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CO_2 enrichment inhibits shoot nitrate assimilation in C_3 but not C_4 plants and slows growth under nitrate in C_3 plants

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Abstract. The CO_2 concentration in Earth's atmosphere may double during this century. Plant responses to such an increase depend strongly on their nitrogen status, but the reasons have been uncertain. Here, we assessed shoot nitrate assimilation into amino acids via the shift in shoot CO_2 and O_2 fluxes when plants received nitrate instead of ammonium as a nitrogen source (ΔAQ). Shoot nitrate assimilation became negligible with increasing CO_2 in a taxonomically diverse group of eight C_3 plant species, was relatively insensitive to CO_2 in three C_4 species, and showed an intermediate sensitivity in two C_3-C_4 intermediate species. We then examined the influence of CO_2 level and ammonium vs. nitrate nutrition on growth, assessed in terms of changes in fresh mass, of several C_3 species and a Crassulacean acid metabolism (CAM) species. Elevated CO_2 (720 μmol CO_2/mol of all gases present) stimulated growth or had no effect in the five C_3 species tested when they received ammonium as a nitrogen source but inhibited growth or had no effect if they received nitrate. Under nitrate, two C_3 species grew faster at sub-ambient (~310 μmol/mol) than elevated CO_2. A CAM species grew faster at ambient than elevated or sub-ambient CO_2 under either ammonium or nitrate nutrition. This study establishes that CO_2 enrichment inhibits shoot nitrate assimilation in a wide variety of C_3 plants and that this phenomenon can have a profound effect on their growth. This indicates that shoot nitrate assimilation provides an important contribution to the nitrate assimilation of an entire C_3 plant. Thus, rising CO_2 and its effects on shoot nitrate assimilation may influence the distribution of C_3 plant species.

Key words: C_3; C_4; climate change; CO_2 acclimation; Crassulacean acid metabolism (CAM); nitrogen; plant distributions.

Introduction

The vast majority of higher plant species conduct carbon fixation solely via the C_3 (Calvin-Benson) pathway, and thus are called C_3 species. In unfertilized soils and agricultural soils receiving standard amounts of fertilizer, exposure of C_3 species to elevated atmospheric CO_2 concentrations initially enhances growth by as much as 35%, but over time, from days to years, this enhancement diminishes, a phenomenon known as CO_2 acclimation (Long et al. 2004, Reich et al. 2006). Previously we demonstrated in the C_3 species wheat and *Arabidopsis* that CO_2 acclimation derives from CO_2 inhibition of nitrate (NO_3^-) assimilation into organic nitrogen (N) compounds (Bloom et al. 2002, 2010, Rachmilevitch et al. 2004). In contrast NO_3^- assimilation in maize (corn), a species that conducts carbon fixation via the C_4 (Hatch-Slack) pathway, was relatively insensitive to atmospheric CO_2 concentration (Cousins and Bloom 2003). Here, we surveyed a taxonomically diverse group of higher plant species, including C_3, C_3-C_4, C_4, and Crassulacean acid metabolism (CAM) plants, to determine the generality of these findings.

We first evaluated the response of shoot NO_3^- assimilation to CO_2 enrichment via measurements of assimilatory quotient (AQ). The assimilatory quotient is the ratio of net CO_2 consumed over net O_2 evolved in a photosynthetically active leaf (Warburg 1948). It varies with NO_3^- vs. NH_4^+ metabolism because NO_3^- assimilation increases the light-dependent splitting of H_2O and generation of O_2. The additional electrons generated during H_2O splitting are transferred first to NO_3^- and then to NO_2^-. This has little effect on net CO_2 consumption, but results in a faster rate of net O_2 evolution, and thus AQ (=CO_2/O_2) decreases (Warburg and Negelein 1920, Cramer and Myers 1948,Van Niel et

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al. 1953, Bloom et al. 1989, Cen and Layzell 2003, Eichelmann et al. 2011). We examined AQ in a taxonomically diverse group of C_3 species (loblolly pine, giant redwood, wheat, barley, *Arabidopsis*, *Flaveria pringlei*, sugar maple, and sweet gum) and C_4 species (maize, *Flaveria bidentis*, and amaranth) and two species that do not fully implement the C_4 pathway and therefore are considered C_3-C_4 intermediates (*Flaveria chloraefolia* and *F. sonorensis*). The results of these experiments reinforced our previous findings about the response of shoot NO_3^- assimilation to CO_2 enrichment.

To assess the importance of this response to overall plant performance, we compared the relative growth rates of several C_3 species (loblolly pine, wheat, *Arabidopsis*, sugar maple, and sweet gum) under NO_3^- or NH_4^+ nutrition at ambient and elevated CO_2 concentrations. The interaction between N form and the two CO_2 treatments were significant for all species, indicating that the performance of C_3 plants in the future may depend on the response of NO_3^- assimilation to CO_2 enrichment. To determine whether recent changes in atmospheric CO_2 concentration may have already influenced the performance of higher plants, we added a third CO_2 treatment, sub-ambient, and grew two of the same tree species as before (loblolly pine and sweet gum) and a CAM species (jade plant). This is, to our knowledge, the first survey of the influence of N form and atmospheric CO_2 concentration on a broad range of higher plant species.

Methods

Plant materials

For this study, we monitored net CO_2 and O_2 gas fluxes as a function of shoot internal CO_2 concentration (C_i) in eight C_3 species, three C_4 species, and two C_3-C_4 intermediates. The C_3 species were the gymnosperms *Pinus taeda* L. (loblolly pine) and *Sequoiadendron giganteum* (Lindl.) Buchholz (giant redwood), the annual monocots *Triticum aestivum* L. (wheat) and *Hordeum vulgare* L. (barley), the annual dicot *Arabidopsis thaliana* L. (thale cress), the herbaceous perennial *Flaveria pringlei* Gand., and the perennial woody dicots *Acer saccharum* Marshall (sugar maple) and *Liquidambar styraciflua* L. (sweet gum). The C_4 species were the annual nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME) monocot *Zea mays* L. (maize), the annual NADP-ME dicot *Flaveria bidentis* L. (coastal plain yellowtops), and the annual nicotinamide adenine dinucleotide-malic enzyme (NAD-ME) dicot *Amaranthus retroflexus* L. (red-root amaranth). The C_3-C_4 intermediates were the herbaceous perennial dicots *Flaveria chloraefolia* A. Gray (clasping yellowtops) and *F. sonorensis* A. M. Powell. Of these 13 species, we previously presented data from two (wheat and *Arabidopsis*) (Bloom et al. 2010).

We surface-sterilized seeds of *Arabidopsis thaliana* (cultivar [cv] Col-0), wheat (*Triticum aestivum* L. cv Veery 10), barley (*Hordeum vulgare* L. cv Steptoe), maize (*Zea mays* L. cv Dekalb), amaranthus (*Amaranthus retroflexus* L.), sweet gum (*Liquidambar styraciflua* L.), and giant redwood (*Sequoiadendron giganteum* (Lindl.) Buchholz) for 1 min in 2.6% NaClO and washed them thoroughly with water.

After surface sterilization, *Arabidopsis* seeds were germinated for 12 d in Magenta boxes (Sigma-Aldrich, St. Louis, Missouri, USA) filled with sand and saturated with a nutrient solution containing 200 μmol/L NH_4NO_3 and 20% strength of a modified Hoagland solution for the other nutrients (Epstein and Bloom 2005). The Magenta boxes were covered with foil for the first 3 d, and the seedlings were gradually acclimated to light over the next 9 d.

We collected seeds of sweet gum (*Liquidambar styraciflua* L.) on the University of California Davis campus. After surface sterilization, the sweetgum seeds were bubbled in 1 mmol/L $CaSO_4$ for 3 h, then planted in a 1:1 sand : vermiculite mix. Germination began within 14 days.

Wheat, barley, maize, amaranthus, and giant redwood seeds were surface sterilized and then germinated for several days on thick paper toweling saturated with 10 mmol/L $CaSO_4$.

We obtained bare-root saplings and seeds of loblolly pine (*Pinus taeda* L.) from the Tree Improvement Program, North Carolina State University, and bare-root saplings of the sugar maple (*Acer saccharum* Marshall) from Musser Forests, Indiana, Pennsylvania, USA. We obtained rooted cuttings of *Flaveria pringlei* Gand., *Flaveria bidentis* L., *Flaveria chloraefolia* A. Gray, and *F. sonorensis* A. M. Powell from Gerald E. Edwards, Washington State University, Pullman, Washington, USA. We collected leaf cuttings of jade plant (*Crassula ovata* Miller) from a plant obtained from a local nursery.

Once the seeds had germinated or the cuttings had developed new roots, we transferred the plants to 19-L opaque polyethylene tubs filled with an aerated nutrient solution similar in composition to the one *Arabidopsis* received. The jade plant's solution also contained 25 mmol/L NaCl because of sodium's role in CAM (Bloom 1979, Brownell 1979). For all species, the nutrient solution was changed twice during the first week and then every 2 or 3 d.

The tubs were placed in controlled environment chambers (Conviron, Winnipeg, Manitoba, Canada) providing photosynthetic photon flux densities (PFD) of 350 and 750 μmol quanta$\cdot m^{-2}\cdot s^{-1}$ at plant height for *Arabidopsis* and the other species, respectively. For *Arabidopsis*, the light period was 9 h at 22°C and 80% humidity, and the dark period was 15 h at 22°C and 60% humidity. For the other species, the light period was 16 h at 24°C and 70% humidity, and the dark period was 8 h at 16°C and 60% humidity. For the gas flux measurements, *Arabidopsis* plants were grown in the chambers until they were ~36 d old, the stage of maturity that has the maximum capacity for NO_3^- assimilation (Rachmi-

levitch et al. 2004); the other species remained in the chambers until they reached an appropriate size for our shoot and root cuvettes. For relative growth rate measurements, plants remained in the chambers for the designated times.

Gas flux measurements

Here we used the difference in assimilatory quotient (ΔAQ) between plants receiving NO_3^- instead of NH_4^+ as a sole N source to assess shoot NO_3^- assimilation. This approach has been verified by multiple independent methods including N isotope labeling (Cen and Layzell 2003, Bloom et al. 2010), N isotope discrimination (Bloom et al. 2010), genotypic and developmental variation (Bloom et al. 1989, Rachmilevitch et al. 2004), accumulation of organic N (Cen et al. 2001, Bloom et al. 2002), and nitrous oxide emissions (Smart and Bloom 2001). Other metabolic processes such as the water–water cycle, fatty acid synthesis, SO_4^{2-} reduction, oxaloacetate reduction, and O_2 uptake by respiration in the light may influence the balance between shoot CO_2 and O_2 gas fluxes (Ort and Baker 2002), but they are unlikely to shift rapidly and dramatically with N source and thus will not influence ΔAQ. For example, O_2 uptake by respiration in the light does not change significantly with N source (Cousins and Bloom 2004). In addition, the number of electrons devoted to NO_3^- assimilation is much larger than those to these other metabolic processes. For example, plants typically assimilate greater than eight times more NO_3^- than SO_4^{2-}. Indeed, NO_3^- assimilation consumes as much as one-quarter of a plant's photosynthetic electron transport (Bloom et al. 1989). The ΔAQ also responds within seconds to changes in CO_2 concentration and light level. This largely excludes processes other than NO_3^- photoassimilation. Lastly, photorespiration does not influence ΔAQ because this process changes neither net CO_2 consumption nor net O_2 evolution (Tolbert 1994, Foyer et al. 2009).

Measurements of ΔAQ are not common because of the difficulty in monitoring relatively small O_2 fluxes against a relatively high background concentration (Hunt 2003). Nonetheless, ΔAQ has proved to be a reliable measure of NO_3^- assimilation in a wide variety of plants (e.g., Warburg and Negelein 1920, Cramer and Myers 1948, Van Niel et al. 1953, Bloom et al. 1989, Cen and Layzell 2003, Eichelmann et al. 2011). Moreover, ΔAQ has the unique advantage that it provides nondestructive and real-time estimates of NO_3^- assimilation.

We first grew the plants under ambient atmospheric conditions (~400 μmol CO_2/mol all gases present) in hydroponic culture with 0.2 mmol/L NH_4NO_3 as the N source. When the plants approached an appropriate size to fill our shoot and root cuvettes, we subjected *Amaranthus retroflexus* to an N-free medium and the other species to a medium containing 0.1 mmol/L $(NH_4)_2SO_4$ for several days to deplete the tissues of free NO_3^-. Such periods of NO_3^- deprivation do not diminish a plant's capacity to assimilate NO_3^- (MacKown 1987). The night before an experiment we placed a split rubber stopper around the stem to seal the shoot and root into separate cuvettes (Bloom 1989, Bloom et al. 1989). Leaves in the shoot cuvette were at their normal orientation. Net gas fluxes from the shoot were monitored with the instrumentation described previously (Bloom et al. 1989, 2002). In brief, an infrared gas analyzer (VIA-500R, Horiba, Kyoto, Japan) measured CO_2 fluxes, a custom O_2 analyzer based on heated zirconium oxide ceramic cells measured O_2 fluxes, and relative humidity sensors (Vaisala, Helsinki, Finland) measured water vapor fluxes. Mass flow controllers (Tylan, Torrance, California, USA) prepared the various gas mixtures, and a pressure transducer (Validyne, North Ridge, California, USA) monitored the gas flows through the shoot cuvette. The shoots received 350 and 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) for *Arabidopsis* and the other species, respectively, and were maintained at 26°C. The roots of all species were in the dark at 20°C and initially received a modified Hoagland solution (Epstein and Bloom 2005) with NH_4^+ as the sole N source and aerated with ambient air.

The next morning, we increased the PAR at plant height over several hours from darkness to 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for *Arabidopsis* and 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for the other species before initiating the measurements. We followed a standard protocol (Bloom et al. 1980) to assess the response of photosynthetic gas fluxes as a function of internal CO_2 concentrations within a leaf (A-C_i measurements). We exposed each plant to between five and seven different external CO_2 concentrations from sub-ambient to elevated, calculated C_i from H_2O exchange, and fit the net CO_2 and O_2 flux data to a biochemical model of photosynthesis that interpolated the values at regular C_i intervals (von Caemmerer 2000, Sharkey et al. 2007). We thus obtained the response of AQ to C_i under NH_4^+ nutrition. At the end of the day, we shifted wheat, barley, and *Arabidopsis* plants to 0.2 mmol/L KNO_3 as a sole N source, and the next day we conducted the same series of measurements to obtain the response of AQ to C_i under NO_3^- nutrition.

We assessed the NH_4^+ treatment first because plants do not store substantial quantities of NH_4^+ within their tissues, whereas they may store substantial amounts of NO_3^- and assimilation of this stored NO_3^- might interfere with assessment of the NH_4^+ treatment if we assessed the NO_3^- treatment first. In the other species, we assessed the NH_4^+ and NO_3^- treatments on separate plants. The AQ under NH_4^+ nutrition minus the AQ under NO_3^- nutrition equals ΔAQ.

Relative growth rate measurements

Five of the C_3 species (*Arabidopsis*, wheat, loblolly pine, sugar maple, and sweet gum) have served as experimental material in free-air CO_2 enrichment

(FACE) studies (Kimball et al. 2001, Holmes et al. 2006, Li et al. 2006, Högy et al. 2009, Natali et al. 2009). These previous studies, however, have not examined the interaction between NH_4^+ vs. NO_3^- as N sources and CO_2 enrichment. Indeed, such experiments would be difficult because rapid microbial transformations among N forms in soil or other solid medium prevent adequate control of a plant's N source (Epstein and Bloom 2005).

We grew the plants in controlled environmental chambers (Conviron) in 19-L opaque polyethylene tubs that contained aerated nutrient solutions with 0.2 mmol/L KNO_3 or 0.2 mmol/L NH_4^+ (in the form of NH_4Cl for wheat and *Arabidopsis* and $NH_4H_2PO_4$ for the other species) as the sole nitrogen source. The environmental chambers were equipped with non-dispersive infrared analyzers and solenoid valves that controlled chamber atmospheric concentration of CO_2. The first year, we grew *Arabidopsis*, wheat, loblolly pine, sugar maple, and sweet gum in two environmental chambers, one set at ambient (~400 μmol/mol) and the other at elevated (~720 μmol/mol) CO_2.

We grew the loblolly pine plants from saplings in the first year and from seed in the second year. Unfortunately, we were only able to obtain sugar maple saplings from the nursery at one time each year because of its growth habit; in the second year, they were damaged in transit, and so most plants did not survive transplanting to solution culture, irrespective of CO_2 or N treatment. As an alternative for the sugar maple during the second year, we examined a CAM species, jade plant (*Crassula ovata*).

During the second year, we equipped a third environmental chamber with soda lime (a mix of $Ca(OH)_2$, NaOH, and KOH) on the air intake and within the chamber in a 19-L tub equipped with fans to achieve sub-ambient CO_2 concentrations (~310 μmol/mol). Thus, we were able to grow loblolly pine, sweet gum, and jade plant under atmospheric conditions that approximate those of 50 years ago (310 μmol/mol), today (400 μmol/mol), and 50 years from today (720 μmol/mol) (IPCC 2007).

Every 3–7 days, we removed each plant from its tub, very gently blotted dry its roots with lint-free tissues (KimWipe, Kimberly Clark, Irving, Texas, USA), put it in a beaker of nutrient solution on a balance to obtain its fresh mass, and then returned it to its tub. We calculated relative growth rate (RGR), perhaps the most common and ecologically significant index of plant growth (Chiariello et al. 1989), from the change in the natural log of fresh mass of each plant over time.

We used fresh masses because this permitted repeated measurements of individual plants, a necessary compromise given the space limitations of our environmental chambers. Moreover, this approach has the advantage of decreasing sample variance. Such an approach may produce measurement artifacts because removal of plants from solution culture can suppress plant processes (Bloom and Sukrapanna 1990). We therefore took great care to minimize such artifacts by blotting the roots very gently and immediately re-submersing the roots in nutrient solution. Under such treatment, any suppression of plant processes disappeared within an hour or two (Bloom and Sukrapanna 1990). Moreover, we subjected the plants to such measurements only every 3–7 d. A key factor is that all N and CO_2 treatments received the same fresh mass measurements. We deemed it unlikely that the N and CO_2 treatments of plants in hydroponic culture would differentially influence their response to the fresh mass measurements.

In the first year, once the influence of the CO_2 and N treatments on the growth of the tree species became apparent (43 d after transplanting for loblolly pine, 74 d for sugar maple, and 63 d for sweet gum), we removed the differential treatments and subjected all the saplings to an ambient CO_2 concentration and NH_4NO_3 as the N source ("Remove" in Fig. 2). After 3 weeks, we discarded from each treatment the three largest loblolly pine saplings and the two largest sweet gum saplings to reduce interplant shading; one week later, we randomized the remaining smaller plants and restored the differential treatments of ambient vs. elevated CO_2 and NH_4^+ vs. NO_3^- ("Restore" in Fig. 2). Sugar maple tends to develop one set of leaves per growing season; by the time we finished assessing the influence of the removing the differential treatments, the sugar maple leaves were fully expanded and less likely to respond to additional experimental manipulations.

We conducted for *Arabidopsis* and wheat, respectively, 10 replicate experiments of 10 plants each and 5 replicate experiments of 6 plants each. For the tree species, we conducted in the first year an experiment with two CO_2 concentrations and 6–10 plants per treatment per measurement date. In the second year, we monitored the growth of 3–10 plants of each species per treatment per measurement date.

At the end of the relative growth measurements on the jade plants, we assessed the diurnal change in titratable acidity, a measure of CAM fixation (Bloom and Troughton 1979). We harvested leaves of each treatment at the beginning and end of the light period, extracted 1.7 mL from each leaf, diluted it in 10 mL of water, and titrated it to pH 6.8 with a 1 mol/L KOH standard.

Statistics

For the gas exchange experiments, we calculated the means and standard errors of ΔAQ at regular C_i intervals (Appendix A). For the relative growth experiments, we took a running average of RGR over three consecutive measurement dates to smooth the data and performed a three-way analysis of variance using the MIXED procedure with repeated measures in SAS (version 9.1, SAS Institute, Cary, North Carolina, USA). Plant age, CO_2 treatment, and N source were the independent variables. Relative growth rate, the dependent variable, was square-root transformed to meet the assumptions of normality and homogeneity of

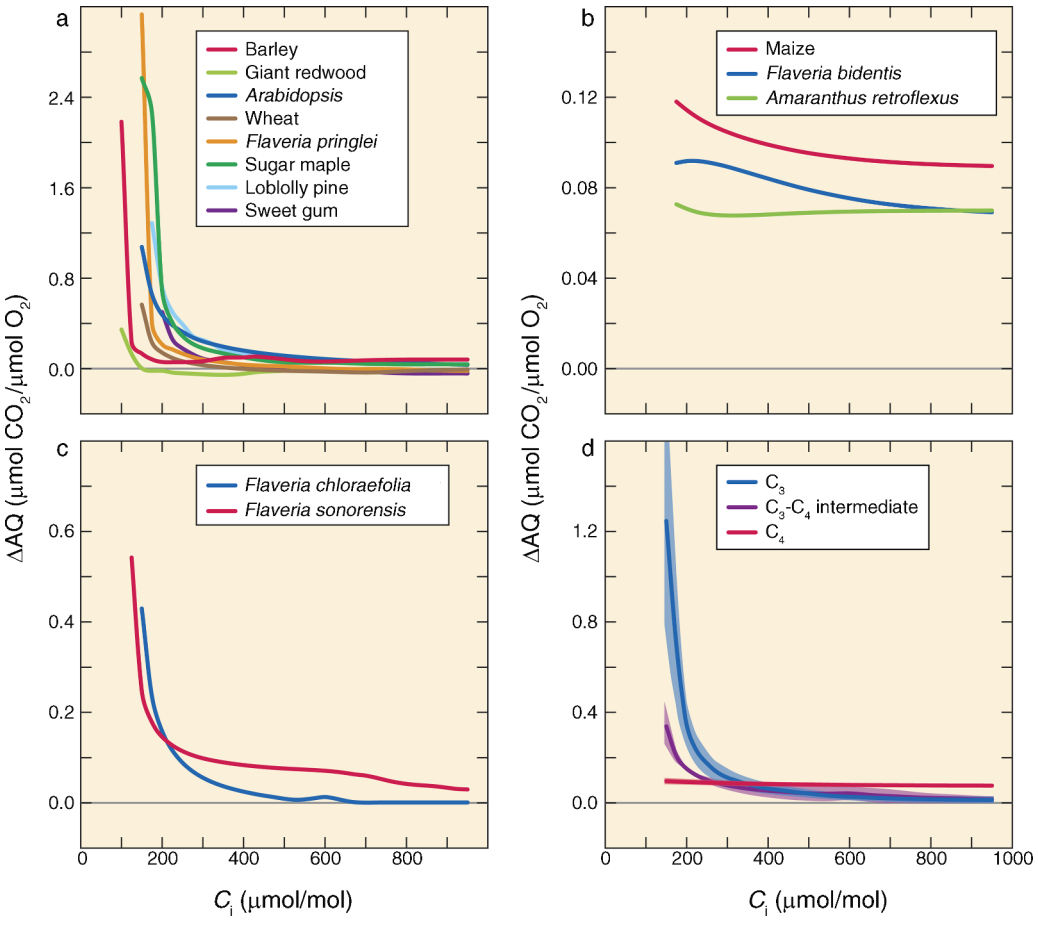

FIG. 1. The ΔAQ, the decrease in the ratio of shoot CO_2 consumption to O_2 evolution with a shift from NH_4^+ to NO_3^- nutrition, as a function of shoot internal CO_2 concentration (C_i) for (a) eight C_3 species, (b) three C_4 species, (c) two C_3-C_4 intermediate species, and (d) the mean ± SE (solid line ± shaded area) of the species conducting a particular carbon fixation pathway. We fit a biochemical model of photosynthesis to 5–7 data points to interpolate values at regular C_i intervals (von Caemmerer 2000, Sharkey et al. 2007). The curve for each individual species is the mean of 4–10 plants. The data for wheat and *Arabidopsis* are from Bloom et al. (2010). Means ± SE for the individual species are presented in Appendix A: Fig. A1.

variance as assessed via the Shapiro-Wilk and Brown-Forsythe tests, respectively, and was repeated over four or more time points for each individual plant. Effects of carbon fixation pathway, CO_2 treatment, N source, or their interactions for a specific age of plant were considered significant when $P < 0.05$. Plotted in Figs. 2 and 3 are the predicted values and standard errors of RGR from the mixed linear model with repeated measures. Appendices B and C provide the output from the statistical analyses of RGR.

RESULTS

The ΔAQ in all eight C_3 species was significantly greater than zero at C_is below ambient atmospheric concentrations (C_i < 250 μmol/mol) and declined exponentially to zero as C_is increased under CO_2 enrichment (Fig. 1a and Appendix A). In contrast, the ΔAQs of the three C_4 species, although they were smaller than the ΔAQs of the C_3 species at C_is below ambient CO_2 concentrations, declined only slightly under CO_2 enrichment and remained significantly greater than zero (Fig. 1b and Appendix A). The two *Flaveria* C_3-C_4 intermediates responded in a manner that was intermediate to the C_3 and C_4 species (Fig. 1c, d).

The annual species *Arabidopsis* and wheat grew about an order of magnitude faster than the three tree species (Fig. 2). *Arabidopsis* and wheat grew faster under NO_3^- nutrition than under NH_4^+ nutrition in both CO_2 treatments, whereas the tree species grew faster under NH_4^+ nutrition in the elevated CO_2 treatment (Fig. 2; Appendix B). Under NH_4^+ nutrition, an elevated atmospheric CO_2 concentration stimulated the growth of *Arabidopsis*, wheat, and loblolly pine and had no significant effect on the growth of sugar maple and sweet gum (Fig. 2; Appendix B).

After several weeks of exposure to NO_3^- nutrition, elevated CO_2 inhibited growth of the tree species, but had no significant effect on *Arabidopsis* and wheat. Removing the differential treatments of the tree species, that is, placing them under ambient CO_2 and feeding

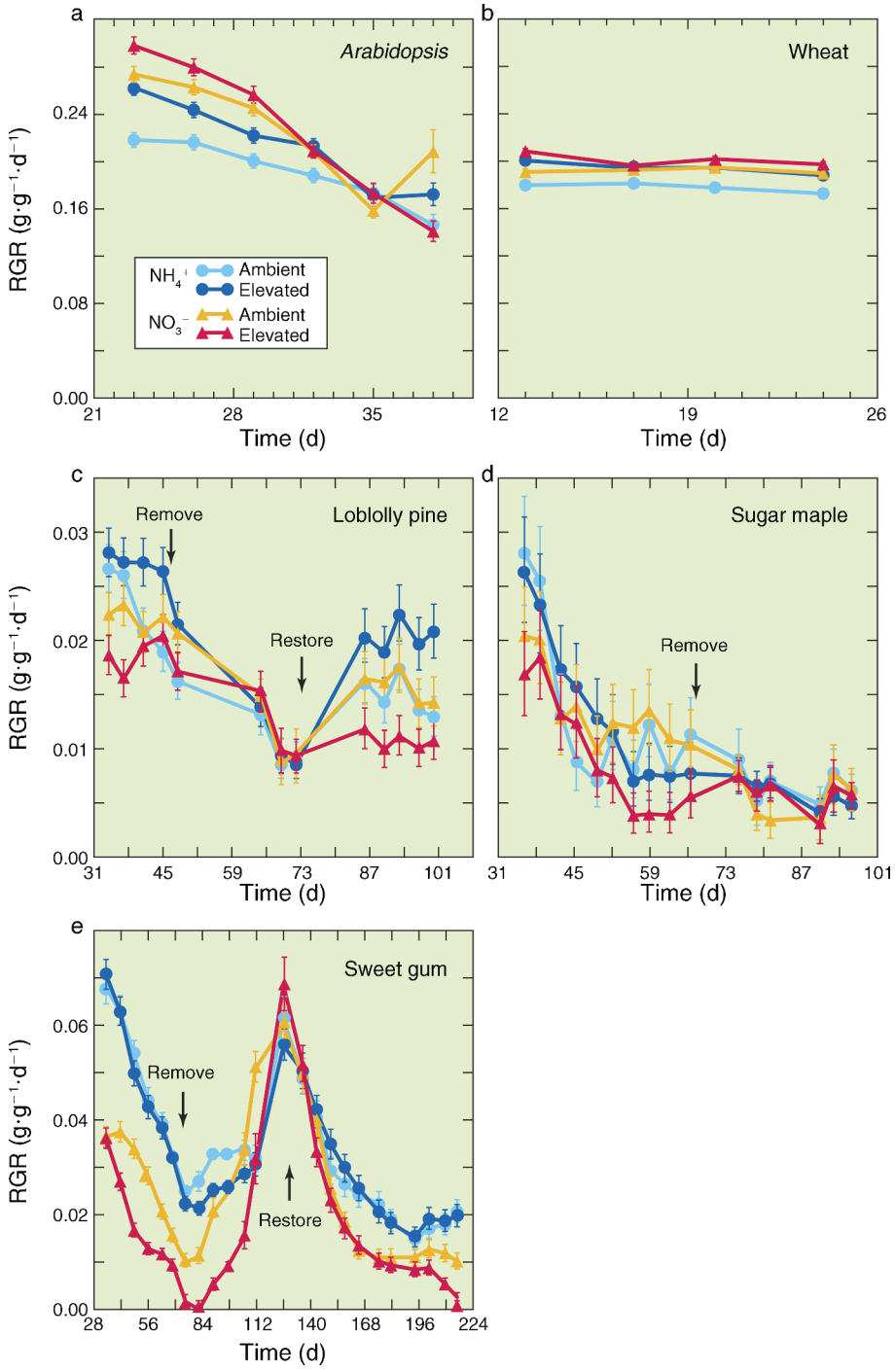

FIG. 2. Relative growth rate of five C_3 species (a) *Arabidopsis thaliana*; (b) wheat, *Triticum aestivum*; (c) loblolly pine, *Pinus taeda*; (d) sugar maple, *Acer saccharum*; and (e) sweet gum, *Liquidambar styraciflua*, in controlled environment chambers under ambient (~400 μmol CO_2/mol all gases present) or elevated (720 μmol/mol) atmospheric CO_2 concentrations and NH_4^+ or NO_3^- nutrition. Time is in days after transplanting to a hydroponic solution. Arrows designate "Remove," when we shifted all tree saplings to ambient CO_2 concentration and NH_4NO_3 nutrition, or "Restore," when we re-imposed the differential CO_2 and N treatments for loblolly pine and sweet gum. Shown are the predicted values and standard errors from mixed linear models with repeated measures on the individual plants. Small error bars are incorporated into the symbols.

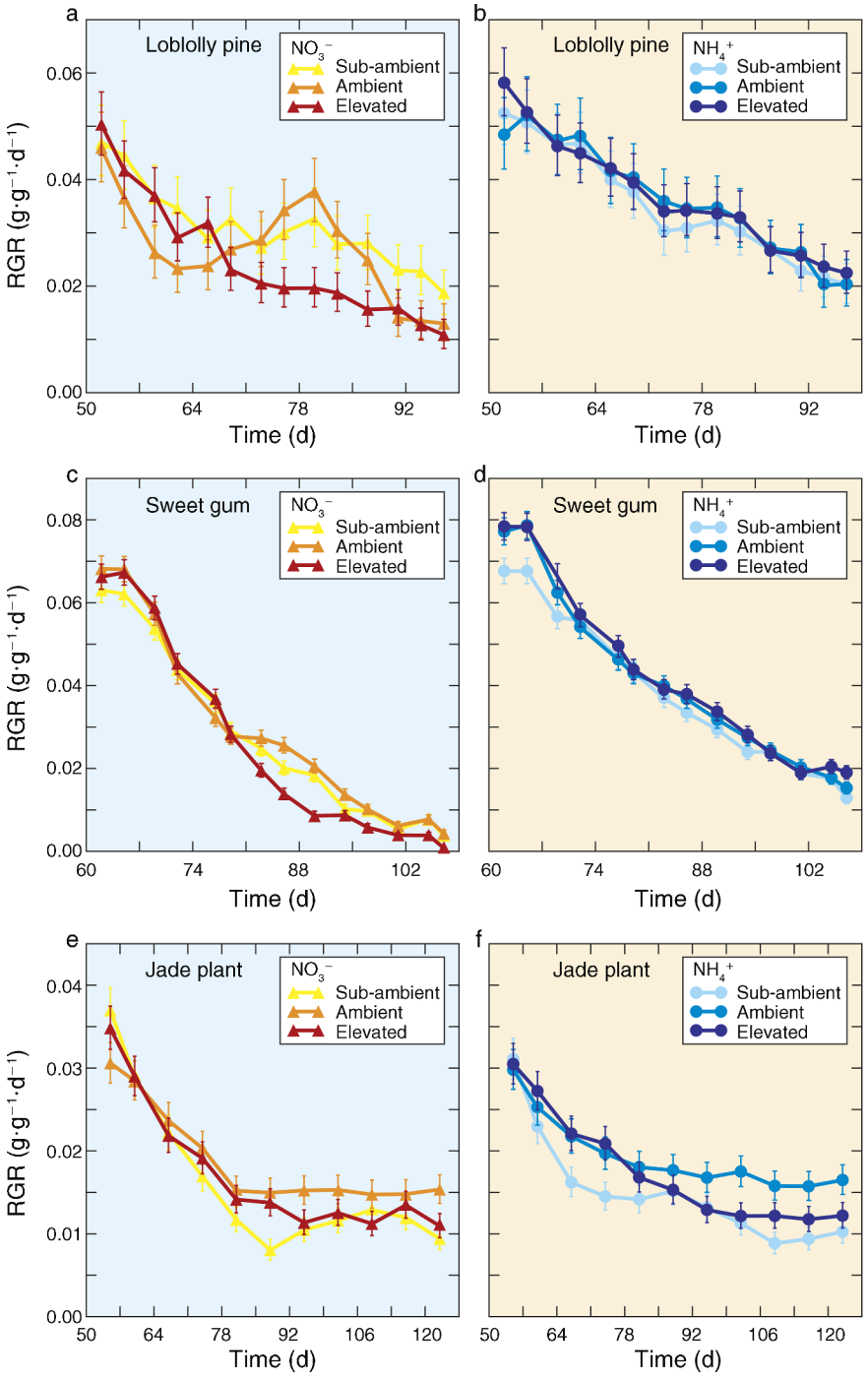

FIG. 3. Relative growth rate of (a, b) loblolly pine, *Pinus taeda*; (c, d) sweet gum, *Liquidambar styraciflua*; and (e, f) jade plant *Crassula ovata* in controlled environment chambers under sub-ambient (~310 μmol/mol), ambient (~400 μmol/mol), or elevated (720 μmol/mol) atmospheric CO_2 concentrations and receiving NO_3^- or NH_4^+ nutrition. Time is in days after transplanting to a hydroponic solution. Shown are the predicted values and standard errors from mixed linear models with repeated measures on the individual plants. Small error bars are incorporated into the symbols.

them NH_4NO_3, eliminated any differences in growth (Fig. 2c, d, e). Restoring the differential CO_2 and N treatments on loblolly and sweet gum saplings for several weeks resulted again in the slower growth of the plants subjected to CO_2 enrichment under NO_3^- nutrition (Fig. 2c, e; Appendix B).

In a separate experiment, loblolly pine, sweet gum, and jade plant (*Crassula ovata*) grew in controlled

environment chambers under sub-ambient (~310 μmol/mol), ambient (~400 μmol/mol), or elevated CO_2 (720 μmol/mol) atmospheric concentrations and received NO_3^- or NH_4^+ nutrition. One to two months after exposure to the differential treatments, the tree saplings that received NO_3^- nutrition grew slowest under the elevated atmospheric CO_2 concentration (Figs. 3a, c and 4; Appendix C). In contrast, growth of the tree saplings that received NH_4^+ nutrition showed no differential response or a slight stimulation of growth under the elevated atmospheric CO_2 concentration (Figs. 3b, d and 4; Appendix C). Growth of the jade plants did not vary significantly with N source and was fastest under the ambient atmospheric CO_2 concentration (Fig. 3e and f; Appendix C). In the jade plant leaves, the change in titratable acidity from dawn to dusk was similar among the two N sources and three CO_2 levels (66.0 ± 3.2 μmol H^+/g fresh mass).

Discussion

The results of these gas flux and growth experiments support the hypothesis that atmospheric CO_2 enrichment interferes with the ability of C_3 species to assimilate NO_3^- into organic N compounds in their shoots and that this impedes their growth. In a diverse collection of C_3 species and C_3-C_4 intermediates, CO_2 enrichment severely decreased photosynthetic O_2 evolution associated with NO_3^- assimilation (Fig. 1a, c). There are obviously alternative mechanisms for NO_3^- assimilation because plants under CO_2 enrichment and NO_3^- nutrition continued to grow, albeit often at a slower pace (Figs. 2 and 3). One such mechanism is root NO_3^- assimilation, which may be enhanced under CO_2 enrichment (Kruse et al. 2003). Unfortunately, relatively little is known about the extent to which the balance between root and shoot NO_3^- assimilation varies within and among species (Epstein and Bloom 2005, Nunes-Nesi et al. 2010). In several species measured at ambient CO_2 concentration, shoots account for the majority of whole-plant NO_3^- assimilation over the entire day (Bloom et al. 1992, Cen and Layzell 2003). This study establishes that CO_2 enrichment inhibits shoot nitrate assimilation in a wide variety of C_3 plants and that this phenomenon influences whole-plant growth; therefore, shoot nitrate assimilation provides an important contribution to the performance of the entire plant.

Several physiological mechanisms may be responsible for the relationship between elevated atmospheric CO_2 concentrations and shoot NO_3^- assimilation (Bloom et al. 2010). One 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 is the biochemical pathway in which the chloroplast enzyme Rubisco catalyzes the oxidation of the high-energy substrate ribulose-1,5-bisphosphate (RuBP) rather than catalyzes the carboxylation of RuBP through the C_3 carbon fixation pathway (Foyer et al. 2009). Photorespiration stimulates the export of malic acid from chloroplasts (Backhausen et al. 1998) and increases the availability of nicotinamide adenine dinucleotide hydride (NADH) in the cytoplasm (Igamberdiev et al. 2001) that powers this first step of NO_3^- assimilation (Robinson 1987, Quesada et al. 2000). CO_2 enrichment decreases photorespiration and thereby decreases the amount of reductant available to power NO_3^- reduction. In contrast, the first carboxylation reaction in the C_4 carbon fixation pathway generates ample amounts of malic acid and NADH in the cytoplasm of mesophyll cells. This may explain why shoot NO_3^- assimilation is relatively independent of CO_2 concentrations in C_4 plants (Fig. 1b).

The dark carboxylation reaction in CAM also generates ample amounts of malic acid that is stored in the vacuoles of leaves. Perhaps this explains the similar responses of jade plant under NO_3^- and NH_4^+ nutrition (Fig. 3e, f). Nonetheless, the relatively few studies on CAM plants usually find that CO_2 enrichment stimulates growth and nocturnal accumulation of titratable acid (Drennan and Nobel 2000). An explanation of our results, the negative effects of elevated or sub-ambient CO_2 on growth and the lack of an effect of the CO_2 or N source treatments on nocturnal accumulation of titratable acid, will require additional study.

Our results also support that relative growth rate of fresh mass was an appropriate measure of plant growth. The relative growth rate for most plants became relatively steady after several weeks of exposure to a particular treatment (Figs. 2 and 3). In the three species having life spans sufficient to allow a shift in conditions (loblolly pine, sugar maple, and sweet gum), removal of the differential CO_2 and N treatments eliminated any differences in the relative growth rates (Fig. 2c, d, e). The sweet gum apparently was experiencing interplant shading because when we discarded the two largest saplings from each treatment, the remaining smaller plants exhibited substantially faster growth (Fig. 2e). In the two species that continually initiated new leaves (loblolly pine and sweet gum), re-imposing the differential CO_2 and N treatments restored the previously observed differences in the relative growth rates (Fig. 2c, e). Treatment differences in the relative growth rate of wheat observed here were consistent with those previously observed in the accumulation of dry mass and leaf area : growth at ambient CO_2 was slightly faster under NO_3^- than NH_4^+ nutrition, and CO_2 enrichment stimulated growth more under NH_4^+ than NO_3^- nutrition (Bloom et al. 2002).

Plant species vary in their dependence on NH_4^+ and NO_3^- as N sources. In this study, some species, such as *Arabidopsis* and jade plant, grew faster under NO_3^- nutrition (Figs. 2a and 3e, f), whereas others such as the tree saplings grew faster under NH_4^+ (Figs. 2c, d, e and 3a–d). The reasons for these differences remain uncertain, but may involve interactions with other mineral nutrients such as potassium and molybdenum (Smart and Bloom 1993). In unfertilized soils, plants tend to

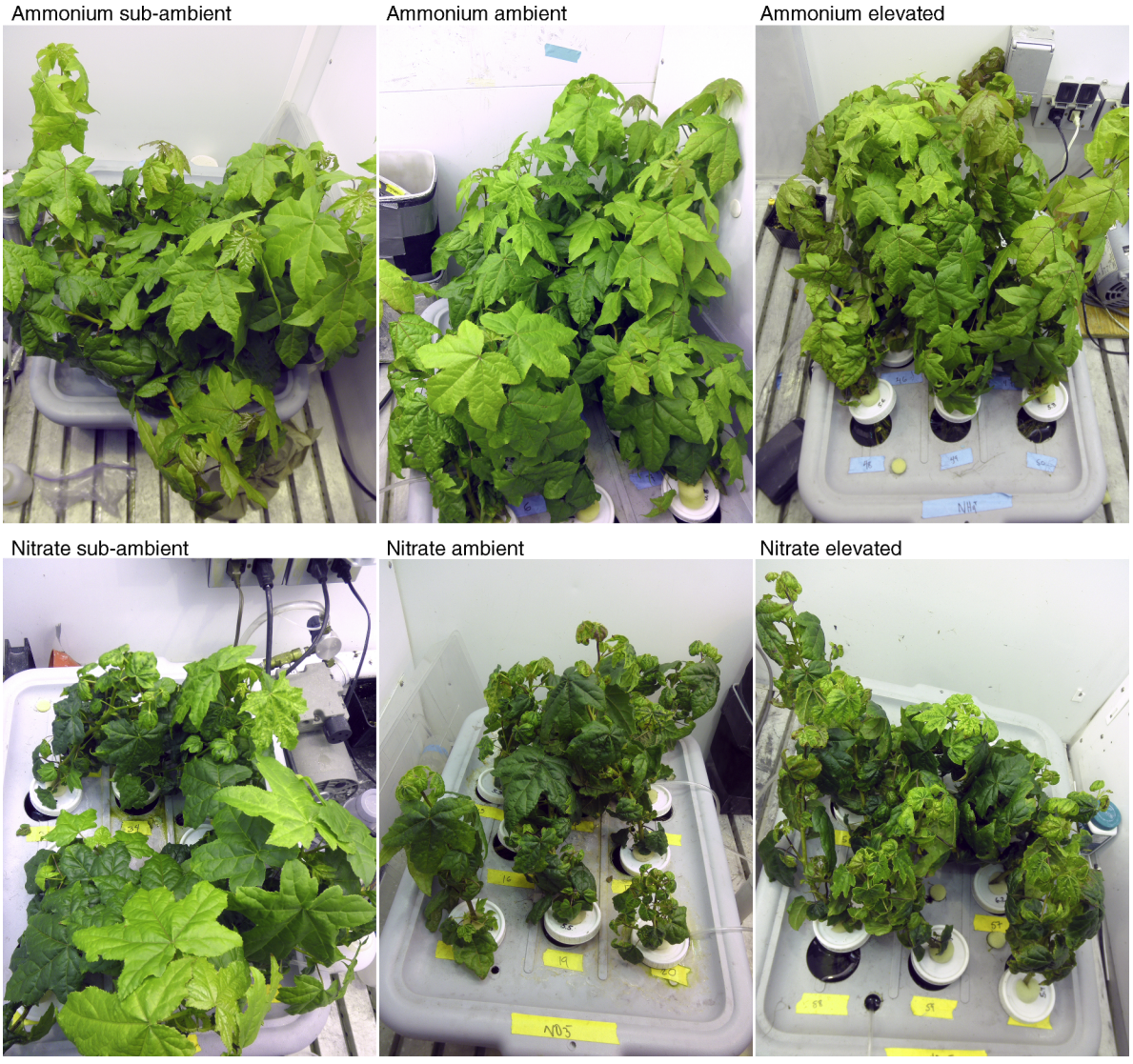

FIG. 4. Sweet gum *Liquidambar styraciflua* in controlled environment chambers under sub-ambient (310 μmol/mol), ambient (~400 μmol/mol), or elevated (720 μmol/mol) atmospheric CO_2 concentrations and receiving NO_3^- or NH_4^+ nutrition.

acquire each N form in relation to its availability in the rhizosphere, which varies with site and season (Epstein and Bloom 2005).

This variation in the relative dependence on NH_4^+ and NO_3^- could explain the observed variation in ecosystem responses to CO_2 enrichment (Bloom et al. 2010, Leuzinger et al. 2011). Net primary productivity diminished under CO_2 enrichment in an annual California grassland for which NO_3^- was the predominant N source (Dukes et al. 2005), presumably because NO_3^- assimilation was inhibited and plant organic N compounds became limiting. In contrast, *Scirpus olneyi*, the dominant C_3 plant in the Chesapeake Bay marsh, an NH_4^+-dominated ecosystem, showed a steady enhancement in photosynthesis and growth under CO_2 enrichment even after a decade of treatment (Rasse et al. 2005). The response of forest ecosystems to CO_2 enrichment has been variable (Korner 2006) and highly sensitive to N fertilization (Oren et al. 2001, Ellsworth et al. 2004). In forest soils, NO_3^- concentrations tend to be low, but high rates of gross nitrification (microbial conversion of NH_4^+ into NO_3^-) indicate a small but ecologically important NO_3^- pool (Stark and Hart 1997).

Sugar maple, a key species in the hardwood forests of northeastern and north-central United States and eastern Canada, is unique among trees in these forests because it grows predominantly in soils that have high rates of NO_3^- production (Lovett and Mitchell 2004). Moreover, the transport systems in sugar maple roots have a higher affinity for NO_3^- than NH_4^+ (Eddy et al. 2008). During the past 50 years, sugar maple has

FIG. 5. Sugar maple *Acer saccharum* in controlled environment chambers after 10 weeks under ambient (~400 μmol/mol) or elevated (720 μmol/mol) atmospheric CO_2 concentrations and receiving NH_4^+ or NO_3^- nutrition.

experienced a population decline characterized by crown dieback and tree mortality throughout its range (Horsley et al. 2008). This decline is usually attributed to acid rain and nutrient imbalances, although the supporting evidence is inconclusive (Minorsky 2003).

Sugar maple also differs from many species in that CO_2 enrichment fails to stimulate its growth under normal soil conditions (Reid and Strain 1994, Kinney and Lindroth 1997, Gaucher et al. 2003, Kubiske et al. 2007, Eller et al. 2011). For example, after seven years of treatment in the FACE experiment at Rhinelander, Wisconsin, USA, sugar maple showed no significant influence of CO_2 treatment on growth, but its mortality tripled under CO_2 enrichment (Kubiske et al. 2007). In the same experiment, aspen and birch grew larger under CO_2 enrichment and the mortality of aspen halved, whereas that of birch did not change.

The results presented here demonstrate that CO_2 enrichment inhibits both NO_3^- assimilation in sugar maple and stunts its growth under NO_3^- nutrition, but has no effect under NH_4^+ nutrition (Figs. 1a, 2d, and 5). This suggests that the change in atmospheric CO_2

concentration from ~320 to 400 μmol/mol experienced over the past 50 years might be a contributing factor to the decline of sugar maple. Indeed, such a change in atmospheric CO_2 concentration corresponds to a change in internal CO_2 concentration from ~180 to 260 μmol/mol, the steepest part of the CO_2 response curve for NO_3^- assimilation in sugar maple (Fig. 1a). If its ability to incorporate NO_3^- were compromised, sugar maple might be at a competitive disadvantage with respect to other tree species that are more reliant on NH_4^+ as a N source. This hypothesis will obviously require much more additional testing.

Loblolly pine (Fig. 3a) and sweet gum (Fig. 4) receiving NO_3^- as a sole N source grew faster under sub-ambient CO_2 than ambient or elevated CO_2 concentrations. This suggests that recent increases in atmospheric CO_2 concentrations might have already compromised the productivity of some forests and other vegetation types and could contribute to the observed higher background (non-catastrophic) mortality rates (van Mantgem et al. 2009).

These results also have implications for the relative distributions of C_3 and C_4 species in the future. Rising CO_2 concentrations in Earth's atmosphere should favor C_3 species on sites where NH_4^+ is the dominant N source and should favor C_4 species on sites where NO_3^- is dominant. Of course, the anticipated changes in temperature and water availability will also influence this balance (Bloom 2010).

C_3 plants under CO_2 enrichment can avoid CO_2 acclimation and sustain enhanced photosynthesis and growth for very long periods of time if they receive N fertilizer applications that far exceed natural mineralization rates and standard agricultural practices (Curtis and Wang 1998, Wand et al. 1999, Ainsworth and Long 2005, de Graaff et al. 2006). These applications of N fertilizer most likely enhance the availability of soil NH_4^+ and thus compensate for lower rates of shoot NO_3^- assimilation. Such fertilizer practices, however, are neither economically nor ecologically viable on a larger scale.

This study highlights a general problem: the difficulty in determining, much less trying to control, the forms and amounts of N available to plants from the rhizosphere. The inorganic forms NH_4^+ and NO_3^- are highly disparate, differing in such fundamental characteristics as charge, oxidation state, toxicity, production rates in the rhizosphere, and mobility through the soil. Here we show that the response of C_3 species to CO_2 enrichment depends on N form, NH_4^+ vs. NO_3^-, but relatively few studies consider this. As atmospheric CO_2 levels continue to rise, the distinction between NH_4^+ and NO_3^- as plant N sources will gain in ecological importance.

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Supplemental Material

Appendix A

A figure showing ΔAQ, the decrease in the ratio of shoot CO_2 consumption to O_2 evolution with a shift from NH_4^+ to NO_3^- nutrition, as a function of shoot internal CO_2 concentration (C_i) for two C_3 grass species, two C_3 herbaceous dicotyledonous species, two C_3 coniferous species, two C_3 dicotyledonous tree species, two C_3-C_4 intermediate species, and three C_4 species (*Ecological Archives* E093-034-A1).

Appendix B

SAS mixed procedure on square-root-transformed relative growth rates of plants exposed to one of two different CO_2 levels (*Ecological Archives* E093-034-A2).

Appendix C

SAS mixed procedure on square-root-transformed relative growth rates of plants exposed to one of three different CO_2 levels (*Ecological Archives* E093-034-A3).