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Nutrient acquisition strategies augment growth in tropical N₂-fixing trees in nutrient-poor soil and under elevated CO₂

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Abstract. Tropical forests play a dominant role in the global carbon (C) cycle, and models predict increases in tropical net primary productivity (NPP) and C storage in response to rising atmospheric carbon dioxide (CO₂) concentrations. The extent to which increasing CO₂ will enhance NPP depends in part on the availability of nitrogen (N) and phosphorus (P) to support growth. Some tropical trees can potentially overcome nutrient limitation by acquiring N via symbiotic dinitrogen (N₂) fixation, which may provide a benefit in acquiring P via investment in N-rich phosphatase enzymes or arbuscular mycorrhizal (AM) fungi. We conducted a seedling experiment to investigate the effects of elevated CO₂ and soil nutrient availability on the growth of two N₂-fixing and two non-N₂-fixing tropical tree species. We hypothesized that under elevated CO₂ and at low nutrient availability (i.e., low N and P), N₂ fixers would have higher growth rates than non-N₂ fixers because N₂ fixers have a greater capacity to acquire both N and P. We also hypothesized that differences in growth rates between N₂ fixers and non-N₂ fixers would decline as nutrient availability increases because N₂ fixers no longer have an advantage in nutrient acquisition. We found that the N₂ fixers had higher growth rates than the non-N₂ fixers under elevated CO₂ and at low nutrient availability, and that the difference in growth rates between the N₂ and non-N₂ fixers declined as nutrient availability increased, irrespective of CO₂. Overall, N₂ fixation, root phosphatase activity, and AM colonization decreased with increasing nutrient availability, and increased under elevated CO₂ at low nutrient availability. Further, AM colonization was positively related to the growth of the non-N₂ fixers, whereas both N₂ fixation and root phosphatase activity were positively related to the growth of the N₂ fixers. Though our results indicate all four tree species have the capacity to up- or down-regulate nutrient acquisition to meet their stoichiometric demands, the greater capacity for the N₂ fixers to acquire both N and P may enable them to overcome nutritional constraints to NPP under elevated CO₂, with implications for the response of tropical forests to future environmental change.

Key words: arbuscular mycorrhizal fungi; elevated CO₂; nitrogen fixation; nutrient acquisition strategies; phosphatase enzymes; tropical trees.

INTRODUCTION

Tropical forests are a critical component of the global carbon (C) cycle. They contain ~25% of the world's terrestrial C in their biomass and soil (Jobbagy and Jackson 2000, Schlesinger and Bernhardt 2013), exchange more carbon dioxide (CO₂) with the atmosphere than any other biome (Foley et al. 2005), and account for at least one-third of terrestrial net primary productivity (NPP; e.g., Roy et al. 2001, Beer et al. 2010, Saatchi et al.

2011). In addition, many Earth system models predict substantial increases in terrestrial NPP in response to increasing atmospheric CO₂ concentrations (Todd-Brown et al. 2014, Wieder et al. 2015). Given the large influence of tropical forests on the global C cycle, increases in tropical NPP could translate into a larger terrestrial C sink, thus enhancing a critical climate benefit provided by tropical forests (Ahlström et al. 2012, Todd-Brown et al. 2014).

Despite model predictions, the extent to which increasing CO₂ will enhance terrestrial NPP is likely to depend on the availability of two key nutrients: nitrogen (N) and phosphorus (P) (Hungate et al. 2003, de Graaff et al. 2006, Cernusak et al. 2013). Though N and P are both essential to all forms of life, multiple lines of evidence suggest tropical NPP may already be limited by

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both N and P, as well as other critical micronutrients like potassium (K). In a fertilization experiment in a lowland tropical forest in Panama, Wright et al. (2011) reported increased wood production with N and K addition and increased litter production with P addition. Wurzburger and Wright (2015) also reported a stand level shift toward the production of fine-roots that are less dense and more nutrient rich with N, P, and K addition. In a similar experiment in Costa Rica, Alvarez-Clare et al. (2013) reported that only small trees with a diameter of 5–10 cm increased in stem growth with P addition. Underscoring the potential importance of nutrients and heterogeneous nutrient limitation, Wieder et al. (2015) demonstrated that current rates of new inputs of N and P may be insufficient to meet the demands of enhanced productivity with elevated CO₂, and highlight the potential for P to ultimately limit terrestrial NPP and C storage not only in the tropics, but globally.

Nevertheless, evidence suggests that some tropical forest tree species have the capacity to meet the nutritional demands of increasing NPP predicted with higher atmospheric CO₂. For example, some members of the Fabaceae family, common canopy trees throughout the Neotropics and African tropics (Gentry 1988, Losos and Leigh 2004, ter Steege et al. 2006), form a symbiotic relationship with bacteria that are capable of acquiring N from the atmosphere in exchange for C (Sprent 2009). This C costly process, known as symbiotic dinitrogen (N₂) fixation, may be promoted under elevated CO₂ as the photosynthetic capacity of trees increase, alleviating N limitation to tree growth (Hungate et al. 1999, Cernusak et al. 2011). Moreover, the ability to fix atmospheric N₂ may also improve P acquisition by enhancing the production of N-rich phosphatase enzymes to hydrolyze organic phosphates (Houlton et al. 2008), or by allowing greater investment of C in arbuscular mycorrhizal (AM) fungi to scavenge greater soil area for inorganic P (Nasto et al. 2014). Together, these two mechanisms may confer a competitive advantage to N₂-fixing trees over non-N₂-fixing trees (Nasto et al. 2017), and elicit greater growth responses to elevated CO₂ in nutrient-poor soils.

Though no study has explicitly addressed the N and P acquisition strategies of N₂ and non-N₂ fixers across a gradient of soil nutrient availability and under elevated CO₂, some previous work highlights the potential for N₂ fixers to regulate nutrient acquisition to maintain high growth responses to elevated CO₂ regardless of the availability of soil nutrients. For example, Cernusak et al. (2011) showed that on N-poor soils, the growth of N₂-fixing tropical tree seedlings responded more strongly than non-N₂ fixers to elevated CO₂, and the growth of the N₂-fixing seedlings was closely related to their mass of nodules, the location of symbiotic N₂ fixation. However, when soil N availability was high, the N₂-fixing and non-N₂-fixing seedlings had similar growth responses to elevated CO₂. The large nodule biomass of the N₂-fixing seedlings at low N availability suggests that they fixed N₂ at relatively high rates, allowing a greater growth

response to elevated CO₂. By contrast, when soil N was plentiful, they likely down-regulated N₂ fixation, as indicated by small nodule biomass, while the non-N₂-fixing seedlings now had equal access to N, reducing their advantage over the non-N₂-fixing seedlings (Thomas et al. 1991, Tissue et al. 1996). In addition, previous research has shown that root phosphatase activity and AM colonization tend to decrease with increasing soil P (Treseder 2004; Marklein and Houlton 2011), suggesting that N₂ fixers would similarly maintain high investment in both P acquisition strategies at low soil P to elicit a greater growth response to elevated CO₂, but down-regulate them at high soil P. Thus, N₂ fixers, through a greater investment in N and P acquisition, may have the capacity to offset nutrient limitation of tropical NPP predicted by some global models.

Here, we examined the role of N and P acquisition strategies (i.e., symbiotic N₂ fixation, root phosphatase activity, and AM colonization) as potential drivers of tropical tree seedling growth under differing soil nutrient availabilities and CO₂. To do this, we grew two N₂-fixing and two non-N₂-fixing tree species in pots and exposed them to three different levels of nutrient availability (0NP, +NP, ++NP) and two different levels of CO₂ (ambient and elevated) in a full-factorial design. We explored how relative growth rate (RGR) and nutrient acquisition strategies of the N₂- and non-N₂-fixing seedlings varied by functional group and/or species, and in response to increased nutrient availability and elevated CO₂ in a high-light, high-water environment. In addition, we determined the strength of the relationships between growth and nutrient acquisition for both functional groups. We hypothesized that, at low soil nutrient availability and under elevated CO₂, N₂ fixers would grow more rapidly than non-N₂ fixers because N₂ fixers have a greater capacity to acquire both N (via N₂ fixation) and P (via phosphatase and/or AM fungi). We also hypothesized that these differences in growth rates between N₂ fixers and non-N₂ fixers would decline as soil nutrient availability increased because N₂ fixers no longer have an advantage in nutrient acquisition.

METHODS

Study site

We conducted this experiment at the Santa Cruz Experimental Field Facility of the Smithsonian Tropical Research Institute (STRI), Gamboa, Panama (9°07' N, 79°42' W). The study site is located within the Panama Canal Watershed at 28 m above sea level. Two glasshouses were used: one with CO₂ similar to ambient conditions (400 ppm) and the other with elevated CO₂ (800 ppm). The elevated CO₂ glasshouse was maintained by releasing CO₂ gas from a high-pressure cylinder when CO₂ declined below 800 ppm. The glasshouses were air-conditioned automatically so that air temperature did not exceed 30°C. In addition, 20%

shade cloths were placed over the glasshouses to reduce surface temperatures of the plant foliage without greatly limiting light.

Species and seedling preparation

We used seedlings of four pioneer tree species that coexist in and are common to lowland moist tropical forests: two N₂ fixers (*Adenanthera pavonina* L. [Fabaceae] and *Inga spectabilis* (Vahl) Willd. [Fabaceae]) and two non-N₂ fixers (*Swietenia macrophylla* King [Meliaceae] and *Tabebuia guayacan* (Seem.) Hemsl. [Bignoniaceae]; Appendix S1: Table S1). Recently, *T. guayacan* has been recognized as a synonym of *Handroanthus guayacan* (Seem.) S.O.Grose [Bignoniaceae] (Grose and Olmstead 2007). These species were chosen because they have a strong tendency to recruit into gaps, and high rates of growth, mortality, and dispersal (Condit et al. 1996). Seeds were collected from mature trees growing in the Panama Canal watershed and germinated in 1.25-L tree pots (Stuewe & Sons, Corvallis, Oregon, USA) containing a 95:5 mixture of silica sand and sieved unsterilized forest soil. The soil was collected from the Santa Rita ridge, is classified as an Oxisol (Typic Kandiodox), and has some of the lowest resin-extractable P (<0.2 mg P/kg) in central Panama (Condit et al. 2013). During the germination phase, tree pots were on plastic benches 1 m above a concrete surface and underneath a rain shelter with a glass roof. The shelter had no sidewalls, so air temperature, wind speed, and relative humidity were similar to ambient conditions. Once each species displayed fully expanded cotyledons, five seedlings per species were harvested for initial seedling mass and leaf area. An additional 15–24 seedlings per species were randomly chosen to be moved into each glasshouse (ambient and elevated CO₂) for the duration of the experiment.

Experimental treatments

At the beginning of the experiment, five to eight seedlings per species in each glasshouse (ambient and elevated CO₂) were randomly chosen to receive a 0NP, +NP, or a ++NP nutrient treatment corresponding to 0, 30, and 60 kg N·ha⁻²·yr⁻¹ as calcium nitrate (Ca(NO₃)₂) and 0, 20, 40 kg P·ha⁻²·yr⁻¹ as potassium phosphate (KH₂PO₄). The N and P fertilizer solution, as well as 75 mL of a N and P free Hoagland's solution (one-fifth strength; to ensure that all other macro- and micronutrients were balanced and non-limiting), were added to each seedling on a weekly basis. Seedlings were watered daily. The nutrient treatments were chosen to represent a wide gradient of nutrient availability and are similar to other seedling studies (e.g., Batterman et al. 2013).

Harvest measurements

We harvested the seedlings of each species in an effort to optimize biomass while avoiding the negative

effect of pot size (Poorter et al. 2012). Seedlings within a species were harvested when the dry plant biomass to pot volume ratios were, on average, <1 g/L in the elevated CO₂, ++NP treatment. Thus, seedlings of different species grew for different periods of time (e.g., 44–60 d), but all species were harvested with similar biomass among the seedlings in the elevated CO₂, ++NP treatment (Appendix S1: Table S1). At the time of harvest, seedlings were separated into root, stem, and leaf fractions. Roots were gently washed with tap water and if the N₂-fixing seedlings contained nodules, they were excised for immediate N₂ fixation analysis using the acetylene reduction assay (ARA). The roots of all seedlings were subsampled and stored overnight at 4°C for phosphatase assays and AM colonization. Total plant dry biomass was measured after drying for 72 h at 60°C.

Plant dry biomass was used to calculate the RGR of each seedling,

$$\text{RGR} = \frac{\ln(M_f) - \ln(M_i)}{dt}$$

where M_f is the final plant dry biomass, M_i is the initial plant dry biomass, and dt is the duration of the experiment in days for each species. The trait is log-transformed as seedling growth is assumed to be exponential and for the convenience of comparing across species (Hunt and Cornelissen 1997; Rees et al. 2010). However, interspecific comparisons may be hampered if growth is size dependent and if size distributions vary among species. We, thus, compared absolute growth rate (AGR) with RGR across all seedlings and found a strong correlation ($r = 0.93$, $P < 0.001$), indicating size-dependent growth does not bias our analyses nor that RGR yields different results than AGR (Poorter et al. 2008).

Dry leaves of each seedling were ground and analyzed for foliar C, N, and P concentrations. Foliar C and N were measured by automated combustion and thermal conductivity detection on a Thermo Flash EA 1112 analyzer (CE Elantech, Lakewood, New Jersey, USA). Foliar P was determined by ignition (550°C, 1 h) and dissolution of the ash in 1 mol/L hydrochloric acid (HCl), with phosphate detection by automated molybdovanadate colorimetry on a Lachat Quikchem 8500 (Hach, Loveland, Colorado, USA).

Root phosphatase activity was determined using *para*-nitrophenyl phosphate (*p*NPP) as an analogue substrate for phosphomonoesterase (Turner et al. 2001). Briefly, ~500 mg of fresh fine roots per seedling were placed in glass vials and immersed in 9 mL of 50 mmol/L sodium acetate-acetic acid buffer (pH 5). The vials were placed in a water bath at 26°C (mean soil temperature in lowland tropical forests of Panama) and shaken for 5 min to equilibrate. While still in the shaking water bath, 1 mL of 50 mmol/L *p*NPP was added to each vial to initiate the assay. After 30 min, the vials were taken out of the shaking water bath and 0.5 mL of solution was

removed and added to 4.5 mL of 0.11 mol/L sodium hydroxide to terminate the assay. The absorbance of each sample was measured at 405 nm against a standard curve of *para*-nitrophenol (*p*NP). Each sample included a negative control for sample and substrate absorbance. Root phosphatase activity was calculated as $\mu\text{mol } p\text{NPP} \cdot (\text{g root dry mass})^{-1} \cdot \text{h}^{-1}$.

Arbuscular mycorrhizal colonization was measured by staining roots with trypan blue (Koske and Gemma 1989). Roots of each seedling were cleared in 10% potassium hydroxide for 3 d, rinsed with tap water, and placed in 3% HCl for 12 h for acidification. The HCl solution was replaced with trypan blue for 24 h and roots were de-stained in water for 12 h. Ten cleared and uniformly stained root pieces per seedling were mounted on slides and root colonization was quantified using the magnified intersection method (McGonigle et al. 1990) on ~50 intersections. Colonization was calculated as the percentage of root length colonized by AM fungi.

Symbiotic N_2 fixation was measured using the ARA method (Hardy et al. 1968). All nodules from each N_2 -fixing seedling (if nodules were present) were excised and incubated in 50-mL clear acrylic tubes with a 10% acetylene (made from reacting calcium carbide with water) atmosphere. After 1 h, 14 mL of headspace gas was removed from each tube with a syringe, injected into 10-mL glass vials, and returned to the laboratory for analysis by gas chromatography using a Shimadzu GC-2014 equipped with a flame ionization detector (Shimadzu, Kyoto, Japan). Background ethylene was accounted for from nodules without acetylene exposure, ethylene produced from tubes and glass vials, ethylene within our acetylene, and ethylene lost as a result of photodegradation during transport. To convert acetylene reduction rates into N_2 fixation rates, an ethylene:N conversion ratio of 2.8:1 was applied (Sullivan et al. 2014). Though this ratio was determined by sampling nodules across a presumably wide variety of N_2 -fixing species in a nearby rain forest, the ratio can vary by species (Batterman et al. 2018). We, thus, urge caution when making inferences about our N_2 fixation rates. Rates of N_2 fixation were calculated on a whole plant basis as $\mu\text{g N/h}$.

Statistical analyses

Data were tested for normality using the Shapiro-Wilk test and homogeneity of variance using the Levene test, and data that did not satisfy those assumptions were log-transformed (e.g., root:shoot, foliar C:N and C:P, N_2 fixation, root phosphatase activity, and AM colonization). We used a nested analysis of variance (ANOVA) to test for effects of CO_2 and nutrient treatments on all harvest measurements. Species were nested into functional groups to test whether the species and/or the functional groups differed in their responses to the CO_2 and nutrient treatments. Multiple linear models were used to determine the influence of nutrient

acquisition strategies (root phosphatase activity, AM colonization, and N_2 fixation) on the RGR of seedlings from each functional group growing at 0NP. All statistical analyses were performed using the open source R software v.2.15.3 (R Development Core Team 2015). For all data, significance was determined when $P < 0.05$.

RESULTS

Growth

Relative growth rates varied across the experimental treatments but were not driven by any specific species (Table 1). While the N_2 fixers had a higher average RGR than the non- N_2 fixers, overall ($P < 0.001$), the difference between the two functional groups depended on nutrient availability (functional group by nutrient treatment effect, $P < 0.001$; Fig. 1a). Specifically, the RGR of the N_2 fixers showed a slight decrease from the low (0NP) to high (++NP) nutrient treatments while the RGR of the non- N_2 fixers increased. As such, the N_2 fixers and non- N_2 fixers had different average RGRs in the low (0NP) and intermediate (+NP) nutrient treatments, but not in the high (++NP). Also, average RGR was higher under elevated CO_2 than under ambient CO_2 , overall ($P < 0.001$; Fig. 1b). There were no interactive effects between the CO_2 treatment and functional group, species, or the nutrient treatment.

Root:shoot allocation and foliar stoichiometry

The variation in root:shoot allocation depended on the combination of the grouping variables and experimental treatments (Table 1), but the variation was primarily due to a single species. While the non- N_2 fixers had a higher average root:shoot than the N_2 fixers, overall ($P = 0.03$), the difference between the two functional groups depended on nutrient availability (functional group by nutrient treatment effect, $P < 0.001$), as well as CO_2 (functional group by CO_2 treatment effect, $P = 0.03$; Fig. 2a). While average root:shoot decreased across the nutrient treatments ($P < 0.001$), it was higher in the non- N_2 fixers only in the low (0NP) nutrient treatment, and this difference was driven by the non- N_2 fixer *Tabebuia guayacana* (species by nutrient treatment effect, $P < 0.001$). Also, while average root:shoot decreased under elevated CO_2 ($P < 0.001$), it was higher in the non- N_2 fixers than the N_2 fixers only under ambient CO_2 , and this difference was driven, again, by *Tabebuia guayacana* (species by CO_2 treatment effect, $P < 0.001$).

The variation in foliar stoichiometry also depended on the combination of the grouping variables and experimental treatments (Table 1). First, while the non- N_2 fixers had a higher average foliar C:N than the N_2 fixers overall ($P < 0.001$), the difference depended on the interaction between nutrient availability and CO_2 (functional group by nutrient by CO_2 treatment effect, $P < 0.001$; Fig. 2b). For example, average foliar C:N was higher in

TABLE 1. *F* table for the nested ANOVAs of the two N₂ fixers and two non-N₂ fixers grown under ambient (400 ppm) or elevated (800 ppm) CO₂, and at low (0NP), intermediate (+NP), and high (++NP) nutrient availabilities.

| Parameter | Functional group | Nutrient | CO ₂ | Species | Functional group × Nutrient | Functional group × CO ₂ | Nutrient × CO ₂ | Species × Nutrient | Species × CO ₂ | Functional group × Nutrient × CO ₂ | Species × Nutrient × CO ₂ |
|---------------------------|------------------|-----------------|-----------------|-----------------|-----------------------------|------------------------------------|----------------------------|--------------------|---------------------------|---|--------------------------------------|
| RGR | 43.9*** | 6.0** | 23.6*** | 0.3 | 10.9*** | <0.1 | 1.9 | 0.5 | 2.4 | 0.2 | 0.5 |
| Root:shoot | 5.0* | 60.5*** | 14.6*** | 2.3 | 8.2*** | 5.1* | 1.0 | 19.6*** | 8.4*** | 1.2 | 7.7*** |
| Foliar C:N | 27.5*** | 484.0*** | 127.5*** | 76.2*** | 5.8** | <0.1 | 0.7 | 5.8*** | 7.7*** | 20.2*** | 5.8*** |
| Foliar C:P | 20.2*** | 153.1*** | 80.0*** | 146.5*** | 34.5*** | 2.6 | 2.5 | 2.1 | 4.0* | 2.4 | 5.6*** |
| Foliar N:P | 3.4 | 5.6** | 11.2** | 68.1*** | 39.7*** | 2.3 | 1.8 | 2.0 | 5.6** | 9.9*** | 3.8** |
| Root phosphatase activity | 217.4*** | 50.0*** | 18.7*** | 1.7 | 3.0 | <0.1 | 0.8 | 3.7** | 3.0 | 1.0 | 1.5 |
| AM colonization | 61.1*** | 64.5*** | 45.2*** | 1.9 | 0.8 | 0.2 | 3.4* | 2.8* | 0.6 | 0.1 | 0.4 |
| N ₂ fixation | | 593.4*** | 56.2*** | 8.3** | | | 14.9*** | 2.2 | 0.3 | | 0.2 |

Notes: Statistically significant results are shown in boldface type (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). Relative growth rate, RGR; arbuscular mycorrhizal (AM) colonization.

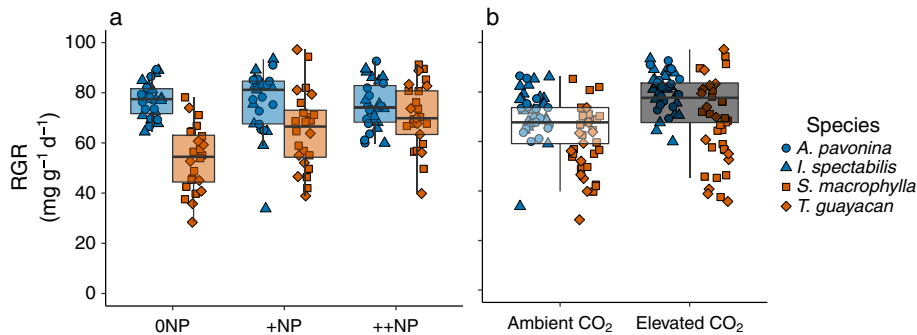


FIG. 1. Relative growth rate (RGR) of (a) the N₂-fixing and non-N₂-fixing seedlings across the low (0NP), intermediate (+NP), and high (++NP) nutrient treatments and (b) all of the seedlings under ambient and elevated CO₂. Box plot mid lines represent medians, boxes represent the 25th and 75th quartile values, whiskers represent 1.5 × the interquartile range, and points represent individual seedlings. Species are *Adenanthera pavonina*, *Inga spectabilis*, *Swietenia macrophylla*, and *Tabebuia guayacan*.

the non-N₂ fixers than the N₂ fixers across all nutrient treatments under elevated CO₂, but only in the intermediate (+NP) and high (++NP) nutrient treatments under ambient CO₂. These differences, however, were driven primarily by the non-N₂ fixer *Swietenia macrophylla* (species by nutrient by CO₂ treatment effect, *P* < 0.001). Despite these differences, average foliar C:N, overall, decreased across the nutrient treatments (*P* < 0.001) and increased under elevated CO₂ (*P* < 0.001).

Second, average foliar C:P of the non-N₂ fixers was higher than the N₂ fixers overall (*P* < 0.001), but the difference between the two functional groups depended on nutrient availability (functional group by nutrient treatment effect, *P* < 0.001; Fig. 2c). Specifically, the average foliar C:P of the non-N₂ fixers decreased more strongly across the nutrient treatments than the N₂ fixers. As such, average foliar C:P in the non-N₂ fixers was higher than the N₂ fixers only in the low (0NP) treatment. Also, while average foliar C:P increased under elevated CO₂ overall (*P* < 0.001), it was driven by both N₂ fixers in the low (0NP) and intermediate (+NP) nutrient treatments and the non-N₂ fixer *Swietenia macrophylla* in the

low nutrient treatment (species by nutrient by CO₂ treatment effect, *P* < 0.001; Fig. 2c).

Last, while average foliar N:P differed across the nutrient (*P* < 0.01) and CO₂ (*P* < 0.01) treatments, this variation was driven by the high foliar N:P of the non-N₂ fixer *Swietenia macrophylla* in the low nutrient treatment under ambient CO₂ (species by nutrient by CO₂ treatment effect, *P* < 0.01; Fig. 2d). As a result, there was no general difference in average foliar N:P between the two functional groups (*P* = 0.07).

Nutrient acquisition strategies

We found little variation among the three nutrient acquisition strategies across the grouping variables and experimental treatments (Table 1). With respect to N acquisition, average whole plant N₂ fixation (μg N/h) differed between the two N₂ fixer species overall and was highest in *Inga spectabilis* (*P* < 0.01; Fig. 3). Also, average whole plant N₂ fixation decreased to zero across the nutrient treatments (*P* < 0.001) and increased under elevated CO₂ (*P* < 0.001). There was a nutrient by CO₂

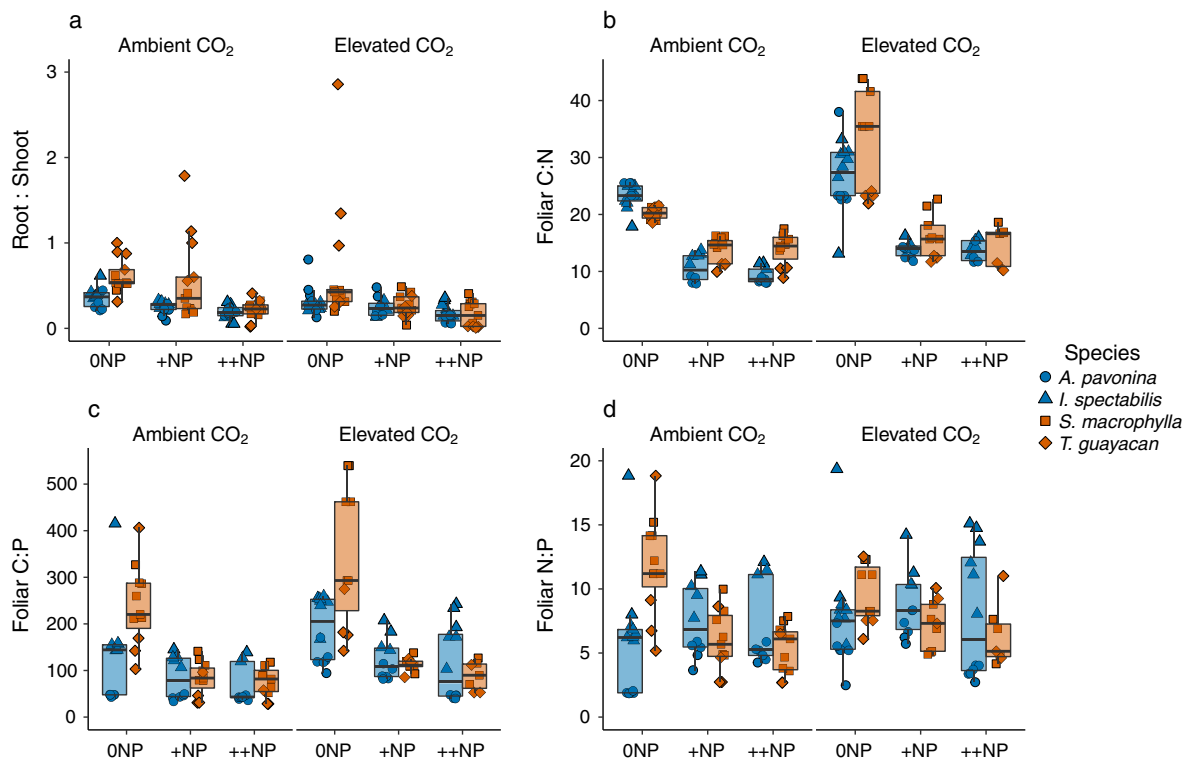


FIG. 2. (a) Root:shoot (a), (b) foliar C:N, (c) foliar C:P, and (d) foliar N:P of the N₂-fixing and non-N₂-fixing seedlings across the low (0NP), intermediate (+NP), and high (++NP) nutrient treatments, and under ambient and elevated CO₂. Box plot mid lines represent medians, boxes represent the 25th and 75th quartile values, whiskers represent 1.5 × the interquartile range, and points represent individual seedlings.

treatment effect, but this was driven by the absence of N₂ fixation in the high (++NP) nutrient treatment under both ambient and elevated CO₂.

Average root phosphatase activity of the N₂ fixers was higher than the non-N₂ fixers ($P < 0.001$), and decreased across the nutrient treatments ($P < 0.001$). However, the decrease in root phosphatase activity across the nutrient treatments was driven by the N₂ fixer *Adenanthera pavonina* and the non-N₂ fixer *Tabebuia guayacan* (species by nutrient treatment effect, $P < 0.01$; Fig. 4a.). Also, average root phosphatase activity was higher under elevated CO₂ overall ($P < 0.001$; Fig. 4b). There were no interactive effects between the CO₂ treatment and functional group, species, or the nutrient treatment (Table 1).

Finally, average AM colonization was higher on the roots of the non-N₂ fixers than the N₂ fixers ($P < 0.001$), decreased across the nutrient treatments ($P < 0.001$), and increased under elevated CO₂ overall ($P < 0.001$). However, the response in AM colonization to the CO₂ treatment depended on nutrient availability (nutrient by CO₂ treatment effect, $P = 0.04$; Fig. 5). Specifically, AM colonization, across all species, was only higher under elevated CO₂ in the intermediate (+NP) and high (++NP) nutrient treatments. There was an interaction between species and the

nutrient treatment in which AM colonization of the non-N₂ fixer *Swietenia macrophylla* did not vary across the nutrient treatments.

Relationships between growth and nutrient acquisition strategies

In the low (0NP) nutrient treatment and across both CO₂ treatments, the RGR of the N₂ fixers was positively correlated with whole plant N₂ fixation ($P = 0.03$, $r = 0.39$; Fig. 6a) and root phosphatase activity ($P = 0.03$, $r = 0.37$; Fig. 6b), but not with AM colonization ($P = 0.53$; Fig. 6c). However, an interactive model that included N₂ fixation and root phosphatase activity accounted for 60% of the variation in the RGR of the N₂ fixers ($P < 0.01$). In the low (0NP) nutrient treatment, and across both CO₂ treatments, the RGR of the non-N₂ fixers was not correlated with root phosphatase activity ($P = 0.06$, Fig. 6d), but was positively correlated with AM colonization ($P = 0.03$, $r = 0.40$; Fig. 6e).

DISCUSSION

Our study demonstrates that N and P acquisition strategies of the two tropical N₂-fixing tree species studied here are capable of augmenting growth when

exposed to elevated CO₂, especially when nutrients are relatively scarce. We hypothesized that, in nutrient-poor soils and under elevated CO₂, N₂ fixers would have higher growth rates than non-N₂ fixers because N₂ fixers have a greater capacity to acquire both N and P. In our study, the non-N₂ fixers had higher levels of AM colonization than the N₂ fixers, but the N₂ fixers had higher root phosphatase activity and the ability to fix atmospheric N₂. Moreover, the latter two strategies were up-regulated in low nutrient soil and under elevated CO₂, which may have enabled higher growth than the non-N₂ fixers. We also hypothesized that the differences in growth between N₂ and non-N₂ fixers would decline as soil nutrient availability increased. Indeed, as soil nutrient availability increased, the difference between the N₂ and non-N₂ fixer growth declined, likely reflecting the observed decrease in nutrient acquisition strategies as the seedlings could invest less in

these strategies and still acquire sufficient soil nutrients. Overall, our results provide compelling evidence that coupled N and P acquisition strategies may enable some tropical tree species to overcome the nutritional constraints on NPP that are likely to be exacerbated with future increases in CO₂.

The N₂ fixers had higher growth than the non-N₂ fixers, but this difference was driven by the disparate growth of the two functional groups in the low and intermediate nutrient soils (Fig. 1a). This implies, at least for the species studied here, that soil nutrients must be relatively scarce in order for the N₂ fixers to realize higher growth rates than the non-N₂ fixers. Previous work suggests that N₂ fixers may have a greater capacity than non-N₂ fixers to acquire both N and P (Houlton et al. 2008). This is especially relevant in the tropics, where N₂ fixers can potentially use fixed N₂ to invest in soil P acquisition (Nasto et al. 2014). A greater capacity to acquire both nutrients, especially P, would confer a competitive advantage in N₂ fixers over non-N₂ fixers in low nutrient soils but not necessarily in high nutrient soils. Our results provide multiple lines of evidence supporting this relationship between nutrient acquisition and the growth of N₂ fixers relative to non-N₂ fixers.

Whole-plant N₂ fixation increased as soil nutrient availability decreased (Fig. 3), reflecting similar patterns in number of nodules (Appendix S1: Fig. S1). Apparently, the N₂ fixers up-regulated their investment in N₂ fixation to meet the nutrient demands of high growth when soil N was insufficient, but down-regulated N₂ fixation when soil N was plentiful. This facultative strategy of N₂ fixation (Menge et al. 2009, Barron et al. 2011, Batterman et al. 2013) may enable N₂ fixer growth to benefit in low N soil and to avoid the C cost of fixation in high N soil as the cost of fixation is higher than direct uptake of soil N (Gutschick 1981, Vitousek et al. 2002, Vance 2008, Fisher et al. 2010). In addition, N₂ fixation was related to growth rates of N₂ fixers in low nutrient soil (Fig. 6a). Though we did not measure the δ¹⁵N of the N₂ fixer biomass (which can be used to estimate the contribution of fixed N₂ to the overall N nutrition of the

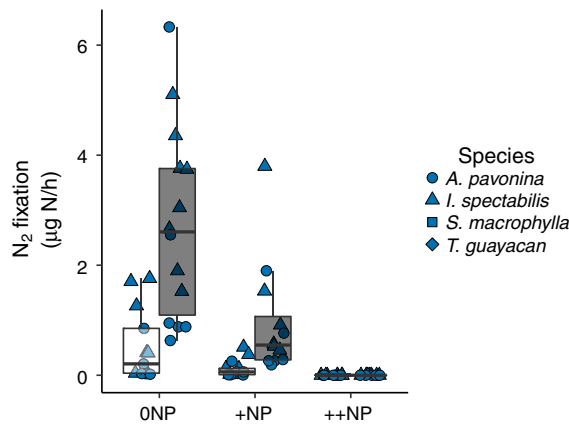


FIG. 3. Whole-plant symbiotic dinitrogen (N₂) fixation of the N₂-fixing seedlings across the low (0NP), intermediate (+NP), and high (++NP) nutrient treatments, and under ambient (white) and elevated (black) CO₂. Box plot mid lines represent medians, boxes represent the 25th and 75th quartile values, whiskers represent 1.5 × the interquartile range, and points represent individual seedlings.

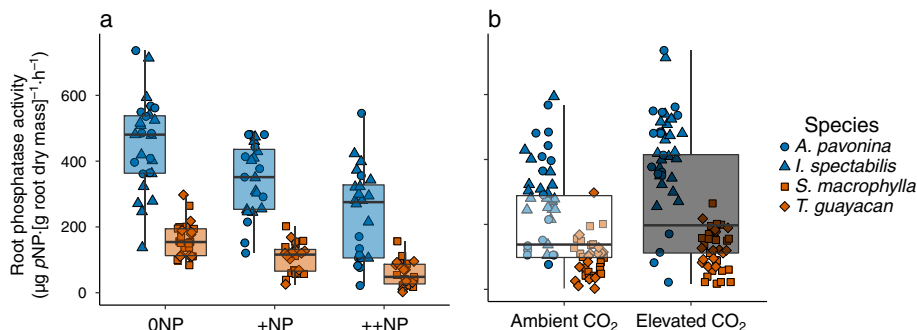


FIG. 4. Root phosphatase activity of the (a) N₂-fixing and non-N₂-fixing seedlings across the low (0NP), intermediate (+NP), and high (++NP) nutrient treatments and (b) all of the seedlings under ambient and elevated CO₂. Box plot mid lines represent medians, boxes represent the 25th and 75th quartile values, whiskers represent 1.5 × the interquartile range, and points represent individual seedlings. The measure pNP is *para*-nitrophenol.

N_2 -fixing seedlings; Shearer et al. 1983), the flexibility of N_2 fixation may help explain, in part, consistent N_2 fixer growth rates across the wide range of soil nutrient availability in this experiment.

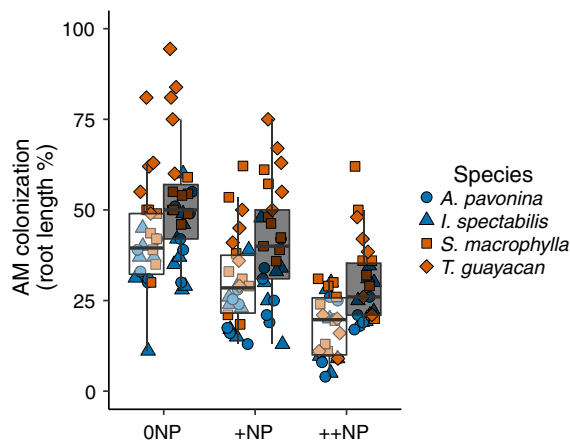


FIG. 5. Arbuscular mycorrhizal (AM) colonization of the N_2 -fixing and non- N_2 -fixing seedlings across the low (ONP), intermediate (+NP), and high (++NP) nutrient treatments, and under ambient (white) and elevated (black) CO_2 . Box plot lines represent medians, boxes represent the 25th and 75th quartile values, whiskers represent $1.5 \times$ the interquartile range, and points represent individual seedlings.

Root phosphatase activity and AM colonization also increased as soil nutrient availability decreased (Fig. 4a and 5, respectively). Such patterns have been reasonably well documented (Treseder 2004, Marklein and Houlton 2011, Png et al. 2017), and suggest an increased demand for P at low fertility. Though root phosphatase activity and AM colonization are not direct measures of P uptake, both strategies are important for P acquisition (Lambers et al. 2008, Smith and Read 2008), and have been linked to the capacity for P uptake (Sanders and Tinker 1971, Ortas et al. 1996, Khaliq and Sanders 2000, Treseder and Vitousek 2001). It is worth noting, however, that this pattern in P acquisition was driven by the responsiveness of one of the two species from each functional group: the N_2 fixer *Adenanthera pavonina* and the non- N_2 fixer *Tabebuia guayacan*. The latter species is a low-P specialist, occurring predominantly on low P soils in the central Panama region (Condit et al. 2013), suggesting that investment in AM fungi might explain its competitive ability at low fertility.

Despite this species-specific response to nutrient availability, root phosphatase activity was higher in the N_2 fixers than the non- N_2 fixers (Fig. 4), and was related to N_2 fixer growth in low nutrient soil (Fig. 6b). Even more, N_2 fixation was positively related to root phosphatase activity (Appendix S1: Fig. S2), and both nutrient acquisition strategies, together, were related to N_2 fixer growth in low nutrient soil. If N_2 fixers were indeed

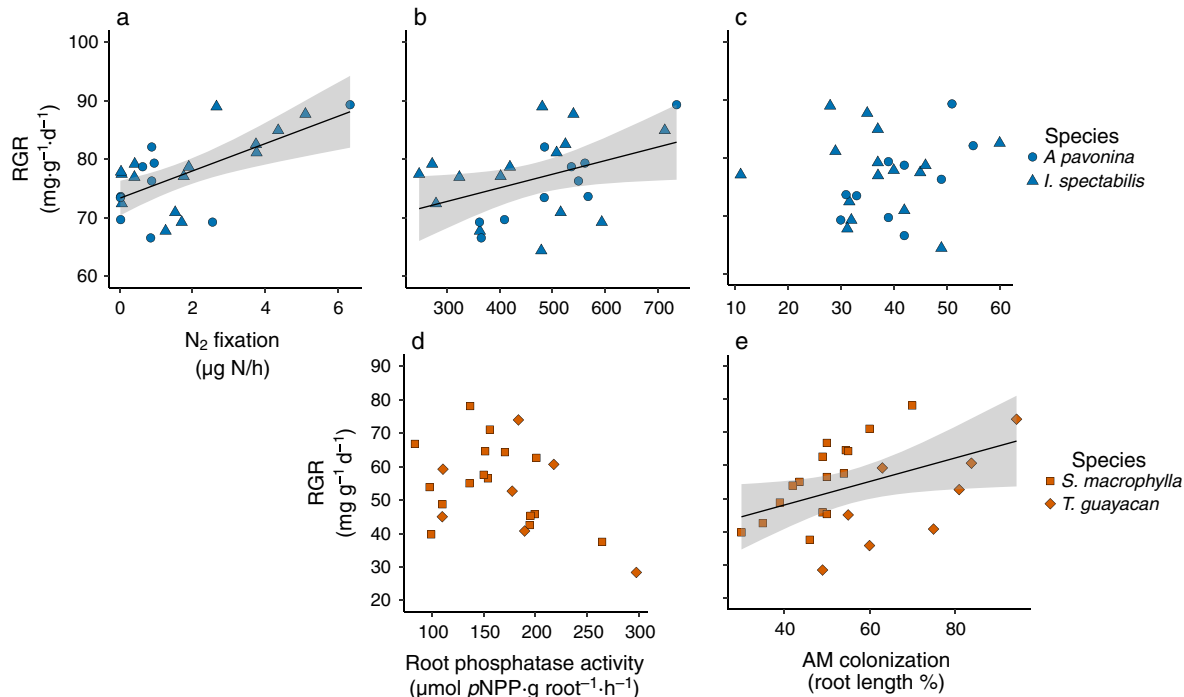


FIG. 6. Relationship between (a) N_2 fixation ($P = 0.03$, $r = 0.39$), (b) root phosphatase activity ($P = 0.03$, $r = 0.37$), and AM colonization ($P = 0.53$), and (c) the relative growth rate (RGR) of the N_2 -fixing seedlings grown in the low nutrient treatment and across both CO_2 treatments. Relationship between (d) root phosphatase activity ($P = 0.06$) and (e) AM colonization ($P = 0.03$, $r = 0.40$), and the relative growth rate (RGR) of the non- N_2 -fixing seedlings grown in the low nutrient treatment and across both CO_2 treatments.

able to invest fixed N into the production of root phosphatases (Houlton et al. 2008), it would help to explain not only the greater rates of root phosphatase activity in N₂ fixers relative to non-N₂ fixers (Nasto et al. 2017), but also their pivotal role in sustaining higher growth than non-N₂ fixers in low nutrient soil. Alternatively, a plant's investment into the production of root phosphatases may not be dependent on N₂ fixation, per se, but the internal N supply of the plant itself (Soper et al. 2018). However, we found no relationship between foliar N:P and root phosphatase activity (Appendix S1: Fig. S3), indicating that N status, in itself, is not a strong control on P acquisition.

By contrast, AM colonization was higher in the non-N₂ fixers than the N₂ fixers (Fig. 5), and was related to non-N₂ fixer growth in low nutrient soil (Fig. 6e). Though it is clear that the non-N₂ fixers (specifically, *Tabebuia guayacana*) were flexible in their ability to adjust levels of AM colonization to appropriately match the soil P conditions, it was not sufficient to allow them to maintain their highest levels of growth across the gradient of nutrient availability. The non-N₂ fixers had no discernible strategy to acquire enough N to keep pace with demand. Thus, it appears that the enhanced capacity for the N₂ fixers to acquire more N and P than the non-N₂ fixers conferred a competitive advantage in the N₂ fixers over the non-N₂ fixers, but only when soil nutrients were scarce.

Nutrient acquisition strategies are not the only mechanisms that could potentially enable the N₂ and non-N₂ fixers to overcome low soil nutrients. Two additional mechanisms could contribute to the growth of both N₂ and non-N₂ fixers in low nutrient soil: greater root:shoot allocation and/or a stoichiometric increase in tissue chemistry. Indeed, root:shoot and foliar C:N and C:P increased as nutrient availability decreased (Fig. 2), but the increases were largely attributable to the non-N₂ fixers (specifically *Swietenia macrophylla*), which also had higher overall root:shoot, foliar C:N, and foliar C:P than the N₂ fixers (Fig. 2). These patterns complement those of the nutrient acquisition strategies in the species from both functional groups in two ways. First, the N₂ fixers had a greater capacity than the non-N₂ fixers to acquire both N and P in low nutrient soil, likely resulting in the observed insensitivity of root:shoot and foliar stoichiometry to variations in soil nutrient availability. Second, the non-N₂ fixers had a relatively limited ability to acquire both N and P in low nutrient soil, and likely relied on the adjustments of root:shoot and foliar stoichiometry (especially for *Swietenia macrophylla* considering its lack of response in root phosphatase activity and AM colonization) to overcome nutrient-poor soil conditions. However, such adjustments were clearly not sufficient, as growth of the non-N₂ fixers declined in the low nutrient soil.

Though the growth of the N₂ and non-N₂ fixers were higher under elevated than ambient CO₂ (Fig. 1b), the effect of CO₂ was not dependent on functional group

(Table 1). In other words, the N₂ and non-N₂ fixers had similar (and positive) growth responses to elevated CO₂. This is a surprising result given that N₂ fixers are thought to respond more strongly to elevated CO₂ than non-N₂ fixers because N₂ fixers have this greater capacity to acquire N and P in low nutrient soil (Thomas et al. 1991, Tissue et al. 1996, Cernusak et al. 2011). Indeed, N₂ fixation (Fig. 3), root phosphatase activity (Fig. 4), and AM colonization (Fig. 5) were all higher under elevated than ambient CO₂. Greater access to C (Tissue et al. 1996, Hungate et al. 1999; Fitter et al. 2000, de Graaff et al. 2006, Cernusak et al. 2011, Jakobsen et al. 2016, Terrer et al. 2016) and stoichiometric demand for N and P (Barrett et al. 1998, Niu et al. 2013) likely led to the observed increase in nutrient acquisition strategies under elevated CO₂. However, these increases were not dependent on functional group, either, and thus did not confer a greater response of one functional group over the other to elevated CO₂.

A greater benefit to the N₂ fixers over the non-N₂ fixers from elevated CO₂ might have appeared if our study had been longer. For example, exposure to elevated CO₂ often increases the photosynthetic C uptake of plants, which increases the availability of carbohydrate and can translate to increased growth (Leakey et al. 2009). However, an increase in carbohydrate alters the C and N metabolism of the plant. As a result, the plant eventually acclimates by reducing synthesis of Rubisco, which ultimately lowers photosynthetic C uptake (Long et al. 2004, Nowak et al. 2004, Ainsworth and Long 2005, Ainsworth and Rogers 2007). Though this also lowers the demand for N, and can constrain the extent to which plants can assimilate C for enhanced growth for prolonged periods of time under elevated CO₂ (Rogers et al. 2009). Dinitrogen fixers can allocate some of the additional carbohydrate gained from an increase in photosynthetic C uptake to their rhizobial partners to stimulate N₂ fixation, allowing N₂ fixers to maximize the stimulation of both photosynthetic C uptake and N₂ fixation to gain a greater benefit in enhanced growth from elevated CO₂ than non-N₂ fixers (Rogers et al. 2009). Thus, if we had conducted a longer study we may have detected not only a competitive advantage of the N₂ fixers over the non-N₂ fixers in nutrient acquisition in low nutrient soil, but also under elevated CO₂.

Finally, we note that broad inferences from our results should be made with caution given the limited number of study species, and in light of a growing number of studies pointing to species-specific results. For example, Cernusak et al. (2011) showed variation in the capacity for nodulation among tropical N₂ fixers, Nasto et al. (2017) showed that differences in nutrient acquisition strategies between non-N₂ and N₂ fixers were driven by individual species belonging to each functional group, Zalamea et al. (2016) and Png et al. (2017) suggested that root phosphatase activity is phylogenetically constrained and not related to functional group or N₂ fixation, and Soper et al. (2018) demonstrated a lack of a

relationship between N₂ fixation, foliar N, and P acquisition among a variety of N₂-fixing legumes, non-N₂-fixing legumes, and non-legumes. Taken together, these studies suggest that larger numbers of species occupying multiple genera and families are needed to generate more robust relationships between nutrient acquisition strategies and growth, and to conclude fundamental differences between N₂ and non-N₂ fixers as functional groups.

Nonetheless, our results provide evidence that at least two tropical N₂-fixing trees have the capacity to maintain relatively high growth in nutrient-poor soil, and augment growth under elevated CO₂, because of their enhanced capacity to acquire N and P. If the mechanisms we invoked here hold true to a wider set of N₂-fixing trees, N₂ fixers could become increasingly important members of tropical forest communities, both in terms of abundance and function. While we note that the experimental conditions in our study replicated a range of conditions likely to be encountered by these species in the rain forest (e.g., the low nutrient treatment simulated a gap environment, or conditions akin to a secondary rain forest, with relatively high irradiance), it remains unclear whether the results from our seedling experiment reflect nutrient acquisition by mature canopy trees in natural forest or even trees of different life history traits. Additionally, it remains unclear if the higher growth rates realized by N₂ fixers are equivocal to higher NPP as respiration and other pathways of C loss may be increasing concurrently. However, it appears that at least two N₂-fixing trees may have the capacity to overcome the nutritional constraints to NPP with predicted increases in atmospheric CO₂, which could help sustain the C sink strength of tropical rain forests.

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M. K. Nasto, K. Winter, B. L. Turner, and C. C. Cleveland conceived of and designed the study. M. K. Nasto and K. Winter conducted the experiment. M. K. Nasto and B. L. Turner conducted the laboratory analyses. M. K. Nasto wrote the manuscript and K. Winter, B. L. Turner, and C. C. Cleveland contributed to revisions.

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SUPPORTING INFORMATION

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