Responses of Arabidopsis and Wheat to Rising CO_2 Depend on Nitrogen Source and Nighttime CO_2 Levels[1][OPEN]

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A major contributor to the global carbon cycle is plant respiration. Elevated atmospheric CO_2 concentrations may either accelerate or decelerate plant respiration for reasons that have been uncertain. We recently established that elevated CO_2 during the daytime decreases plant mitochondrial respiration in the light and protein concentration because CO_2 slows the daytime conversion of nitrate (NO_3^-) into protein. This derives in part from the inhibitory effect of CO_2 on photorespiration and the dependence of shoot NO_3^- assimilation on photorespiration. Elevated CO_2 also inhibits the translocation of nitrite into the chloroplast, a response that influences shoot NO_3^- assimilation during both day and night. Here, we exposed Arabidopsis (*Arabidopsis thaliana*) and wheat (*Triticum aestivum*) plants to daytime or nighttime elevated CO_2 and supplied them with NO_3^- or ammonium as a sole nitrogen (N) source. Six independent measures (plant biomass, shoot NO_3^-, shoot organic N, ^{15}N isotope fractionation, $^{15}NO_3^-$ assimilation, and the ratio of shoot CO_2 evolution to O_2 consumption) indicated that elevated CO_2 at night slowed NO_3^- assimilation and thus decreased dark respiration in the plants reliant on NO_3^-. These results provide a straightforward explanation for the diverse responses of plants to elevated CO_2 at night and suggest that soil N source will have an increasing influence on the capacity of plants to mitigate human greenhouse gas emissions.

The CO_2 concentration in Earth's atmosphere has increased from about 270 to 400 μmol mol^{-1} since 1800, and may double before the end of the century (Intergovernmental Panel on Climate Change, 2013). Plant responses to such increases are highly variable, but plant nitrogen (N) concentrations generally decline under elevated CO_2 (Cotrufo et al., 1998; Long et al., 2004). One explanation for this decline is that CO_2 inhibits nitrate (NO_3^-) assimilation into protein in the shoots of C_3 plants during the daytime (Bloom et al., 2002, 2010, 2012, 2014; Cheng et al., 2012; Pleijel and Uddling, 2012; Myers et al., 2014; Easlon et al., 2015; Pleijel and Högy, 2015). This derives in part from the inhibitory effect of CO_2 on photorespiration (Foyer et al., 2009) and the dependence of shoot NO_3^- assimilation on photorespiration (Rachmilevitch et al., 2004; Bloom, 2015).

A key factor in global carbon budgets is plant respiration at night (Amthor, 1991; Farrar and Williams, 1991; Drake et al., 1999; Leakey et al., 2009). Nighttime elevated CO_2 may inhibit, have a negligible effect on, or stimulate dark respiration, depending on the plant species (Bunce, 2001, 2003; Wang and Curtis, 2002), plant development stage (Wang et al., 2001; Li et al., 2013), experimental approach (Griffin et al., 1999; Baker et al., 2000; Hamilton et al., 2001; Bruhn et al., 2002; Jahnke and Krewitt, 2002; Bunce, 2004), and total N supply (Markelz et al., 2014). The current study is, to our knowledge, the first to examine the influence of N source, NO_3^- versus ammonium (NH_4^+), on plant dark respiration at elevated CO_2 during the night.

Plant organic N compounds account for less than 5% of the total dry weight of a plant, but conversion of NO_3^- into organic N expends about 25% of the total energy in shoots (Bloom et al., 1989) and roots (Bloom et al., 1992). During the day, photorespiration supplies a portion of the energy (Rachmilevitch et al., 2004; Foyer et al., 2009), but at night, this energetic cost is borne entirely by the respiration of C substrates (Amthor, 1995) and may divert a substantial amount of reductant from the mitochondrial electron transport chain (Cousins and Bloom, 2004). The relative importance of NO_3^- assimilation at night versus the day, however, is still a matter of intense debate (Nunes-Nesi et al., 2010). Here, we estimated NO_3^- assimilation using several independent methods and show in Arabidopsis (*Arabidopsis thaliana*) and wheat

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(*Triticum aestivum*), two diverse C_3 plants, that NO_3^- assimilation at night can be substantial, and that elevated CO_2 at night inhibits this process.

RESULTS

Exposure to elevated CO_2 only at night decreased biomass accumulation for Arabidopsis and wheat under NO_3^- nutrition (Fig. 1). For Arabidopsis, exposure to elevated CO_2 only during the day also increased biomass accumulation under NO_3^- nutrition (Fig. 1). Under NH_4^+ nutrition, plants exposed to elevated CO_2 day and night accumulated more biomass than those exposed to ambient CO_2 during the day or night (Fig. 1). Both species accumulated more biomass when they received NO_3^- as an N source rather than NH_4^+, but this was statistically significant in wheat only when all of the CO_2 treatments were treated as a group (Table I). These results are similar to those of previous experiments (Bloom et al., 2012) and may derive from a cation-anion imbalance that develops in plants receiving NH_4^+ as a sole N source (Epstein and Bloom, 2005). For example, plants under NH_4^+ nutrition may become potassium deficient or may accumulate too much molybdenum (Smart and Bloom, 1993).

Elevated CO_2 at night increased free NO_3^- concentrations in shoots under NO_3^- nutrition (Fig. 2), but in Arabidopsis, this was statistically significant only when all of the CO_2 treatments were treated as a group (Table I). In plants receiving NO_3^- nutrition, elevated CO_2 both day and night decreased shoot organic N concentration (Fig. 2). We observed similar trends in earlier experiments (Bloom et al., 2002, 2010, 2012). Plants receiving NO_3^- nutrition had lower shoot and root organic N concentrations than those receiving NH_4^+ nutrition (Fig. 2), although P was less than 0.25 for wheat at ambient CO_2 day and night. Total organic N per shoot or root was similar under both N forms, however (data not shown), because of the biomass differences under the two forms (Fig. 1).

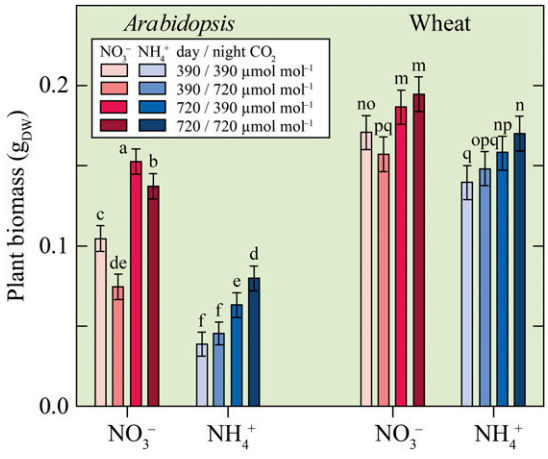

Figure 1. Total biomass (grams dry weight [g_{DW}]) for Arabidopsis (left) and wheat (right) receiving NO_3^- or NH_4^+ and exposed to one of four CO_2 treatments: ambient during the day and night (390/390 μmol mol^{-1}), ambient during the day and elevated at night (390/720 μmol mol^{-1}), elevated during the day and ambient at night (720/390 μmol mol^{-1}), or elevated during the day and night (720/720 μmol mol^{-1}). Shown are the mean ± SE (n = 11–15). Bars for one species labeled with different letters differed by $P < 0.10$.

Ambient CO_2 during the day and night increased the $\delta^{15}N$ (‰) of organic N in shoots (Fig. 2). If NO_3^- availability does not limit assimilation, shoots preferentially assimilate ^{14}N-NO_3^- (Carlisle et al., 2014). Therefore, the higher shoot $\delta^{15}N_{organic}$ signatures under ambient rather than elevated CO_2 indicate that the availability of free NO_3^- in the shoot was more limiting under ambient than elevated CO_2 because shoot NO_3^- assimilation was faster (Bloom et al., 2010, 2014).

About one-half of the $^{15}NO_3^-$ absorbed was assimilated during the night (Fig. 3). Elevated CO_2 at night decreased plant $^{15}NO_3^-$ assimilation, although P was less than 0.30 for Arabidopsis receiving elevated CO_2 during the day (Fig. 3). The rates of $^{15}NO_3^-$ assimilation at night were about two-thirds of the daytime rates that we reported previously (Bloom et al., 2010).

In Arabidopsis and wheat receiving NH_4^+ as their sole N source, neither net CO_2 evolution nor net O_2 consumption changed significantly with CO_2 treatment (Fig. 4). Under NO_3^- nutrition, elevated CO_2 decreased net CO_2 evolution and net O_2 consumption in Arabidopsis genotypes with the capacity to assimilate NO_3^- and increased net O_2 consumption in wheat (Fig. 4, A, B, and D), but had no effect in an Arabidopsis double mutant with limited NO_3^- assimilation (Fig. 4C). The change in respiratory quotient (RQ) with a shift from NO_3^- to NH_4^+ nutrition (ΔRQ; where RQ is the ratio of net CO_2 evolution to net O_2 consumption) was insensitive to the CO_2 treatment in the Arabidopsis double mutant with a limited capacity to assimilate NO_3^- (Fig. 4C), but decreased at elevated CO_2 in Arabidopsis genotypes with the capacity to assimilate NO_3^- and in wheat (Fig. 4, A, B, and D).

Carbohydrate concentrations at the end of the day, except for those of Suc, were higher in the elevated CO_2 treatment than the ambient CO_2 treatment (Fig. 5). At the end of the night, the carbohydrate concentrations were lower than at the end of the day (Fig. 5). Carbohydrate concentrations at the end of the night were generally higher in the elevated CO_2 treatment than the ambient CO_2 treatment (Fig. 5).

DISCUSSION

Here, six independent measures, plant biomass accumulation under NO_3^- nutrition (Fig. 1), shoot free NO_3^- concentration (Fig. 2), shoot organic N concentrations (Fig. 2), shoot $\delta^{15}N$ in the organic fraction (Fig. 2), plant ^{15}N-NO_3^- assimilation (Fig. 3), and ΔRQ (Fig. 4), indicated that elevated CO_2 at night inhibited nighttime NO_3^- assimilation in Arabidopsis and wheat. Plants that received NH_4^+ as their sole N source or had mutations that limited NO_3^- assimilation did not respond to elevated CO_2 at night (Figs. 1, 2, and 4).

Table I. *A mixed-model ANOVA on the effects of N source (NO_3^- or NH_4^+) and atmospheric CO_2 concentration regime (ambient day and night, 390/390 μmol mol^{-1}; ambient during the day and elevated at night, 390/720 μmol mol^{-1}; elevated during the day and ambient at night, 720/390 μmol mol^{-1}; or elevated day and night, 720/720 μmol mol^{-1}) on various parameters of Arabidopsis and wheat (n = 10–16)*

Dash, No data; ns, nonsignificant differences; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

	Arabidopsis					Wheat				
Main Effects	Plant Biomass	Shoot NO_3^-	Shoot Organic N	Root NO_3^-	Root Organic N	Plant Biomass	Shoot NO_3^-	Shoot Organic N	Root NO_3^-	Root Organic N
N	***	—	***	—	***	***	—	***	—	***
CO_2	***	**	***	***	***	***	***	***	***	ns
N*CO_2	***	—	***	—	ns	*	—	**	—	***

Several physiological mechanisms could be responsible for these responses. Elevated CO_2 inhibited the activity of the mitochondrial enzymes succinate dehydrogenase and cytochrome c oxidase (Gonzalez-Meler et al., 1996; Drake et al., 1999; Gonzàlez-Meler and Siedow, 1999). These may interfere with carbohydrate catabolism and decrease the energy available for NO_3^- assimilation. Elevated CO_2 also inhibited the translocation of nitrite from the cytoplasm into the chloroplast, the site where the subsequent conversion into amino acids occurs (Bloom et al., 2002).

Our finding that elevated CO_2 inhibited nighttime NO_3^- assimilation and that this inhibition could impede the growth of plants exposed to elevated CO_2 at night is consistent with several other studies. In soybean (*Glycine max*) receiving both NH_4^+ and NO_3^- as N sources, elevated CO_2 inhibited nighttime respiration, carbohydrate translocation, and NO_3^- assimilation as monitored from the disappearance of NO_3^- from leaf discs (Bunce, 2004). This most likely explains the slower growth of soybean exposed to elevated CO_2 at night (Bunce, 2003). Plants may compensate to some extent for elevated CO_2 during the day or night by increasing the proportion of NO_3^- assimilated in the roots (Kruse et al., 2002, 2003) because root NO_3^- assimilation is relatively insensitive to CO_2 concentration (Bloom et al., 2010, 2014). Nonetheless,

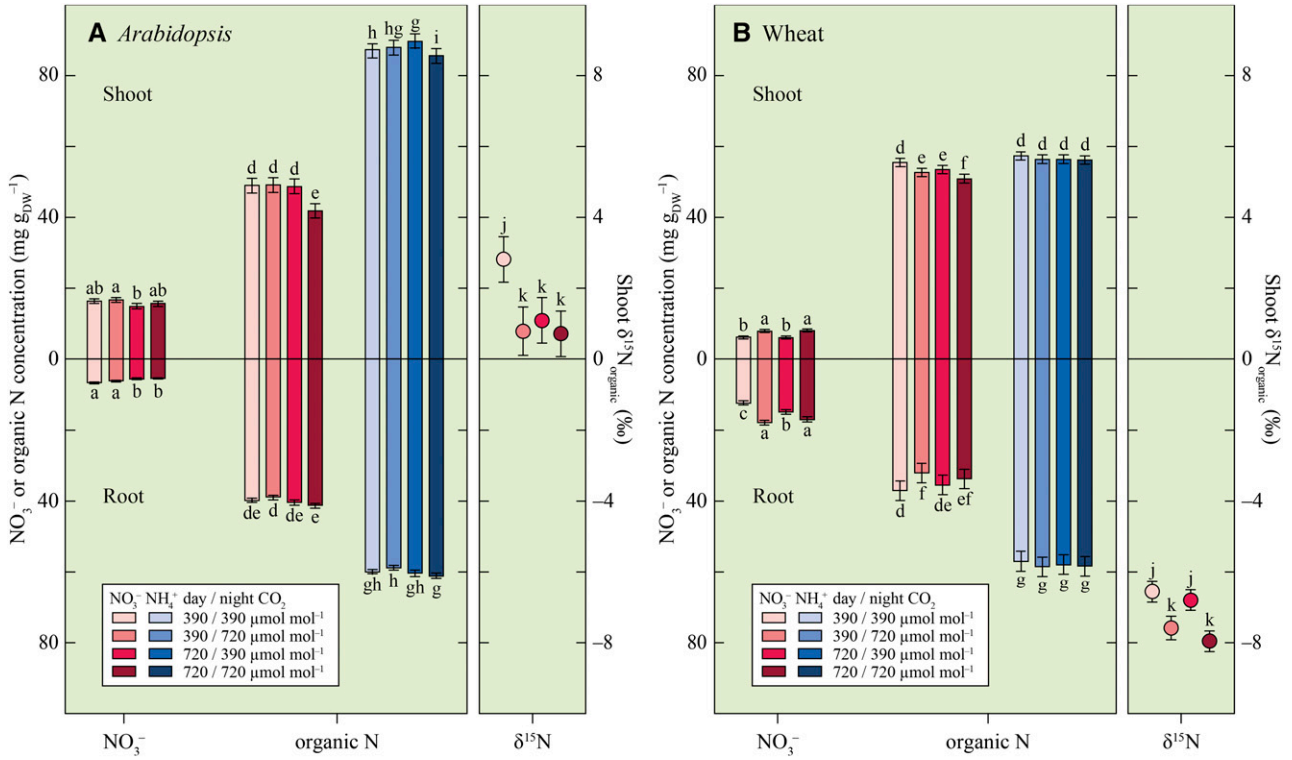

Figure 2. NO_3^- and organic N concentration (milligrams per grams dry weight [mg g_{DW}^{-1}]) in the shoot (top left) and root (bottom left) and shoot $\delta^{15}N$ (‰) of organic N (right) for Arabidopsis (A) and wheat (B) receiving NO_3^- or NH_4^+ and exposed to one of four CO_2 treatments: ambient during the day and night (390/390 μmol mol^{-1}), ambient during the day and elevated at night (390/720 μmol mol^{-1}), elevated during the day and ambient at night (720/390 μmol mol^{-1}), or elevated during the day and night (720/720 μmol mol^{-1}). Shown are the mean ± SE (n = 8–10). Bars and symbols for one species labeled with different letters differed by $P < 0.10$.

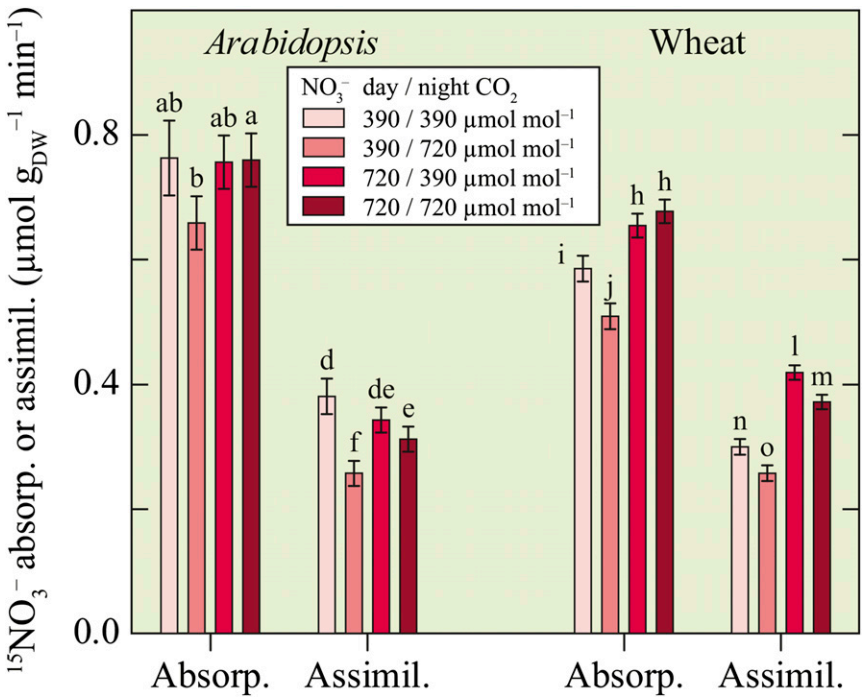

Figure 3. Whole-plant $^{15}NO_3^-$ absorption and assimilation (μmol NO_3^- g_{DW}^{-1} [for grams per dry weight] min^{-1}) in the dark for Arabidopsis and wheat exposed to one of four CO_2 treatments: ambient during the day and night (390/390 μmol mol^{-1}), ambient during the day and elevated at night (390/720 μmol mol^{-1}), elevated during the day and ambient at night (720/390 μmol mol^{-1}), or elevated during the day and night (720/720 μmol mol^{-1}). Shown are the means ± SE (n = 5–14). Bars for one species labeled with different letters differed by $P < 0.10$.

roots did not fully compensate for slower shoot NO_3^- assimilation in the current study because plant biomass (Fig. 1) and shoot organic N (Fig. 2) both tended to decline when nighttime CO_2 was elevated.

The relative dependence of a plant on NO_3^- versus NH_4^+ varies with species, physiological state of the plant, and availability of each N form from the medium. The availability of each N form depends on many factors in the rhizosphere, including microbial activity, water status, pH, and cation exchange capacity (Epstein and Bloom, 2005). The wide range in plant responses to nighttime elevated CO_2 that others have encountered

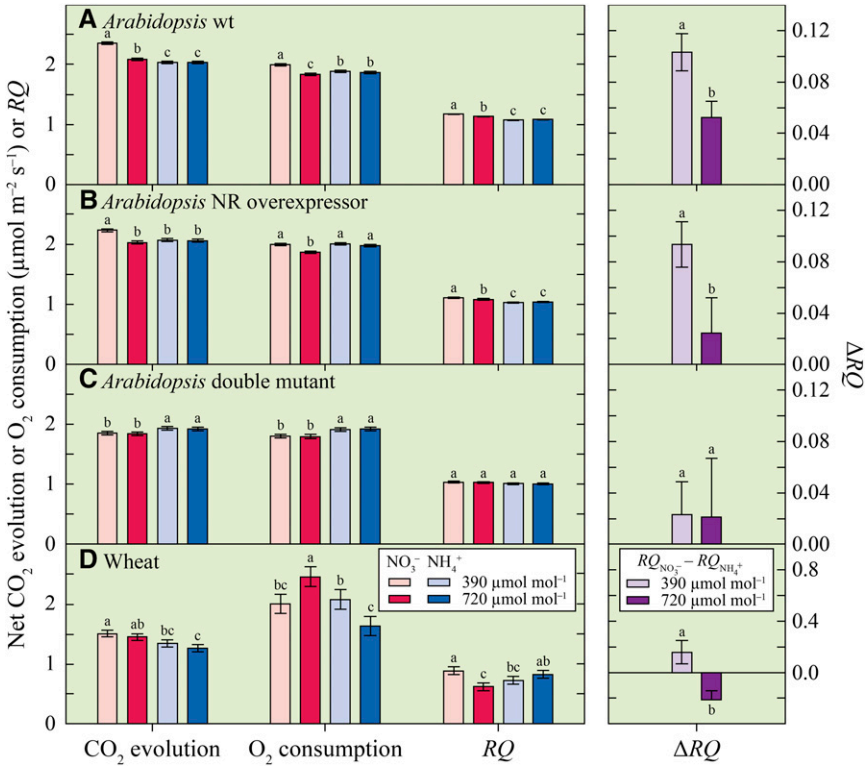

Figure 4. Shoot net CO_2 evolution, net O_2 consumption, and RQ at night under ambient (390 μmol mol^{-1}) or elevated (720 μmol mol^{-1}) CO_2 atmosphere for three Arabidopsis genotypes and wheat receiving NO_3^- or NH_4^+ (left). The effect was measured in the Arabidobsis wild type (wt), a transformant overexpressing nitrate reductase (NR overexpressor), and a double mutant lacking detectable NR activity (double mutant). Shown are the mean ± SE (n = 6–14). Changes in the shoot RQ (ΔRQ) with the shift from NO_3^- to NH_4^+ as an N source (right). For each parameter within one genotype, bars labeled with different letters differed by $P < 0.10$.

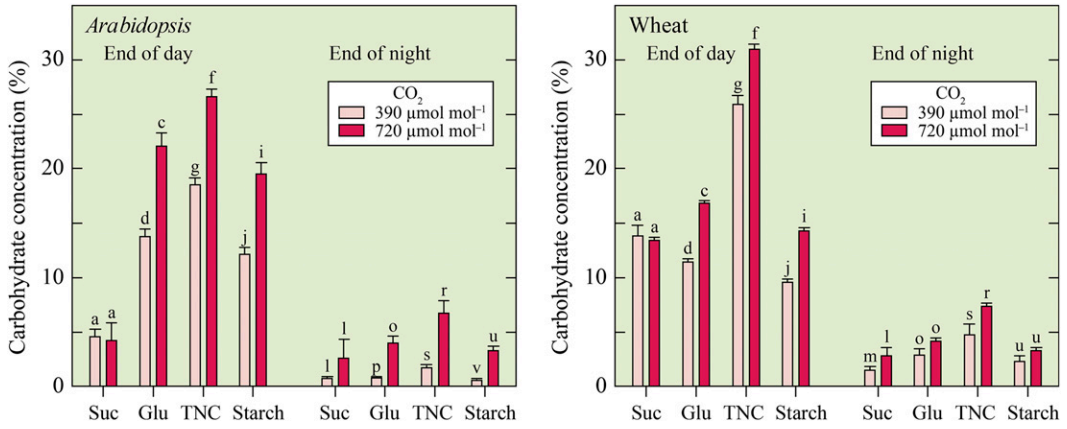

Figure 5. Free Suc, total Glc (Glu), total nonstructural carbohydrates (TNC), and starch (Starch) concentrations in shoots of Arabidopsis and wheat at the end of the day and at the end of the night in plants exposed to ambient (390 μmol mol^{-1}) or elevated (720 μmol mol^{-1}) CO_2 and NO_3^- nutrition. Concentrations are in percentage (w/w). Shown are the mean ± SE (n = 3–4). For each parameter within one species, bars labeled with different letters differed by $P < 0.05$.

(e.g. Bunce, 2003), therefore, may derive from differences in the relative dependence of plants on NO_3^- and NH_4^+.

The relative dependence of Arabidopsis and wheat on NO_3^- and NH_4^+ was not a factor in the current experiments because plants received either NO_3^- or NH_4^+ as a sole N source. Indeed, Arabidopsis and wheat showed similar differential responses to the N source: they both grew larger when receiving NO_3^- rather than NH_4^+ as an N source (Fig. 1), both had lower organic N concentrations when receiving NO_3^- rather than NH_4^+ as an N source (Fig. 2), and both respired more CO_2 when assimilating NO_3^- at relatively high rates (ambient CO_2, NO_3^- nutrition) than when assimilating NH_4^+ (Fig. 4).

Dark respiration, both net CO_2 evolution and net O_2 consumption, decreased at elevated CO_2 under NO_3^- nutrition and did not change under NH_4^+ nutrition (Fig. 4). Assimilation of NO_3^- expends about 25% of carbon catabolism, whereas that of NH_4^+ expends about 3% (Bloom et al., 1992; Cousins and Bloom, 2004). Therefore, it is expected that CO_2 inhibition of NO_3^- assimilation would produce observable changes in respiratory gas fluxes.

Our gas-exchange system sealed plants into shoot and root cuvettes with a stopper around the stem, so the surface area of the seal was minimal. Any leaks that might have occurred would have equally influenced the gas fluxes of plants receiving the two N forms and the various genotypes. Therefore, the differences in gas fluxes that we observed among N forms and genotypes did not derive from the measurement artifacts that others have encountered (e.g. Jahnke and Krewitt, 2002).

The RQ, ratio of net CO_2 evolved to net O_2 consumed, was generally higher in Arabidopsis than wheat (Fig. 4). This suggests that Arabidopsis and wheat were catabolizing different substrates. For example, catabolism of malic acid results in an RQ of 1.33, whereas that of carbohydrates results in an RQ of 1.0, that of lipids results in an RQ of 0.70, and that of ketones results in an RQ of 0.66 (Stiles, 1994). Differences between Arabidopsis and wheat in the substrates supporting dark respiration will require further examination, but carbohydrate concentrations showed similar changes overnight in both species (Fig. 5).

Plant carbohydrate concentrations can influence the concentrations of other plant constituents such as NO_3^- and organic N. Nonetheless, plant material for the NO_3^- and organic N analyses was collected in the morning when the differences in carbohydrates between the CO_2 treatments were relatively small, only a few percent of dry mass (Fig. 5). This could decrease the difference in NO_3^- between the CO_2 treatments and contribute to the increase in organic N, but would not be sufficient to account for all of the observed differences between the CO_2 treatments.

Our results have profound implications for research on plant responses to elevated CO_2. Some free-air CO_2 enrichment experiments expose plants to elevated CO_2 only during the day and let CO_2 return to ambient levels at night. This is to avoid the added expense of applying concentrated CO_2 at night and to avoid the difficulties of controlling atmospheric CO_2 concentrations when photosynthesis does not provide a strong sink for the CO_2. The large variation in plant responses to elevated CO_2 among field experiments (Ainsworth and Long, 2005) may derive in part from differences in nighttime CO_2 concentrations.

Few studies on plant responses to elevated CO_2 have attempted to define the form of N that the plants are using. Our data demonstrate that N form and nighttime atmospheric CO_2 concentration are critical factors in determining plant performance under the environmental conditions anticipated during the next few decades. Indeed, the future of food quality, in terms of protein and other nutrients (Myers et al., 2014), and the extent to which plants serve as sinks for human CO_2 emissions (Bloom, 2010) will depend on the relationship between elevated CO_2 and N form.

MATERIALS AND METHODS

Plant Material

We used Arabidopsis (*Arabidopsis thaliana*) 'Columbia' and wheat (*Triticum aestivum*) 'Veery.' For the gas exchange experiments, we also used two Arabidopsis 'Columbia' genotypes that exhibited different levels of NR activity (Rachmilevitch et al., 2004): a transgenic line harboring the chimeric gene Lhch1*3::Nia1*2 (the Arabidopsis nitrate reductase gene under the regulation of the light-harvesting chlorophyll *a/b* protein promoter) that had about twice the NR activity of the wild type (Heimer et al., 1995) and a genotype with mutations in both structural genes for NR, *nia1 nia2*, which had little detectable NR activity (Wilkinson and Crawford, 1993).

Growth Conditions

Arabidopsis seeds were germinated and grown in Magenta boxes for 12 d. For the first 3 d, the boxes were covered with foil, and then over the next 9 d, the plants were gradually acclimated to light. A pool of seedlings (60 per tub) were transplanted and kept for 3 d in 5-L opaque polyethylene tubs filled with an aerated nutrient solution, and the shoots were covered with transparent plastic trays. During this period (12 d + 3 d), all plants received the same nutrient solution: macronutrients (mM) at 1.25 $CaSO_4$, 0.2 KNO_3, 0.2 NH_4Cl, 0.75 $MgSO_4$, 0.25 KH_2PO_4, and 0.75 K_2HPO_4; micronutrients (μM) at 50 KCl, 25 H_3BO_3, 2 $MnSO_4H_2O$, 2 $ZnSO_4$, 7 H_2O, 0.5 $CuSO_4$, 5 H_2O, and 0.5 Na_2MoO_4; and Fe-NaDPTA (Sequestrene 330, Becker Underwood) at 0.2 g L^{-1}. The most uniform seedlings were transplanted to 5-L tubs (10 plants per tub) and placed in controlled environmental growth chambers (Conviron PGR15), four tubs per chamber. The chambers had a 9-h-light period of 350 μmol m^{-2} s^{-1} of photosynthetic active radiation at plant height, 21°C, and 80% humidity, and a 15-h-dark period, 21°C, and 60% humidity. Nutrient solution was changed twice during the first week, three times during the second week, and every other day thereafter.

Wheat seeds were surface sterilized with 20% (v/v) NaOCl and then washed thoroughly with water. Healthy seeds were rolled up in a paper towel soaked with 10 mM $CaSO_4$ for 4 d at 25°C in the dark, with the bottom one-fourth of the rolled towel sitting in a 10 mM $CaSO_4$ solution. The most uniform wheat seedlings were transplanted to 20-L opaque polyethylene tubs (10 seedlings per tub) filled with a nutrient solution containing: macronutrients (mM) at 1.0 $CaSO_4$, 0.2 KNO_3, 0.2 NH_4Cl, 1.0 $MgSO_4$, 0.5 KH_2PO_4, and 0.5 K_2HPO_4; micronutrients (μM) at 50 KCl, 25 H_3BO_3, 2 $MnSO_4H_2O$, 2 $ZnSO_4$, 7 H_2O, and 0.5 H_2MoO_4; and Fe-NaDPTA (Sequestrene 330, Becker Underwood) at 0.2 g L^{-1}. Plants grew in controlled environmental chambers (Conviron PGR15) with 15 h of 500 μmol m^{-2} s^{-1} of photosynthetic active radiation at plant height, 25°C, and 70% humidity, and with 9 h of dark, 16°C, and 60% humidity. Nutrient solution was changed every 3 d during the first week and every other day thereafter.

Growth and N Balance Experiments

Arabidopsis and wheat plants were exposed for 20 and 10 d, respectively, to either 0.2 mM KNO_3 or 0.2 mM NH_4Cl as an N source and one of four CO_2 treatments: ambient CO_2 during the day and night (390/390 μmol mol^{-1}), ambient CO_2 during the day and elevated CO_2 at night (390/720 μmol mol^{-1}), elevated CO_2 during the day and ambient CO_2 at night (720/390 μmol mol^{-1}), or elevated CO_2 during the day and night (720/720 μmol mol^{-1}). Two controlled environmental chambers were equipped with nondispersive infrared analyzers (Horiba APBA-250E) and control systems that added CO_2 (filtered through a $KMnO_4$ column to remove contaminating hydrocarbons such as ethylene) to maintain one at ambient (390 μmol mol^{-1}) and the other at elevated (720 μmol mol^{-1}) CO_2 concentrations. The chambers were shifted to the alternative CO_2 concentration in replicate experiments. The lids of the 5- and 20-L tubs were cut into halves; this allowed us to shift one-half of the plants from one chamber to the other chamber for the treatments that had different CO_2 concentrations during the day and night. The shift occurred within 30 min of the chamber lights turning on or off. At this time, even plants that remained in the same chamber were briefly lifted out of the nutrient solution tub.

After 35 d for Arabidopsis and 14 and 30 d, respectively, for the growth and N balance of wheat, plants were harvested in the morning soon after the lights turned on when shoot carbohydrate levels were relatively low. The roots were separated from shoots and rinsed in a chilled solution containing 1 mM $CaSO_4$. Then, both shoots and roots were placed in a forced-air drying oven for 3 d at 60°C. Shoots and roots were ground to a fine powder in a ball mill.

Total N and total N isotope ratios were determined by a PDZ Europa ANCA-GSL elemental analyzer interfaced to an isotope ratio mass spectrometer (Sercon Ltd.) at the University of California Davis Stable Isotope Facility. During analysis, samples were interspersed with several replicates of at least two different laboratory standards. These laboratory standards, selected to be compositionally similar to the samples being analyzed, were previously calibrated against National Institute of Standards and Technology standard reference materials (IAEA-N1, IAEA-N2, IAEA-N3, IAEA-CH7, and NBS-22). The final delta values were expressed relative to air. The NO_3^- concentration of the diluted extracts was determined spectrophotometrically (Doane and Horwath, 2003). Organic N was estimated from the difference between total N and unassimilated NO_3^- because NH_4^+ concentrations in these species are low and do not vary significantly with CO_2 treatment (Bloom et al., 2002). We conducted two replicate experiments for each species.

Natural Abundance of Organic ^{15}N

^{15}N-organic N was estimated from the difference between total ^{15}N and ^{15}N-NO_3^-. The N isotopic composition of plant NO_3^- extracts was analyzed from N_2O generated by denitrifying bacteria lacking N_2O reductase (Sigman et al., 2001) at the UC Davis Stable Isotope Facility. In brief, *Pseudomonas chlororaphis* were grown in a tryptic soy broth amended with NO_3^- for 7 d. During this time, the O_2 in the headspace of the medium bottles and the NO_3^- in the medium were consumed. Concentrated 2-mL aliquots of this culture were then divided into 20-mL headspace vials that were sealed and purged for 2 h with N_2 gas to remove N_2O and O_2. Samples of the plant tissue extracts containing 0.1 μmol NO_3^--N were injected through the septae of the vials. The conversion of NO_3^- to N_2O was complete within less than 1 h. The N_2O was flushed from the vials with helium, trapped cryogenically, and then released into the isotope ratio mass spectrometer. Standards of KNO_3 (IAEA-N1, IAEA-N2, and IAEA-N3), having $\delta^{15}N$ values that bracketed the values of our samples, were processed in the same manner as the plant tissue extracts and converted to N_2O by the bacteria. A linear regression between measured versus known $\delta^{15}N$ values of the standards was used to adjust the $\delta^{15}N$ values of the samples. The adjustments were typically between 1‰ and 2‰ $\delta^{15}N$. The final delta values were expressed relative to air.

^{15}N-NO_3^- Labeling Experiments

Measurements of NO_3^- uptake and assimilation were made on 35- to 36-d-old Arabidopsis plants and 14-d-old wheat plants. These plants were grown on the nutrient solution described earlier but with both NO_3^- and NH_4^+ as N sources. The night before ^{15}N-NO_3^- labeling, 12 plants were transferred from the controlled environment chamber to a multiplant measurement system in the laboratory (Kosola and Bloom, 1994). The root of each plant was sealed by a rubber stopper around the stem into cuvettes supplied with a continuous flow of nutrient solution. The nutrient solution contained 0.2 mM KNO_3, 1 mM $CaSO_4$, 5 μM KH_2PO_4, and 5 μM K_2HPO_4.

The plants were kept in the laboratory for two night periods interrupted by one light period. In the first night period (8 h), the plants were allowed to recover from any transplant shock. During the following light period, the shoots were exposed to either an ambient (390 μmol mol^{-1}) or elevated (720 μmol mol^{-1}) CO_2 concentration. This light period was 9 h for Arabidopsis and 12 h for wheat; light intensity was 350 and 500 μmol m^{-2} s^{-1} for Arabidopsis and wheat, respectively; and the temperature was set at 25°C and 22°C for Arabidopsis and wheat, respectively. The next night period and after 1 h of acclimation to darkness, the nutrient solution containing natural abundance levels of ^{15}N-NO_3^- was switched to one containing 25 atom % ^{15}N-NO_3^-. During this second night period, NO_3^- uptake and assimilation were assayed using the tracer ^{15}N in plants exposed to either an ambient (390 μmol mol^{-1}) or elevated (720 μmol mol^{-1}) CO_2 concentration. This night period was 12 and 8 h for Arabidopsis and wheat, respectively, and the temperature was controlled at 20°C.

Before beginning the labeling period, we harvested five plants, and after the labeling period, we harvested seven plants. The roots were rinsed in a chilled solution with 1 mM $CaSO_4$. Shoots and roots were dried at 60°C for 3 d and ground to a fine powder in a ball mill. Total N and NO_3^- tissue concentration and its ratio, $^{15}N/^{14}N$, were analyzed as described earlier. Plant absorption of ^{15}N-NO_3^- was calculated from the difference in total ^{15}N between the plants harvested after the labeling period and those harvested before. Plant assimilation of ^{15}N-NO_3^- was calculated from the difference in ^{15}N-organic N between the plants harvested after the labeling period and those harvested before.

Gas Exchange Experiments

We monitored net CO_2 evolution and net O_2 consumption simultaneously from the whole canopy and calculated the RQ (ratio of net CO_2 evolved to net O_2 consumed). The differences in the RQ between NH_4^+-fed and NO_3^--fed plants (ΔRQ) reflect NO_3^- assimilation in the nighttime because electrons generated from the catabolism of carbohydrates to CO_2 are transferred to NO_3^- or nitrite rather than O_2 (Bloom et al., 1992). Thus, ΔRQ (change in RQ with a shift in N source) has provided real-time, nondestructive estimates of NO_3^- assimilation for nearly a century (Warburg and Negelein, 1920; Van Niel et al., 1953). Measurements of net CO_2 assimilation and net O_2 evolution were made on 35- to 36-d-old Arabidopsis and 14-d-old wheat plants.

Two days before gas exchange measurements, a plant was switched from a nutrient solution containing 0.2 mM NH_4NO_3 to one containing 0.2 mM NH_4Cl to deplete NO_3^- from the plant tissue. To monitor gas fluxes, the stem of an intact plant was wrapped with Teflon plumber's tape, and a thin layer of silicon vacuum grease was applied to the outside. A slit rubber stopper with an appropriately sized hole was fit around the taped stem. The stopper sealed the root system of this intact plant into a root cuvette made of acrylic plastic and stainless steel for both Arabidopsis and wheat and its shoot system into a shoot cuvette made of glass and Teflon-coated aluminum for Arabidopsis and into a gold-plated cuvette with a glass top for wheat (Bloom et al., 1989). Roots remained in the dark at 18°C and were supplied with a continuous flow of an aerated nutrient solution containing 1 mM $CaSO_4$, 0.5 μM K_2HPO_4, and either 200 μM KNO_3 or 200 μM NH_4Cl. The pH of the solution was 6.0. The leaves in the shoot cuvette retained their natural orientation to the light source (1,000-W metal-halide lamp; Wide-Lite). The light levels during the light cycles were 350 and 500 μmol m^{-2} s^{-1} for Arabidopsis and wheat, respectively. The light-dark cycle was the same as in the controlled environmental chambers. Two 0.07-mm copper-constantan thermocouples were placed on the abaxial side of two leaves to monitor leaf temperatures.

The plant was kept in the lab for 2 d, the first day with NH_4^+ as the sole N source and the second day with NO_3^- as the sole N source. During the first light period, the plant was allowed to recover from any transplant shock (Bloom and Sukrapanna, 1990). During the subsequent dark period, the gas exchange of the NH_4^+-fed plant was monitored. At the start of the second light period, the nutrient solution was switched to one containing NO_3^- as the sole N source. Finally, during the second dark period, the gas exchange of the NO_3^--fed plant was monitored. Gas exchange measurements began 1 h into the dark period. Plants were subjected to each CO_2 concentration, 390 and 720 μmol mol^{-1} CO_2, for 30 min before taking a measurement, shifting back and forth three times between the two concentrations. The rate of respiration at a given CO_2 concentration did not change significantly during the 2 h between the measurements ($P > 0.9$). After the 2-d measurement period, we determined the leaf area and calculated specific respiration rates on a leaf area basis (either CO_2 evolution or O_2 consumption in μmol m^{-2} s^{-1}; Bloom et al., 1980). In addition, shoot and root dry weights were determined.

An open gas exchange system (Bloom et al., 1989) monitored net CO_2 assimilation, net O_2 evolution, and transpiration using a commercial nondispersive infrared CO_2 analyzer (Horiba model VIA-500R), a custom-designed O_2 analyzer, and relative humidity sensors (Vaisala), respectively. The custom O_2 analyzer contained two cells of calcia-stabilized zirconium oxide ceramic similar to those found in an Applied Electrochemistry model N-37 M. When heated to 752 ± 0.01°C in an electric furnace, these cells become selectively permeable to O_2, and at the ambient O_2 concentration (20.97% O_2), generate a Nernst potential of 106 nV per μmol mol^{-1} difference in O_2 concentration. The oxygen analyzer resolves 2 μmol mol^{-1} O_2 partial pressure difference on a background of 209,490 μmol mol^{-1}. Mass flow controllers (Tylan) mixed 2% CO_2 in air from a compressed gas cylinder and CO_2-free air from a 100-L storage tank to obtain the 390 and 720 μmol mol^{-1} CO_2 concentrations. The flow rate through the shoot chamber was 10 cm^3 s^{-1}. A pressure transducer (Validyne) monitored the pressure drop across a capillary to measure the gas flow through the shoot chamber. The leaf vapor pressure deficit was maintained at approximately 10 mbar. To check for leaks, we periodically confirmed that the net flux rates of CO_2 and O_2 in an empty cuvette were zero through a range of known cuvette CO_2 concentrations.

Carbohydrate Analysis

Shoots of plants used in the dark ^{15}N-labeling experiments were also analyzed for carbohydrates. Samples were extracted by hot deionized water, and the extract was analyzed for free sugars (Glc, Fru, and Suc). The samples for total Glc were enzymatically hydrolyzed at 55°C with amyloglucosidase for 12 h and analyzed for free Glc. The analyses were conducted by HPLC with mass selective detection (Johansen et al., 1996) using a Phenomenex Luna NH2 HPLC column (250 × 4.6 mm) at a flow rate of 2.75 mL min^{-1} acetonitrile: water (78:22). The method has a detection limit of 0.2% and is reproducible within 10% (relative). Total nonstructural carbohydrate was calculated from the sum of total Glc, free Fru, and free Suc, whereas starch was calculated from total Glc minus free Glc multiplied by 0.9 (Smith, 1969).

Statistics

An ANOVA was conducted (PROC MIXED in SAS 9.3, SAS Institute). All of the data met the assumptions of normality and homogeneity of variance as evaluated via the Shapiro-Wilks and Levene's tests, respectively. We determined the effects of N form and CO_2 treatment and their interaction on the different parameters evaluated. Tukey's post hoc test was conducted on the differences between means.

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