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Nitrogenase activity and N_2 fixation are stimulated by elevated CO_2 in a tropical N_2-fixing tree

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Abstract Seeds of *Gliricidia sepium*, a fast-growing woody legume native to seasonal tropical forests of Central America, were inoculated with N_2-fixing *Rhizobium* bacteria and grown in environmentally controlled glasshouses for 67–71 days under ambient CO_2 (35 Pa) and elevated CO_2 (70 Pa) conditions. Seedlings were watered with an N-free, but otherwise complete, nutrient solution such that bacterial N_2 fixation was the only source of N available to the plant. The primary objective of our study was to quantify the effect of CO_2 enrichment on the kinetics of photosynthate transport to nodules and determine its subsequent effect on N_2 fixation. Photosynthetic rates and carbon storage in leaves were higher in elevated CO_2 plants indicating that more carbon was available for transport to nodules. A $^{14}CO_2$ pulse-chase experiment demonstrated that photosynthetically fixed carbon was supplied by leaves to nodules at a faster rate when plants were grown in elevated CO_2. Greater rates of carbon supply to nodules did not affect nodule mass per plant, but did increase specific nitrogenase activity (SNA) and total nitrogenase activity (TNA) resulting in greater N_2 fixation. In fact, a 23% increase in the rate of carbon supplied to nodules coincided with a 23% increase in SNA for plants grown in elevated CO_2, suggesting a direct correlation between carbon supply and nitrogenase activity. The improvement in plant N status produced much larger plants when grown in elevated CO_2. These results suggest that *Gliricidia*, and possibly other N_2-fixing trees, may show an early and positive growth response to elevated CO_2, even in severely N-deficient soils, due to increased nitrogenase activity.

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Introduction

Current atmospheric partial pressures of CO_2 (35 Pa) have risen dramatically in the past 120 years since the industrial revolution, primarily due to the combustion of fossil fuels and deforestation, and are expected to double by the end of the next century (Keeling et al. 1989). Plants maintained at elevated CO_2 often exhibit enhanced growth and photosynthesis, especially when other environmental resources such as light, water and nutrients are not limiting (Bazzaz 1990; Ceulemans and Mousseau 1994; Gunderson and Wullschleger 1994). However, most terrestrial ecosystems are nitrogen limited (Vitousek and Howarth 1991) and may not respond to elevated CO_2 with increased plant growth unless there is a concomitant increase in nitrogen availability (Kramer 1981). Biological N_2-fixation currently accounts for 60% of the "new" nitrogen deposited on land annually (Schlesinger 1991) and symbiotic N_2-fixing trees may be expected to improve the fertility of N-deficient soils in a high CO_2 environment by gradually increasing soil N content (Boring et al. 1988; Chapin et al. 1994). Thus, N_2-fixing trees may stimulate photosynthesis and growth under elevated CO_2 conditions, inducing positive feedback on rates of carbon sequestration in forests.

The acquisition of carbon and nitrogen are tightly linked in N_2-fixing plants. For example, nitrogen is a primary component of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme that catalyzes photosynthetic reduction of CO_2 to carbohydrate, while photosynthesis supplies organic carbon to nodules, where it is used in the nitrogenase enzyme system as a source of energy and reducing power to fix N_2. Because of this coupling, nitrogenase activity in plants is regulated by aspects of both carbon and N processes (Vance and Heichel 1991; Hunt and Layzell 1993; Hartwig and Nosberger 1994), including photosynthesis (rate of carbon

supply), nitrogen availability (N source strength), and nitrogen demand (N sink strength). It has been argued that the demand for symbiotically fixed N directly governs nitrogenase activity (Hartwig and Nosberger 1994) and the supply rate of carbon determines the degree to which the energy requirements of N_2-fixation are met (Vance and Heichel 1991; Hunt and Layzell 1993). In particular, it is the supply of current photosynthate rather than stored carbon that is most important, since stored carbohydrates are frequently in excess and do not generally affect rates of nitrogenase activity (Hartwig et al. 1990). Although elevated CO_2 has been shown to stimulate symbiotic N_2-fixation in agricultural crops and herbs (Hardy and Havelka 1976; Phillips et al. 1976; Masterson and Sherwood 1978; Williams et al. 1981; Finn and Brun 1982; Allen et al. 1988; Ryle et al. 1992), it is not clear to what extent increased N_2-fixation is accompanied by increased carbon consumption. Previous studies of the effects of elevated CO_2 on N_2-fixation in trees have quantified nodule activity based on the rate that molecular N_2 is converted to organic N, and assumed that higher rates of N_2-fixation were due to higher rates of photosynthate transport to nodules (Norby 1987; Arnone and Gordon 1990; Thomas et al. 1991).

Gliricidia sepium (Jacq.) Walp. is a fast-growing woody legume native to seasonal tropical forests of Central America that forms nodules in association with *Rhizobium* bacteria. In a previous study, *Gliricidia* grown in elevated CO_2, and supplied with exogenous N, increased the percentage of plant N provided by N_2 fixation by increasing nodule mass and specific nitrogenase activity (Thomas et al. 1991); an increase in photosynthate transport to nodules was assumed, but not demonstrated. The primary objective of our study was to quantify the effect of CO_2 enrichment on the kinetics of photosynthate transport to nodules and determine its subsequent effect on N_2 fixation. Plants were grown without soil N to maximize the demand for symbiotically fixed N. We used a $^{14}CO_2$ pulse-chase method to quantify the kinetics of carbon translocation from leaves to nodules.

Materials and methods

Plant material and growth conditions

Seeds of *G. sepium* were obtained from a single seed source in the municipality of Santa Cruz Guanacaste, Costa Rica from the Latin American Seed Bank of the Centro Agronomico Tropical de Investigacion y Ensenanza (CATIE), Turrialba, Costa Rica. Seedlings were grown in 3.3-l pots in disinfected coarse sand. At the time of planting, all seeds were inoculated with *Rhizobium* by mixing seeds with a concentrated sucrose solution containing three strains of *Rhizobium* (NifTAL, Paia, Hawaii, USA). Nodulation was evident in all plants harvested at 67–71 days. All pots were watered to saturation with a N-free nutrient solution each morning and with distilled water each afternoon. The nutrient solution was adjusted to pH 6.1 and contained 1.0 mmol l^{-1} P, 3.0 mmol l^{-1} K, 3.5 mmol l^{-1} Ca, 1.5 mmol l^{-1} Mg, 3.0 mmol l^{-1} S, 0.14 mmol l^{-1} Fe, 0.05 mmol l^{-1} B, 0.01 mmol l^{-1} Mn, 0.001 mmol l^{-1} Zn, 0.001 mmol l^{-1} Cu, 0.05 μmol l^{-1} Mo and 0.16 μmol l^{-1} Co.

Plants were germinated and grown in two glasshouses in the Duke University Phytotron. Chamber CO_2 partial pressures were automatically monitored and controlled (Hellmers and Giles 1979) at 35 Pa or 70 Pa. Plants were exposed to natural light intensity and photoperiod in the glasshouses during the experimental period of June through August; photosynthetic photon flux density (PPFD) at mid-day was usually > 1500 μmol m^{-2} s^{-1}. Temperature was controlled in the glasshouses so that average day/night air temperatures were 30/24°C. Relative humidity was approximately 70% during the day and nearly 100% at night. Treatments were rotated every week between glasshouses to minimize possible glasshouse effects. Due to restrictions prohibiting the use of radioisotopes in the glasshouses, plants were moved from glasshouses to growth chambers in the Duke University Phytotron 3 days prior to labeling. Environmental conditions in the growth chambers were controlled to closely match those in the glasshouses: approximately 70/100% relative humidity (day/night), 30/24°C air temperature (day/night), 14/10 h photoperiod and thermoperiod, and PPFD of approximately 1000 μmol m^{-2} s^{-1} supplied by a combination of high pressure sodium vapor and metal halide high intensity discharge lamps. Glasshouses and growth chambers were closely monitored daily, and therefore environmental conditions were assumed to be similar between CO_2 treatments except for differences in atmospheric CO_2 partial pressures.

Gas exchange

One day prior to labeling plants with $^{14}CO_2$, gas exchange was measured on leaves of 15 plants per CO_2 treatment in the growth chambers using a LICOR 6200 portable photosynthesis system (LICOR Inc., Lincoln, Neb.). Net photosynthesis and night-time dark respiration rates of leaves were measured at the growth CO_2 partial pressure under ambient environmental conditions in the growth chambers.

Carbon supply to nodules

A carbon radiotracer was used to quantify the effect of growth CO_2 concentration on the kinetics of photosynthetically fixed carbon supplied to nodules from leaves. $^{14}CO_2$ was generated as described by Tissue and Nobel (1990) and was stored in a gas cylinder at 35 Pa CO_2. Plants were removed from the growth chambers and labeled in a flow-through hood under environmental conditions similar to those in the growth chambers. Attached leaves (approximately 12 cm^2 total leaf area) were placed into a clear acrylic cuvette and exposed to $^{14}CO_2$ at 35 Pa CO_2 for 5 min. Three plants from each CO_2 treatment were harvested immediately after labeling and analyzed for ^{14}C; these data were used to normalize initial ^{14}C activities to 500 kBq for plants in both CO_2 treatments. Exposure to $^{14}CO_2$ was terminated by flushing the cuvette with $^{12}CO_2$ for 2 min and then removing the leaves from the cuvette. Plants were returned to the growth chambers immediately after labeling.

Plants were harvested and nodules collected at five time intervals: 0, 12, 24, 48 and 96 h after labeling. Nodules were dried at 70°C for 3 days, weighed and then ground to a powder in a Wiley mill. Duplicate 5–10 mg samples of nodules from each plant were solubilized for 2 days at 45°C with a tissue solubilizer (PROTOSOL, New England Nuclear) before addition of the scintillation cocktail (BioSafe II, Research Products International, Mount Prospect, Ill.). Samples were counted in a Beckman LS 6000 scintillation counter (Beckman Instruments, Irvine, Calif.). Carbon supply to nodules was calculated as ^{14}C specific activity (amount of ^{14}C per unit dry weight of nodule). The initial rate at which ^{14}C was supplied to nodules, termed "filling", was calculated as the slope of the best fit linear regression of points between 0 h and 24 h (peak ^{14}C specific activity). The rate at which ^{14}C decreased in nodules, termed "emptying", was calculated as the slope of the best fit linear regression of points between 24 h and 96 h. Total residence time of ^{14}C in the nodules was estimated by extrapolating the "emptying" linear regression to its *x*-intercept.

Biomass and nitrogen fixation

Fifteen plants in each CO_2 treatment were harvested at 67–71 days after planting and the biomass of each plant part (leaf, stem, root and nodule) and the proportion of total biomass in each plant part was determined. Plant parts were dried at 70°C for at least 3 days before measuring their mass. Leaf surface area was determined using a LICOR 3100 leaf area meter (LICOR Inc., Lincoln, Neb.) and specific leaf mass (SLM; leaf mass per unit leaf area) was calculated and used as an indirect measure of starch accumulation.

Tissue nitrogen concentration was determined after digestion using a microKjeldahl technique and analyzed colorimetrically (Lowther 1980) using a Technicon TRAACS-800 autoanalyzer (Bran and Luebbe Inc., Elmsford, N.Y.). The average specific nitrogenase activity (SNA) was calculated as the total amount of N in the plant divided by the average mass of all of the nodules on that plant divided by the age of the plant when it was harvested [mg N (g DW nodule)$^{-1}$ day^{-1}]; the average mass of nodules was determined by dividing final nodule mass by two and assuming that nodule mass increased linearly during the experiment. Total nitrogenase activity (TNA) was determined to be equal to total plant N content because bacterial N_2 fixation was the only source of N available to the plant.

Statistical analyses

A one-way analysis of variance (ANOVA) was used to test for main effects of CO_2 treatment on gas exchange, biomass, leaf characteristics and nitrogen. A Scheffé post hoc multiple comparison test was used to determine whether means of the dependent variable were significantly different at the 0.05 probability level. An analysis of covariance (ANCOVA) was used to determine if slopes of lines representing "filling" and "emptying" of radioactive carbon in nodules were significantly different due to CO_2 treatment; significant differences were indicated by significant interactions with the covariate (time; Data Desk 4.0, Data Description Inc., Ithaca, N.Y.).

Results

Leaf photosynthetic rates were 49% higher in plants grown and measured at elevated CO_2 (P = 0.007) compared with plants grown and measured at ambient CO_2 (Table 1). However, there was no change in night-time dark respiration rates (P = 0.601; Table 1), indicating that total leaf carbon uptake was increased for plants grown at elevated CO_2. Growth in elevated CO_2 increased total leaf area by 36% (P = 0.045) and SLM by 40% (P = 0.001), indicating that approximately 90% more carbon was stored in leaves of 70 Pa CO_2 plants compared with 35 Pa CO_2 plants (Table 1). Total plant biomass increased 84% for plants grown in elevated CO_2 (P = 0.008), with significant increases observed in the biomass of leaves, stems and roots (Fig. 1). Because nodule biomass was unchanged by growth in elevated CO_2, nodules represented a smaller percentage of total plant biomass for plants grown at 70 Pa CO_2 compared with 35 Pa CO_2. Biomass partitioning to stems was also reduced at elevated CO_2 (Fig. 1).

Table 1 Leaf net photosynthesis and night-time dark respiration rates were measured under growth conditions after 66 days of CO_2 treatment. Total leaf area and specific leaf mass (SLM) were measured on plants harvested after 67–71 days growth in ambient (35 Pa) or elevated (70 Pa) CO_2. Values are means (± SE) for 15 plants per treatment. An *asterisk* within a row indicates a statistical difference at P<0.05

Measurement	Ambient CO_2	Elevated CO_2
Photosynthesis (μmol m^{-2} s^{-1})	7.6 (0.7)	11.3 (1.1) *
Respiration (μmol m^{-2} s^{-1})	–0.88 (0.06)	–0.84 (0.05)
Total leaf area (cm^2)	278 (27)	378 (52) *
Specific leaf mass (g m^{-2})	48.5 (1.3)	67.8 (2.7) *

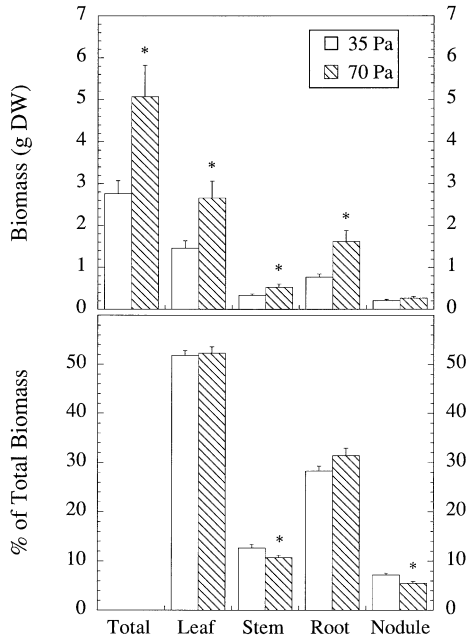

Fig. 1 Dry weight biomass of the whole plant and different plant parts (leaf, stem, root and nodule) and biomass partitioning between plant parts after 67–71 days growth in ambient or elevated CO_2. Values are means (+ SE) for 15 plants per treatment. An *asterisk* within a plant part indicates a statistical difference at $P < 0.05$

The ^{14}C specific activity of nodules [Bq (g DW nodule)$^{-1}$] was significantly higher in plants grown at 70 Pa CO_2 compared with 35 Pa CO_2 at 12 h (86% higher), 24 h (61% higher) and 48 h (113% higher) after leaves were labeled with $^{14}CO_2$ (Fig. 2). After 96 h, ^{14}C specific activities were not different between CO_2 treatments. The apparent peak of ^{14}C specific activity occurred at 24 h in both CO_2 treatments. There was a significant increase in the rate of "filling" of nodules with ^{14}C for plants grown at elevated CO_2 (P = 0.016), with plants at 70 Pa CO_2 exhibiting 61% higher rates than those at 35 Pa CO_2. "Filling" of nodules with ^{14}C is described by the following linear regressions: ambient CO_2, $y = 14.74x–4.92$, $r^2 = 0.854$ and elevated CO_2, $y = 23.79x + 5.26$, $r^2 = 0.934$. There was also a significant difference in the rate of "emptying" of nodules due to CO_2 treatment (P = 0.027), with plants at elevated CO_2 exhibiting 148% higher rates than those at ambient CO_2 (Fig. 2). "Emptying" of nodules is described by the following linear regressions: ambient CO_2, $y = –2.37x + 375.18$, $r^2 = 0.444$ and elevated CO_2, $y = –5.88x + 717.15$, $r^2 = 0.828$). Total residence time of

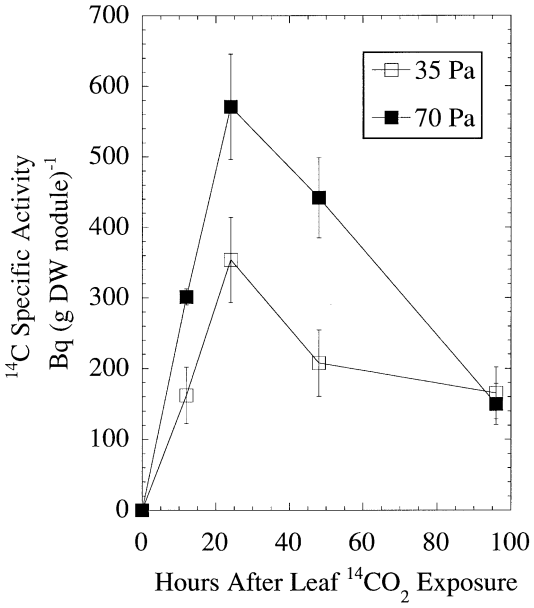

Fig. 2 ^{14}C specific activity of nodules collected at 0, 12, 24, 48 and 96 h after leaves were labeled with $^{14}CO_2$. Leaves were labeled after 67 days of growth in ambient (35 Pa) or elevated (70 Pa) CO_2. Values are means (± SEs) for three plants per treatment and time period

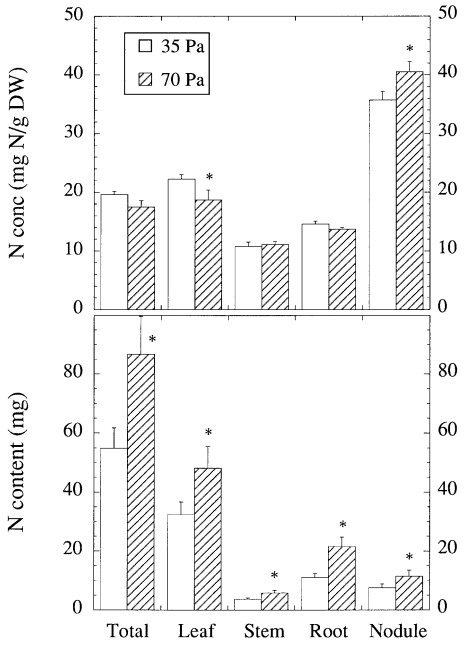

Fig. 3 Nitrogen concentration and nitrogen content of the whole plant and different plant parts (leaf, stem, root and nodule) after 67–71 days of growth in ambient or elevated CO_2. Values are means (+ SEs) for 15 plants per treatment. An *asterisk* within a plant part indicates a statistical difference at $P < 0.05$

^{14}C in nodules was estimated to be 158 h in ambient CO_2 plants and 122 h in elevated CO_2 plants, indicating that photosynthetically fixed carbon supplied by leaves to nodules was consumed 23% more rapidly in plants grown in elevated CO_2 compared with plants grown in ambient CO_2.

Total plant N concentration did not change with elevated CO_2, but there was a decrease of 16% in leaf N concentration ($P = 0.048$), apparently due to dilution of N caused by increased SLM, and an increase of 13% in nodule N concentration ($P = 0.049$) at 70 Pa CO_2 compared with 35 Pa CO_2 (Fig. 3). However, due to increased total plant biomass, total plant N content increased 58% in plants grown in elevated CO_2 ($P = 0.037$) with individual plant parts exhibiting 48–94% increases in N content (Fig. 3). Average SNA [mg N (g nodule)$^{-1}$ day^{-1}] was 8.70 + 0.40 (mean ± SE, $n = 15$) in 70 Pa CO_2 plants and 7.08 ± 0.50 (mean ± SE, $n = 15$) in 35 Pa CO_2 plants, a significant increase of 23% ($P = 0.005$) that indicated a stimulation in the nitrogenase enzyme system in plants grown in enriched atmospheric CO_2.

Discussion

In biological N_2 fixation, the host plant supplies carbohydrate to the microsymbiont to support the N_2 fixation reaction in root nodules and the microsymbiont supplies organic nitrogen compounds to the host plant. Factors that stimulate photosynthate production in the host plant generally enhance N_2 fixation, if the demand for symbiotically fixed nitrogen is high, because the supply rate of photosynthate to the nodule is one of the primary factors limiting N_2 fixation (Vance and Heichel 1991; Hunt and Layzell 1993). A doubling of atmospheric CO_2 increased leaf carbon assimilation in *Gliricidia* by stimulating leaf photosynthesis, as has been observed in other N_2-fixing species (Arnone and Gordon 1990; Ryle et al. 1992; Vogel and Curtis 1995). Concomitant increases in total leaf area and SLM indicated that approximately 90% more carbon was stored in leaves of plants grown at 70 Pa CO_2 compared with 35 Pa CO_2. Clearly, exposure to elevated CO_2 stimulated photosynthate production and increased the amount of carbon available for export to root nodules.

Although it has often been presumed that elevated CO_2 increased carbon supply to nodules (Wilson et al. 1933; Hardy and Havelka 1976; Phillips et al. 1976; Norby 1987; Arnone and Gordon 1990; Ryle et al. 1992; Vogel and Curtis 1995), this study was the first to directly demonstrate that the rate of carbon supplied to nodules was increased by plant growth in enriched atmospheric CO_2. The increased rate of photosynthetically fixed carbon supplied to nodules in *Gliricidia* supports the hypothesis that growth in elevated CO_2 will produce greater rates of carbon assimilation and, therefore, greater rates of carbon export to active carbon sinks, such as nodules. More rapid consumption of labeled carbon in nodules of elevated CO_2 plants indicated that nodules were a greater sink for carbon when grown under elevated CO_2 conditions.

Carbon was supplied at a greater rate to nodules of *Gliricidia* grown in elevated CO_2, but this did not affect nodule mass per plant, in contrast to the more frequent

observation of increased nodule mass in N_2-fixing trees exposed to elevated CO_2 (Norby 1987; Arnone and Gordon 1990). In a previous study of *Gliricidia*, it was shown that nodule mass did not increase with exposure to elevated CO_2 unless exogenous N was added to the soil (Thomas et al. 1991), suggesting that nodule development is reduced under initial, severe N deficiency. Despite no change in nodule mass, increased carbon supply to nodules of *Gliricidia* did stimulate TNA and resulted in greater total N_2 fixation. Because all N accreted in the plant was supplied by N_2 fixation, and final nodule biomass did not change, the significant increase in total plant N content indicated a substantial increase in SNA in plants grown at elevated CO_2. SNA increased 23% in plants grown at elevated CO_2, compared with plants grown at ambient CO_2, indicating that the increase in TNA was most likely due to increased SNA. Alternatively, it is possible that nodule mass increased more rapidly in elevated CO_2 plants than in ambient CO_2 plants, despite similar nodule mass at the final harvest. In that case, greater average nodule mass during the experiment could have increased total plant N accretion in elevated CO_2 without an increase in SNA. However, CO_2-mediated differences in nodule mass over time are unlikely given that other plant parts (leaves and roots) of *Gliricidia* showed similar responses to CO_2 after 31 days and 71 days (Thomas et al. 1991).

Studies with an actinorhizal N_2-fixing tree, *Alnus glutinosa*, have shown that elevated CO_2 increased TNA, but that the effect of elevated CO_2 on SNA has been variable, with effects either positive (Arnone and Gordon 1990; Vogel and Curtis 1995) or non-existent (Norby 1987). Regardless of the mechanism, higher TNA in an elevated CO_2 atmosphere should increase nitrogen availability in ecosystems where N_2-fixing plants are present. For example, if we apply our observed increase in nitrogenase activity of 23% to an estimate of global symbiotic N_2-fixation on land (96 Tg year^{-1}; Schlesinger 1991), a doubling of the CO_2 partial pressure from present-day levels would increase nitrogen inputs by 22 Tg year^{-1} or 1.5 kg ha^{-1} year^{-1}. Such a perturbation is equivalent to 25% of the present-day increase in N_2 fixation caused by human activities such as fertilizer manufacturing (Schlesinger 1991), and is likely to promote an increase in the net primary production of ecosystems exposed to future levels of atmospheric CO_2.

In conclusion, we demonstrated that growth in elevated CO_2 directly increased the supply of photosynthetically fixed carbon from leaves to nodules. Increased carbon supply to nodules in plants grown in elevated CO_2 stimulated SNA and TNA. Indeed, a 23% faster rate of carbon supplied to nodules coincided with a 23% increase in SNA for plants in elevated CO_2, suggesting a direct correlation between carbon supply and nitrogenase activity. The stimulation of SNA and TNA increased whole plant N accretion. The improvement in plant N status produced much larger plants when grown in enriched CO_2 atmospheres even without exogenous soil N. These results suggest that *Gliricidia*, and possibly other N_2-fixing trees, may show an early and positive growth response to elevated CO_2, even in severely N-deficient soils, due to increased nitrogenase activity.

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