated CO_2 was only

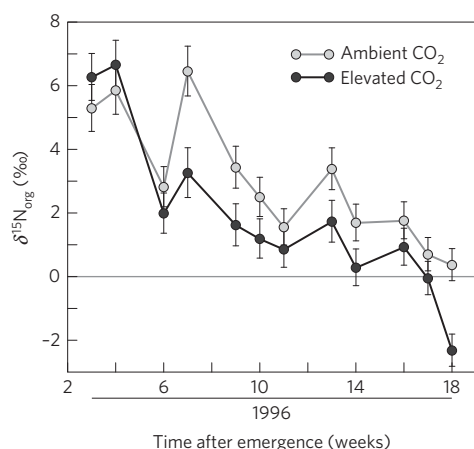


Figure 3 | Isotopic signature of organic N ($\delta^{15}\text{N}_{\text{org}}$) in wheat leaves as a function of time after emergence (weeks). Shown are data from the 1996 field season for plants grown under ambient ($363 \mu\text{mol mol}^{-1}$) and elevated ($548 \mu\text{mol mol}^{-1}$) CO_2 atmospheres in the high-N fertilization treatment at Maricopa, Arizona. Plotted are the estimated means and standard errors (SAS Proc Mixed version 9.3).

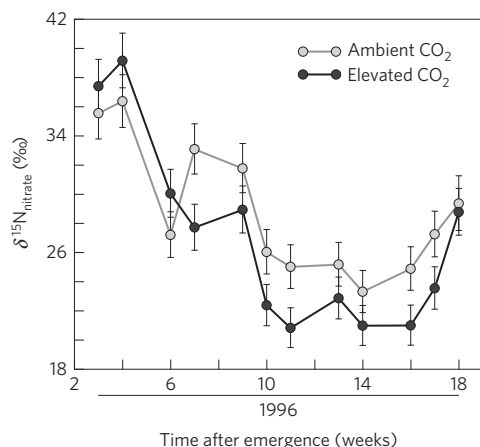


Figure 4 | Isotopic signature of nitrate ($\delta^{15}\text{N}_{\text{nitrate}}$) in wheat leaves as a function of time after emergence (weeks). Shown are data from the 1996 field season for plants grown under ambient ($363 \mu\text{mol mol}^{-1}$) and elevated ($548 \mu\text{mol mol}^{-1}$) CO_2 atmospheres in the high-N fertilization treatment at Maricopa, Arizona. Plotted are the estimated means and standard errors (SAS Proc Mixed version 9.3).

270 kg N ha^{-1} , indicating that nearly half of the N applied was not retained in the crop^{16,33}. Thus, such high fertilization rates would be undesirable because of higher costs, greater NO_3^- leaching into groundwater, and greater N_2O emissions.

Obviously, plants have alternative strategies for acquiring organic N. One such strategy is the use NH_4^+ as a N source. Undoubtedly, the wheat at Maricopa absorbed NH_4^+ as part of its mineral N supply, but nitrification at this site was rapid¹⁷, and the soils contained relatively little NH_4^+ as the growing season progressed (S. A. Prior and H. A. Torbert personal communication, 2013). Another strategy is root NO_3^- assimilation, which may be enhanced under elevated CO_2 ²¹. Unfortunately, relatively little is known about the extent to which the balance between root and leaf NO_3^- assimilation varies within and among species³⁴. Breeding crops for enhanced root NO_3^- and NH_4^+ assimilation has the potential to compensate for lower shoot NO_3^- assimilation rates and likely losses in food quality as atmospheric CO_2 rises, but this approach is yet untapped.

Methods

Wheat (*Triticum aestivum* L.) leaves were obtained from the 1996 and 1997 FACE experiment at the Maricopa Agricultural Center near Phoenix, Arizona¹⁵. Briefly, high- and low-N treatments at this site were assigned in four replicates under ambient and FACE rings 25 m in diameter. Within the rings, ambient and elevated CO_2 were controlled at 363 and $548 \mu\text{mol CO}_2 \text{ mol}^{-1}$ in 1996 and 370 and $559 \mu\text{mol CO}_2 \text{ mol}^{-1}$ in 1997 by releasing air containing different CO_2 concentrations from 2.5-m-high vertical pipes spaced every 2 m around the periphery. Certified seed of Yecora Roho, a cultivar still widely used in the region³⁵, was planted on 15 December 1995 or 1996 and seedlings emerged 1 January 1996 or 1997. The soil at the experimental site is classified as Trix clay loam, fine-loamy, mixed (calcareous), hyperthermic Typic Torrifluvents. Nitrogen fertilizer in the form of NH_4NO_3 was applied in the drip irrigation water: the high-N treatment received four applications (50 kg N ha^{-1} at 4 weeks after emergence, 125 kg N ha^{-1} at 8 weeks, 125 kg N ha^{-1} at 12 weeks, and 50 kg N ha^{-1} at 16 weeks) for a total rate of 350 kg N ha^{-1} ; the low-N treatment received a total of 70 and 15 kg N ha^{-1} for 1996 and 1997, respectively, in three increments^{16,35}. In addition to the fertilizer applied, substantial residual inorganic N was present at sowing (80 kg N ha^{-1} in 1996 and 145 kg N ha^{-1} in 1997).

Plant harvests were made at 10–14 day intervals through the season¹⁵. At each harvest, 24 plants were sampled within each replicate of a treatment. The plants were stored on ice and transported to the laboratory. Green leaf tissue was oven dried at 70°C and stored in evacuated plastic bags that were sealed in paint cans. Subsequently, this leaf tissue was ball-milled at UC Davis, and total N and total N isotope ratios were determined using an elemental analyser interfaced to an isotope ratio mass spectrometer (Sercon) at the UC Davis Stable Isotope Facility. During analysis, samples were interspersed with two or more different $\delta^{15}\text{N}$ standards.

Nitrate was extracted with 1 mM CaSO_4 from subsamples of the pulverized leaves by using an orbital shaker, followed by centrifugation. The NO_3^- concentration of the diluted extracts was determined spectrophotometrically³⁶. The nitrogen isotopic composition of plant NO_3^- extracts was analysed from N_2O generated by denitrifying bacteria lacking N_2O reductase¹⁹. Briefly, 2 ml aliquots of *Pseudomonas chlororaphis* culture were sealed into 20 ml headspace vials that were purged for 2 h with N_2 gas to remove N_2O and O_2 . Samples containing $0.1 \mu\text{mol NO}_3^- \text{ N}$ of the plant tissue extracts or standards were injected through the septae of the vials. The N_2O was flushed from the vials with He, trapped cryogenically, and then released into the isotope ratio mass spectrometer (Sercon) at the UC Davis Stable Isotope Facility. Samples were interspersed with $\delta^{15}\text{N}$ KNO_3 standards that were processed like the plant tissue extracts.

The leaf samples collected in the second year (1997) seemed to have become contaminated with the heavy nitrogen isotope because the $\delta^{15}\text{N}$ values were highly variable and reached up to 250 ‰ in individual samples. Therefore, we report N isotope ratios for only the first year (1996).

Leaf organic N was estimated from the difference between leaf total N and leaf unassimilated NO_3^- because NH_4^+ concentrations in wheat leaves are low and do not vary significantly with CO_2 treatment¹². The isotope ratio of the leaf organic nitrogen ($\delta^{15}\text{N}_{\text{organic}}$) was thus calculated by dividing the mass of ($^{15}\text{N}_{\text{total}} - ^{15}\text{N}_{\text{NO}_3^-}$) by the mass of ($^{14+15}\text{N}_{\text{total}} - ^{14+15}\text{N}_{\text{NO}_3^-}$).

The first fertilization, applied 4 weeks after plant emergence, increased leaf NO_3^- concentration an average of fivefold and twofold in 1996 and 1997, respectively, and therefore, we considered only data collected after week 6 in our statistical analysis. An analysis of variance using the MIXED procedure with repeated measures in SAS (version 9.3, SAS Institute) assessed the effects of year (1 or 2 years), CO_2 treatment (2 treatments), week after emergence (11 weeks), blocks (4 blocks), and their interactions on total N, the ratio of NO_3^- to total N, $\delta^{15}\text{N}_{\text{nitrate}}$ and $\delta^{15}\text{N}_{\text{organic}}$ (see Supplementary Tables for the SAS program used and the resulting analysis of variance tables). All of the data met the assumptions of normality and homogeneity of variance as evaluated using the Shapiro–Wilks and Levene's tests, respectively. The dependent variables (total N, ratio of NO_3^- to total N, $\delta^{15}\text{N}_{\text{nitrate}}$ and $\delta^{15}\text{N}_{\text{organic}}$) were repeated for each experimental block. The independent variables and their interactions were considered significant when $P \leq 0.05$.

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Author contributions

All authors contributed to the data set, discussed the results and commented on the manuscript. A.J.B. and M.B. designed the study. M.B. conducted the chemical analyses. A.J.B. carried out the statistical analysis and wrote the paper.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to A.J.B.

Competing financial interests

The authors declare no competing financial interests.