

# Phosphorus limitation of early growth differs between nitrogen-fixing and nonfixing dry tropical forest tree species

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## Summary

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- Tropical forests are often characterized by low soil phosphorus (P) availability, suggesting that P limits plant performance. However, how seedlings from different functional types respond to soil P availability is poorly known but important for understanding and modeling forest dynamics under changing environmental conditions.
- We grew four nitrogen (N)-fixing Fabaceae and seven diverse non-N-fixing tropical dry forest tree species in a shade house under three P fertilization treatments and evaluated carbon (C) allocation responses, P demand, P-use, investment in P acquisition traits, and correlations among P acquisition traits.
- Nitrogen fixers grew larger with increasing P addition in contrast to non-N fixers, which showed fewer responses in C allocation and P use. Foliar P increased with P addition for both functional types, while P acquisition strategies did not vary among treatments but differed between functional types, with N fixers showing higher root phosphatase activity (RPA) than nonfixers.
- Growth responses suggest that N fixers are limited by P, but nonfixers may be limited by other resources. However, regardless of limitation, P acquisition traits such as mycorrhizal colonization and RPA were nonplastic across a steep P gradient. Differential limitation among plant functional types has implications for forest succession and earth system models.

## Introduction

Lowland tropical soils have been described as phosphorus (P) limited, which is attributed in part to their advanced weathering state (Walker & Syers, 1976; Vitousek *et al.*, 2010; Cleveland *et al.*, 2011). Once initial soil P stocks are depleted, the remaining P is either occluded by secondary minerals or present in organic forms, both of which are difficult for plants to access (Lambers *et al.*, 2008; Turner, 2008). Therefore, plants growing in low-P soils invest in either mining (e.g. enzymes that hydrolyze organic compounds) or scavenging traits (symbiosis with mycorrhizal fungi, rapid root growth, or root hair proliferation) to optimize P uptake (Lambers *et al.*, 2008; Zemunik *et al.*, 2015). These P acquisition traits have different resource costs. For instance, root exudates can account for 50% of belowground carbon (C) allocation, while mycorrhizal fungi can demand up to 25% of total C assimilated by plants (Lynch & Ho, 2005). Construction of fine roots has a low C cost, although their maintenance is higher compared with coarse roots (Laliberté *et al.*, 2015; McCormack *et al.*, 2015). Even though trait costs vary, some traits may be more efficient than others depending on soil P availability (Treseder & Vitousek, 2001; McCormack &

Iversen, 2019) and their multifunctionality for acquiring other limiting resources (e.g. water).

Plants with high foliar P tissue concentrations in low-P soils can invest more in P acquisition traits (Hayes *et al.*, 2014). They optimize resource use by investing in only a subset of P acquisition traits and downregulate these traits when inorganic forms of P are more abundant (i.e. show plasticity; Ryan *et al.*, 2012; Ushio *et al.*, 2015). However, for most species and functional types, it is still unclear which combination of traits (strategy) is the most efficient for acquiring P, in part because most experiments often measure only a handful of root chemical and morphological traits (McCormack *et al.*, 2017), which precludes characterizing an overall plant strategy, especially with regard to symbiotic and physiological traits. Moreover, comparisons across gradients of soil P availability that would elucidate plasticity in investment are limited (Laliberté, 2017). Understanding patterns of plant P use, investment in different P acquisition traits and the coordination among them is important for improving our predictions of plant species abundances and distributions across successional gradients, assessing plant responses to global changes, and defining plant functional types in earth system models (Bardgett *et al.*, 2014; Hanbury-Brown *et al.*, 2022).

In addition to P, plants require multiple other nutrients (Kaspari & Powers, 2016) many of which may also be in short supply and thus affect seedling performance (Santiago *et al.*, 2012). While multiple studies have suggested that tropical forest soils can have an excess of nitrogen (N) relative to P (Jenny, 1950), N has still been shown to play a role in nutrient limitation in many cases (e.g. late succession; Ostertag & DiManno, 2016; Wright, 2019). Nitrogen and P concentrations are mechanistically linked by the physiological requirements of organisms (McGroddy *et al.*, 2004), and even though their stoichiometry is constrained, variation in these ratios occurs among species, soil types, and ecosystems (Güsewell, 2004; Townsend *et al.*, 2008).

Plant N and P requirements might be especially linked in leguminous species that acquire N through biological N fixation, common to some Fabaceae subfamilies (Caesalpinioideae and Papilionoideae) and nine other plant families (Soltis *et al.*, 1995; Augusto *et al.*, 2013). Nitrogen fixation is a P-demanding process, and many empirical and theoretical investigations have suggested that N fixation may thus be coupled with P acquisition (Wang *et al.*, 2007; Marklein & Houlton, 2012). This alternative N acquisition pathway may allow N-fixer species to access more P using this 'extra' N, for example by investing it in phosphatase enzyme production (Marklein & Houlton, 2012; Nasto *et al.*, 2014; Png *et al.*, 2019). However, this link may be species-specific and could be phylogenetically conserved (i.e. a characteristic of the Fabaceae family, rather than mechanistically linked to N fixation *per se*; Wurzburger & Hedin, 2016; Png *et al.*, 2017; Batterman *et al.*, 2018; Soper *et al.*, 2019), which hinders broad generalizations. To date, most studies exploring how P addition impacts species performance and nutrient acquisition traits have explored few traits and used a small number of species to represent tropical diversity (Huante *et al.*, 1995; Siqueira & Saggini-Júnior, 2001; Batterman *et al.*, 2013; Zalamea *et al.*, 2016), thus limiting the breadth of conclusions that can be drawn.

Nevertheless, broad patterns suggesting the distinctiveness of N-fixing Fabaceae species in terms of growth, development, and physiology are starting to emerge. Tropical forest N fixers have faster germination rates, higher water use efficiency (WUE), and higher seedling growth rates compared with nonfixers (Powers & Tiffin, 2010; Vargas *et al.*, 2015; Adams *et al.*, 2016; Smith-Martin *et al.*, 2017). However, multiple studies conducted in wet forests have suggested that traits such as N fixation and root phosphatase activity (RPA) can differ among N-fixing species (Cernusak *et al.*, 2011; Png *et al.*, 2017; Batterman *et al.*, 2018; Soper *et al.*, 2019). Therefore, the applicability of the N-fixer and non-fixer functional type does not seem appropriate to characterize species functional roles in wet forests (Batterman *et al.*, 2018). By contrast, findings from Waring *et al.* (2019) suggest that N-fixing and nonfixing trees in tropical dry forests (TDFs) respond differently to N and P addition. Model simulations confirm that soil variability influences functional type (fixer vs nonfixer) composition and processes during succession (Medvigy *et al.*, 2019). Therefore, exploring how seedlings from these functional types acquire and use P in a biome that represents 40% of tropical forests (Murphy & Lugo, 1986), experience high nutrient limitation due to its intense seasonality (Read & Lawrence, 2006), and have

a high abundance of Fabaceae species during early succession (Baribault *et al.*, 2012; Gei *et al.*, 2018) is important for understanding how projected changes in rainfall regimes may affect C storage in TDFs (Chadwick *et al.*, 2015; Norby *et al.*, 2017; Hanbury-Brown *et al.*, 2022).

To compare the extent to which early growth, performance, and trait values of N-fixing and nonfixing TDF seedlings respond to P, we grew plants across a gradient of P availability in a shade house experiment. We measured a range of physiological, morphological, and chemical traits. Specifically, we addressed the following questions: (1) How do N-fixing and non-N-fixing seedlings respond to P availability? (2) How does P addition affect P acquisition traits? (3) How does P addition impact photosynthetic phosphorus-use efficiency (PPUE) of N-fixing and non-N-fixing seedlings? (4) What are the correlations among foliar P concentrations, P use, and acquisition traits? We predicted that: (1) N fixers show greater growth responses to P addition than non-N fixers because N fixers are faster growers. (2) Investment in P acquisition traits varies inversely as a function of soil P availability, and because traits for acquiring P are 'expensive', these traits are negatively correlated with each other reflecting trade-offs. (3) Photosynthetic P-use efficiency decreases with P addition for both functional types. (4) Foliar P concentrations are positively correlated with P acquisition traits.

## Materials and Methods

### Site description

Our study was conducted in a shade house located at Estación Experimental Forestal Horizontes (hereafter 'Horizontes'), part of Área de Conservación Guanacaste (ACG) in northwestern Costa Rica (10.71°N, 85.59°W). Horizontes has a mean annual temperature of 25°C and a mean annual precipitation of *c.* 1730 mm that mostly falls between late May and early November (Waring *et al.*, 2019; Werden *et al.*, 2020).

### Soil collection

Local forest soil from 5 to 30 cm depth was collected in May 2019 at Horizontes and then left in the sun for 4 d covered with plastic to kill any live roots. Thereafter, soils were homogenized and sieved to 5 mm. Soils at the site are Andic and Typic Haplustepts (Alfaro *et al.*, 2001), with a high clay content (38 ± 1%) and total N : P of 8.3, suggesting P limitation (Waring *et al.*, 2019).

### Species selection

We initially selected seeds of 30 species that commonly occur across successional and soil fertility gradients (30–1272 total P mg kg<sup>-1</sup>) in ACG and Área de Conservación Arenal Tempisque (ACAT)-Parque Nacional Palo Verde (10.35°N, 85.35°W; Powers & Tiffin, 2010; Werden *et al.*, 2018), and had seeds available at the time the experiment was established. Due to logistical constraints (i.e. low germination rates, pests, and high

mortality), only 11 species of the original 30 were used in the experiment. We included four N-fixing species from the Papilionoideae subfamily, the most abundant Fabaceae subfamily in the area (Werden *et al.*, 2018) and the one with the highest species richness (Koenen *et al.*, 2020). Papilionoideae species primarily associate with arbuscular mycorrhizal (AM) fungi. We caution here that our results should not be extended beyond the Papilionoideae subfamily; however, the fixers and nonfixers used in this study do not differ significantly in distribution characteristics across successional or soil gradients compared with the regional species pool (Methods S1; Table S1; Fig. S1). Nonfixers included one ectomycorrhizal and six AM species from seven different families, including one Fabaceae (Table S2; Fig. S2). We collected most seeds in the ACG and ACAT and purchased seeds of *Tabebuia impetiginosa* (Mart. Ex DC.) Standl. from the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), Costa Rica.

The experiment was conducted during the rainy season from May to October in a 36 m<sup>2</sup> shade house that excluded *c.* 55% of PAR (J. Powers, unpublished data). Before planting, all seeds were soaked in water overnight. *Hymenaea courbaril* L. seeds were scarified to accelerate germination (Smith-Martin *et al.*, 2017); seeds were placed in 100°C water for 30 s and then in ice water for 1 min, this process was repeated three times. Then, between 2 and 15 seeds (depending on seed size and availability) of each species were planted per pot (11 species × three treatments × seven replicates = 231 pots). Pots were big enough to have a biomass volume ratio < 1 g l<sup>-1</sup> (10 × 10 cm and 20 cm deep containing *c.* 2 kg of soil; Poorter *et al.*, 2012). Pots were randomly arranged within plastic crates to avoid confounding positioning species and treatment effects (eight pots per crate). One week after germination, seedlings were thinned to one per pot, because we obtained low germination rates for *Lonchocarpus rugosus* Benth. And *H. courbaril* additional seeds were added. To minimize differences in soil microbial communities, all seedlings were inoculated with microbial inoculum from three different forest soils collected across the regional P gradient (Smith-Martin *et al.*, 2017; Waring *et al.*, 2019). We prepared the inoculum by combining equal weights of the three soils in 1 l water, shaking them, and adding 10 ml of this solution to each pot 3 wk after sowing. This treatment was repeated the following week to ensure microbial colonization. When seedlings were *c.* 1 month old, they were fertilized every 2 wk from July to October with 10 ml of either 0.0016 g ml<sup>-1</sup> of phosphoric acid (H<sub>3</sub>PO<sub>4</sub>; 45 kg P ha<sup>-1</sup> yr<sup>-1</sup>; P++; Waring *et al.*, 2019), 0.00016 g ml<sup>-1</sup> H<sub>3</sub>PO<sub>4</sub> (4.5 kg P ha<sup>-1</sup> yr<sup>-1</sup>; P+), or tap water (P0) to emulate the regional soil P gradient and P application rates used in other fertilization experiments (Siqueira & Saggin-Júnior, 2001; Alvarez-Clare *et al.*, 2013; Waring *et al.*, 2019). Soil moisture was measured in each pot to 5 cm depth three times over the course of the experiment with a SM150 Soil Moisture Sensor (Delta-T Devices, Cambridge, UK). When rainfall was scarce, that is lack of rain for > 24 h, seedlings were hand-watered every other day to prevent desiccation. At the conclusion of the experiment, we measured soil pH in distilled water on air-dried soils from all pots using a 1 : 2.5 soil-to-solution ratio. Finally, to quantify the

distribution of soil P among different P pools, a modified Hedley fractionation was conducted on a subset of soils from the pots at the soil laboratory at CATIE.

### Harvesting and biomass

We harvested all seedlings 4 months after germination over a 10-d period. None of the individuals showed evidence of being pot bound. At the time of harvest, each seedling was separated into root, stem, and leaf fractions. Roots were carefully removed, gently washed in water, and weighed. In the case of nodulating species, nodules were removed from the roots (before weighing), counted, and weighed. Leaves were immediately scanned to calculate leaf area using IMAGEJ software (Schneider *et al.*, 2012). Leaves, stems, roots, and nodules of each seedling were dried at *c.* 60°C for 5 d and then weighed to calculate total dry biomass (TDB), leaf mass fraction (LMF), stem mass fraction (SMF), root mass fraction (RMF; Table S3), and nodule mass fraction (NMF). Root biomass included the tap roots if they were present. Dry weights of leaves were used to calculate specific leaf area (SLA, cm<sup>2</sup> g<sup>-1</sup>). All statistical analyses were performed using dry weights. Specific leaf area did not vary between functional types and treatments (Fig. S3, SLA *P* > 0.05) and will not be further discussed.

### Plant performance and nutrient limitation

We measured relative height growth rate (RGR), photosynthetic capacity, and WUE as metrics of plant performance. Stem height was measured from the base of the stem to the apical meristem on each seedling every 2 wk after the first true leaves emerged. We used the classic RGR (cm d<sup>-1</sup>) equation: (log<sub>e</sub>[final height] – log<sub>e</sub>[initial height]) / (final day – initial day; Hunt & Cornelissen, 1997; Rees *et al.*, 2010). Photosynthetic capacity was quantified by measuring photosynthetic responses to carbon dioxide concentration (A<sub>c</sub> curves). Gas exchange measurements were performed on one recently matured leaf of three seedlings per species per treatment *in situ* with a LI-6400XT portable photosynthesis system (Li-Cor, Lincoln, NE, USA) at a constant temperature (30°C). Measurements were made at CO<sub>2</sub> concentrations ranging between 0 and 1200 ppm (nine points total), irradiance was 1000 μmol m<sup>-2</sup> s<sup>-1</sup> (unlikely to cause photoinhibition of photosynthesis when leaves are measured below or above optimum temperatures), and relative humidity ranged between 60% and 80%. Measurements were made between 07:00 and 14:00 h, and the ambient air temperature generally increased from 25°C to 32°C. We then obtained maximum photosynthetic rates (A<sub>max</sub>) and rubisco maximum carboxylation rate (V<sub>cmax</sub>) using the PLANTECOPHYS R package (Duursma, 2015).

Finally, we analyzed foliar nutrients, WUE, and photosynthetic PPUE. We bulked all leaves per individual and transported them to the University of Minnesota (UMN) where they were ground and analyzed for total C, N, and C isotopic ratio (δ<sup>13</sup>C) on an Isoprime 100 isotope ratio mass spectrometer coupled with an Elementar Pyrocube combustion elemental analyzer

(Elementar Americas Inc., Ronkonkoma, NY, USA). Other foliar nutrients (aluminum, boron, calcium, chromium, copper, iron, P, potassium, magnesium, manganese, sodium, and nickel) were analyzed at the UMN Research Analytical Lab using standard methods in an iCAP<sup>TM</sup> 7600 ICP-OES (inductively coupled argon plasma optical emission spectrometer) Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA) following digestion in HNO<sub>3</sub>. We calculated PPUE from  $A_{\max}$  and foliar P concentrations (Veneklaas *et al.*, 2012).

### Belowground traits

We quantified plant investment in P acquisition using RMF and specific root length (SRL) – a morphological response, RPA – a physiological response, and % arbuscular mycorrhizal colonization (AMC) – a biotic response representing degree of collaboration with symbionts. Specific root length was calculated after scanning all roots with a transparency scanner (Epson Perfection V800; Suwa, Japan; 1–4 images per individual). We placed the roots in a clear polycarbonate tray filled with water to ensure no root overlap and used the RHIZOVISION software (Seethepalli *et al.*, 2020) to calculate root length of fine root fractions ( $\leq 2$  mm diameter), that is excluding any tap root. After scanning, root systems were separated into two subsamples: one sample for RPA and the other for scoring AMC. Root phosphatase activity was determined using para-nitrophenyl phosphate as an analog substrate for phosphomonoesterase using standard methods (Methods S2; Turner *et al.*, 2001). Finally, we scored AMC by staining the roots with alanine blue (Koske & Gemma, 1989) and quantifying colonization microscopically using the magnified intersection method (McGonigle *et al.*, 1990) on *c.* 100 intersections per root fragment. We calculated colonization as the percentage of root length colonized by AM fungi (Methods S3). Ectomycorrhizal colonization was measured using the grid-line intersection method (Brundrett *et al.*, 1996). This variable was not included in any of the analyses as only one species was ectomycorrhizal, and these data are not directly comparable to AMC.

While quantifying N-fixation activity was not a goal of this experiment, we assessed plant C construction investment in fixing structures by quantifying nodule mass and NMF (Methods S4; Table S4; Fig. S4).

### Statistical analysis

First, we tested the effects of our experimental treatments on soil pH and soil P fractions using linear models, pooling data by P treatment across all species. Then, to determine how N-fixing and nonfixing seedlings respond to P availability, we analyzed the effects of P addition on TDB, RGR,  $A_{\max}$ , and  $\delta^{13}\text{C}$  as a metric of WUE with linear mixed-effects models. In these models, trait values were the response variable. Functional type (N-fixer or nonfixer), treatment (control; P0, low; P+, or high; P++), and their interaction were included as fixed effects. Species identity was included as a random effect to account for variation among species. Seedling initial height was included as a covariable to

control for differences in species' sizes. We used a similar model with a beta distribution for SMF and LMF. We checked the distribution of the residuals for all models to ensure that normality and homoscedasticity assumptions were met. When assumptions were not met (TDB, RGR, and  $\delta^{13}\text{C}$ ), we used a generalized linear mixed-effects model (GLMM) assigning an independent variance structure (varIdent) as a function of species. Finally, we obtained the *F*-values of a two-way analysis of variance (ANOVA, type III) for the fixed effects and performed multiple comparisons among treatments and/or functional types using a Tukey *post hoc* test (Table 1; Methods S5).

Our second question explored how P addition affects P acquisition traits. The statistical models used to answer this question were similar to the ones used in question 1. Because the residuals did not meet the normality and homoscedasticity assumptions, we used a GLMM with a variance structure (varIdent) as a function of species (RPA, foliar N : P, foliar P, and foliar N) or log-transformed (SRL) the response variable. Arbuscular mycorrhizal colonization and RMF were modeled with a beta distribution (colonization values range from 0 to 1). To answer how PPUE changed with P addition, we ran the same statistical model used in question 1. Then, following Messier *et al.* (2010), we performed a variance partitioning analysis for each trait independently. Importantly, the variance explained for our models corresponds to the group-level variation, that is functional type, species identity, individual, and treatment. A nested linear model was used for each trait (Messier *et al.*, 2010).

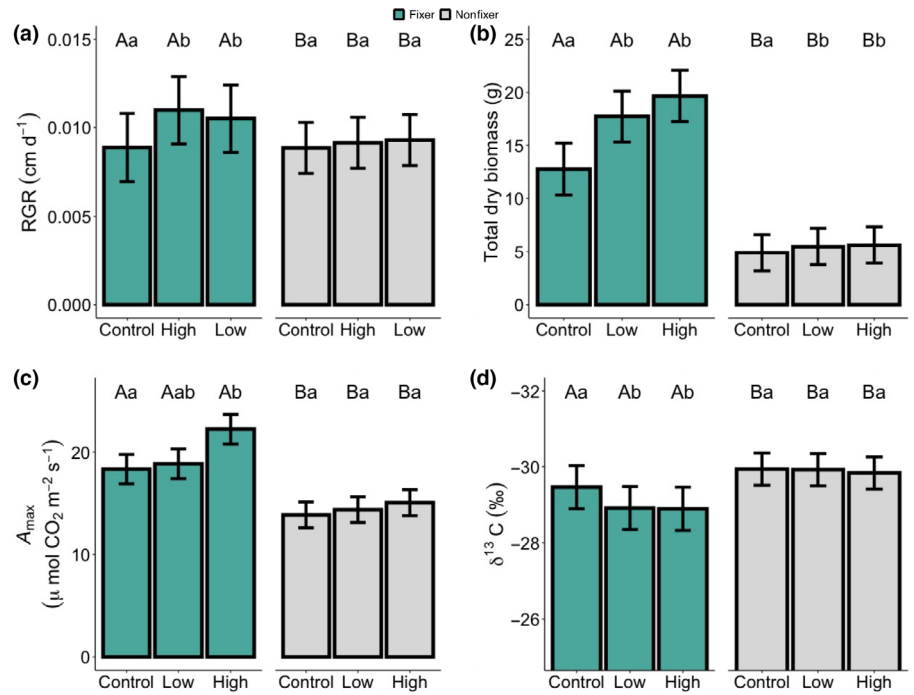
Last, we explored whether there were correlations among foliar N and P, PPUE, and nutrient acquisition traits (RPA, AMC, RMF, and SRL) conducting a pairwise Pearson correlation analysis with Bonferroni corrections for each functional type separately. We excluded the mycorrhizal colonization data of *Q. oleoides* from the nonfixer correlation because this species had

**Table 1** *F*-values from linear mixed models with species as a random effect, and functional type (N-fixer or non-N-fixer) and treatment (control (P0), low (P+), and high (P++) phosphorus (P)) as fixed effects.

Response	Functional type	Treatment	Functional type × Treatment
Relative growth rate	0.0001	<b>13.87***</b>	<b>6.68***</b>
Total dry biomass	<b>17.11***</b>	<b>6.98*</b>	<b>13.09***</b>
$A_{\max}$	<b>5.37*</b>	<b>3.99*</b>	1.24
$V_{C_{\max}}$	<b>5.87**</b>	1.27	1.42
$\delta^{13}\text{C}$	0.44	<b>5.38**</b>	<b>3.35*</b>
Root phosphatase activity	<b>12.89**</b>	0.58	0.65
Specific root length	<b>26.93*</b>	0.46	0.16
Foliar P	0.71	<b>15.28***</b>	<b>5.99**</b>
Foliar N	<b>55.05***</b>	1.19	0.86
Foliar N : P	<b>34.99***</b>	<b>70.49***</b>	<b>19.87***</b>
PPUE	0.56	<b>3.57*</b>	<b>3.27*</b>

Statistically significant results are shown in boldface type (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).  $A_{\max}$ , maximum photosynthetic rate; PPUE, photosynthetic phosphorus-use efficiency;  $V_{C_{\max}}$ , maximum rate of rubisco carboxylase activity.

**Fig. 1** Comparison of biomass and growth responses among nitrogen (N)-fixing and nonfixing seedlings to three levels of phosphorus (P). Reported results come from a one-way analysis of variance for each functional trait. (a) Relative growth rate (RGR). (b) Total dry biomass (TDB). (c) Maximum photosynthetic rate ( $A_{\max}$ ). (d) Water use efficiency (WUE)  $\delta^{13}\text{C}$  (a proxy for WUE) of four N-fixing and seven non-N-fixing species of seedlings across control (P0), low (P+), and high (P++) P treatments. Green bars represent N-fixing species, and gray bars represent non-N-fixing species. Bar heights represent the marginal means extracted from the linear mixed models, and error bars represent the standard error. Capital letters represent significant differences between functional types, while lowercase letters represent differences among treatments within a functional type.



a different mycorrhizal type. Results were considered statistically significant at  $P < 0.05$ . All statistical analyses were done using R software for statistical computing v.4.0.3 (R Core Team, 2021) and the following packages: NLME (Pinheiro *et al.*, 2017), LME4 (Bates *et al.*, 2011), GLMMTMB (Brooks *et al.*, 2017), HMISC (Harrell, 2019), and CAR (Fox & Weisberg, 2020).

## Results

### Soil pH and phosphorus

At the end of the experiment, soil pH ranged from 5.56 to 7.07. Phosphorus addition decreased soil pH by 0.06 (P+) and 0.14 (P++) units compared with the control ( $P < 0.01$ ). However, the differences in pH between the control and low-P treatment were not statistically significant ( $P > 0.05$ ). Total P in the Hedley fractions ranged from 548.7 to 1165.9  $\text{mg kg}^{-1}$  and confirmed that P fertilization increased soil total P by a factor of 1.4 (P+) and 2.1 (P++) compared with the control ( $P < 0.05$ ). As expected, inorganic fractions experienced the highest increases compared with organic fractions. While P in the Hedley fraction was consistently higher in the high-P treatment than in the other two treatments, the  $\text{NaHCO}_3$  organic P was the only fraction that did not change among treatments ( $P > 0.05$ ; Table S5).

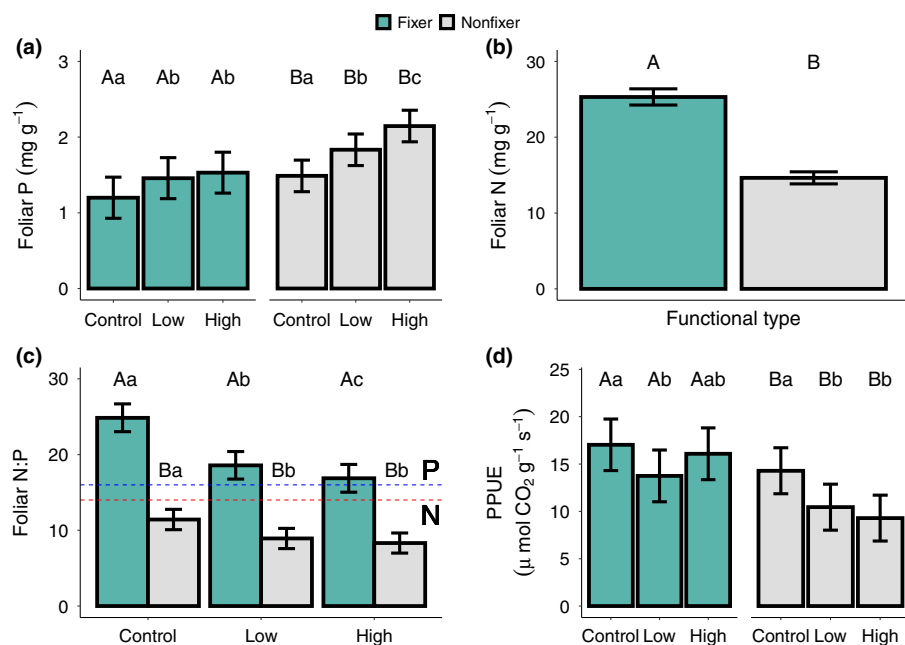
### Growth and biomass

Early growth of N fixers was more responsive to P addition than nonfixers (functional type by treatment effect,  $P < 0.01$ ; Tables 1, S6). Nitrogen fixers that received P increased their RGR by 18% (P+) and 24% (P++) compared with the control ( $P < 0.001$ ), but differences between the two P addition rates were not significant

( $P > 0.05$ ). Relative growth rates for nonfixers were similar among P addition treatments ( $P > 0.05$ ; Figs 1a, S5). Similar to RGR, N fixers had a mean TDB three times higher compared with nonfixers across treatments ( $P < 0.001$ ). The differences in TDB depended on the interaction between treatment and functional type ( $P < 0.001$ ). The mean TDB of N fixers was 39% (P+) and 54% (P++) higher when P was added compared with the control ( $P < 0.001$ ). Mean TDB of nonfixers showed more modest increases of 12% (P+) and 15% (P++), respectively, compared with the control ( $P < 0.05$ ). Mean TDB did not significantly differ between the low and high treatments for either functional type ( $P > 0.05$ ; Figs 1b, S6). Finally, LMF did not differ between functional types and treatments ( $P > 0.05$ ), and allocation to stems differed across P treatments ( $P < 0.05$ ) but not between functional types (Fig. S7).

### Photosynthesis and WUE

Nitrogen fixers on average had a 36% higher maximum photosynthetic rate ( $A_{\max}$ ) compared with nonfixers ( $P < 0.05$ ). Mean  $A_{\max}$  increased by 21% for N fixers when P was added in high quantities (P++) compared with the control ( $P < 0.05$ ; Figs 1c, S8). The rubisco carboxylation rate ( $V_{c_{\max}}$ ) also differed between functional types. Nitrogen fixers had a higher mean  $V_{c_{\max}}$  compared with nonfixers ( $P < 0.05$ ). However,  $V_{c_{\max}}$  values did not vary significantly across P treatments for either functional type ( $P > 0.05$ ). Finally,  $\delta^{13}\text{C}$  values (interpreted as a proxy for mean WUE), ranged from  $-25.9\text{‰}$  to  $-32.42\text{‰}$  among individuals. The variation in WUE depended upon the interaction between functional types and treatment ( $P < 0.05$ ). Nitrogen fixers increased WUE with P addition ( $P < 0.05$ ), while nonfixers had similar WUE across treatments ( $P > 0.05$ ; Figs 1d, S9, S10).



**Fig. 2** Comparison of foliar and photosynthetic P-use efficiency (PPUE) responses of nitrogen (N)-fixing and nonfixing seedling to three levels of phosphorus (P). Reported results come from a one-way analysis of variance for each functional trait. (a) Foliar P. (b) Foliar N. (c) Foliar N : P; dashed lines indicate P (blue) and N limitation (red) thresholds based on Reich & Oleksyn (2004). (d) Photosynthetic P-use efficiency of four N-fixing and seven non-N-fixing species of seedlings across control (P0), low (P+), and high (P++) P treatments. Green bars represent N-fixing species, and gray bars, non-N-fixing species. Bar heights represent the marginal means extracted from the linear mixed models, and error bars represent the standard error. Capital letters represent differences between functional types, while lowercase letters represent differences within a functional type.

### Foliar P, N, and stoichiometry

Nitrogen fixers had lower mean foliar P than nonfixers, and the variation in foliar P depended on the combination of functional type and treatment ( $P < 0.01$ ). Phosphorus addition increased foliar P of N fixers by 22% (P+) and 28% (P++) compared with the control ( $P < 0.001$ ), with no significant differences between the two fertilization levels ( $P > 0.05$ ). Foliar P of nonfixers increased by 23% (P+) and 44% (P++), respectively, compared with the control ( $P < 0.001$ ). High doses of P increased foliar P by 17% compared with the low treatment ( $P < 0.001$ ; Figs 2a, S11). By contrast, foliar N was 72% higher for N fixers compared with nonfixers ( $P < 0.001$ ) and did not vary across treatments for either functional type ( $P > 0.05$ , Figs 2b, S12). Mean foliar N : P of N fixers was higher across treatments relative to nonfixers, 20.4 for N fixers compared with 9.8 for non-N fixers ( $P < 0.001$ ; Figs 2c, S13). The variation in N : P depended on the interaction between functional type and treatment ( $P < 0.001$ ). When P was added, N fixers had lower N : P compared with the control (24.8 P0, 18.6 P+, and 16.9 P++;  $P < 0.001$ ). High rates of P addition had lower N : P compared with the low rates ( $P < 0.05$ ). Nonfixers also showed large changes in N : P with P addition. N : P decreased when P was added (11.4 P0, 8.3 P+, and 8.9 P++;  $P < 0.001$ ). The low and high fertilization treatments had similar N : P ( $P > 0.05$ ). Results for other foliar nutrients appear in the supplement section (Table S7): only boron, chromium, and potassium differed among treatments and functional types.

### Photosynthetic P-use efficiency

Photosynthetic P-use efficiency depended upon the interaction between functional types and treatment ( $P < 0.05$ ). Nitrogen fixer PPUE was 38% higher than nonfixer PPUE ( $P < 0.05$ ). However, both functional types decreased PPUE with P addition.

Fertilized nonfixers decreased PPUE by 27% (P+) and 35% (P++) compared with the control ( $P < 0.01$ ; no difference between P treatments;  $P > 0.05$ ). Nitrogen fixers, on the contrary, decreased their PPUE by 19% (P+) compared with the control ( $P < 0.05$ ). However, PPUE for the high P treatment was similar to PPUE of the control and low P ( $P > 0.05$ ; Fig. 2d).

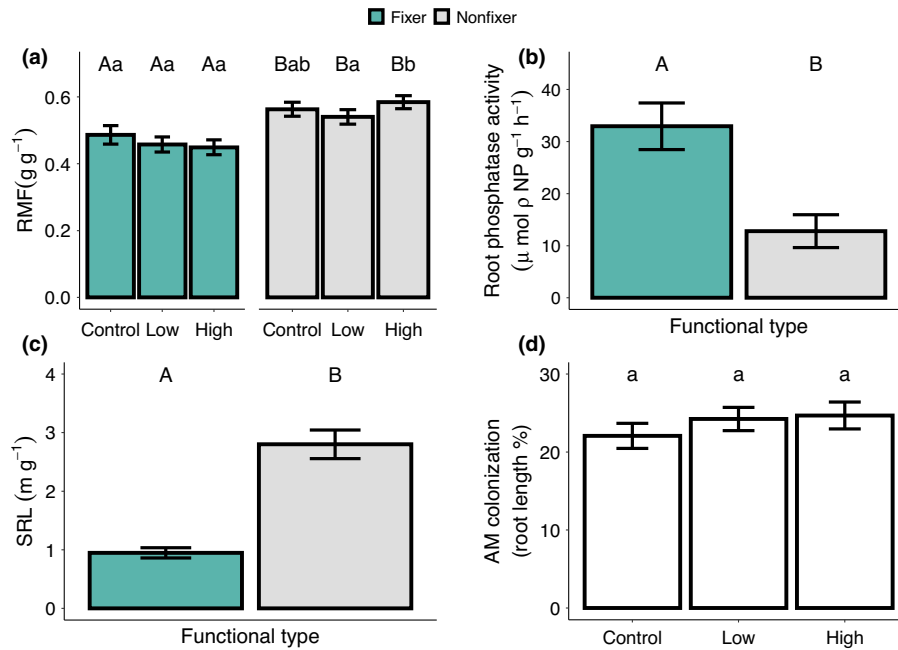
### P acquisition traits

Phosphorus acquisition traits differed primarily by functional type, rather than in response to P addition (Table 2). Root biomass allocation depended on the combination of functional type and treatment ( $P < 0.05$ ). Nitrogen fixers had lower RMF compared with nonfixers. Root mean fraction of nonfixers only changed between the low and high P addition rates ( $P < 0.001$ ). Nitrogen fixers had similar RMF across treatments ( $P > 0.05$ ; Figs 3a, S14). On the contrary, mean RPA of N fixers was three times higher compared with nonfixers ( $P < 0.01$ ) and did not decline with P addition for either functional type ( $P > 0.05$ ;

**Table 2** Chi-squared values from linear mixed models with a beta distribution with species as a random effect, and functional type (N-fixer or non-N-fixer) and treatment (control (P0), low (P+), and high (P++) phosphorus (P)) as fixed effects.

Response	Functional type	Treatment	Functional type × Treatment
%AMC	3.48	3.30	0.33
Root mass fraction	1.62	3.01	<b>7.22*</b>
Stem mass fraction	2.98	<b>6.88*</b>	2.44
Leaf mass fraction	0.18	0.085	3.20

Statistically significant results are shown in boldface type (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ); % AMC, arbuscular mycorrhizal colonization.



**Fig. 3** Comparison of phosphorus (P) acquisition strategy responses of nitrogen (N)-fixing and nonfixing seedling to three levels of P. Reported results come from a one-way analysis of variance for each functional trait. (a) Root mass fraction (RMF). (b) Root phosphatase activity (RPA). (c) Specific root length (SRL). (d) Arbuscular mycorrhizal colonization (AMC) of four N-fixing and seven non-N-fixing species of seedlings across control (P<sub>0</sub>), low (P<sub>+</sub>), and high (P<sub>++</sub>) P treatments. Green bars represent N-fixing species, and gray bars, non-N-fixing species. Arbuscular mycorrhizal colonization was similar for both functional types. Bar heights represent the marginal means extracted from the linear mixed models for the RPA. The raw data means were used for the other three variables since the model outputs were not biologically meaningful. Error bars represent the standard error. Capital letters represent differences between functional types, while lowercase letters represent differences within a functional type.

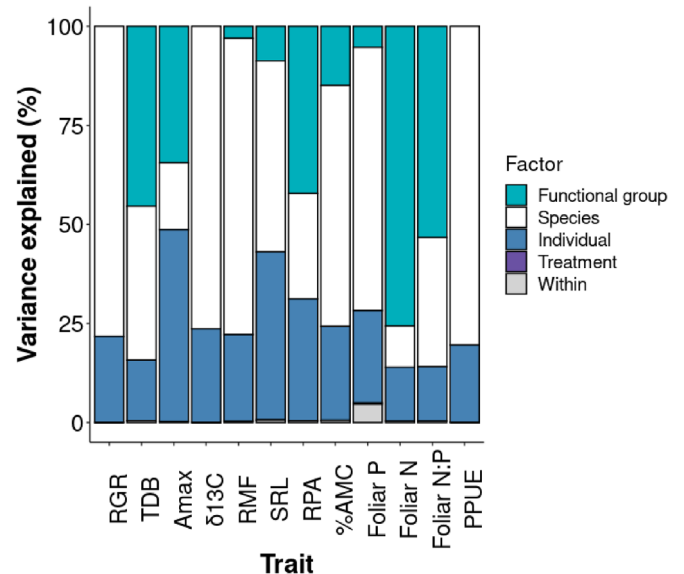
Figs 3b, S15). Similar to RPA, the average SRL did not change with P addition for either functional type ( $P > 0.05$ ). However, SRL of nonfixers was three times higher compared with N fixers ( $P < 0.05$ ; Figs 3c, S16). Finally, AMC varied substantially across species (from 0% to 45%; Fig. S17), though the mean ( $c. 26\%$ ) was similar for both functional groups across treatments ( $P > 0.05$ , Fig. 3d).

### Variance partitioning

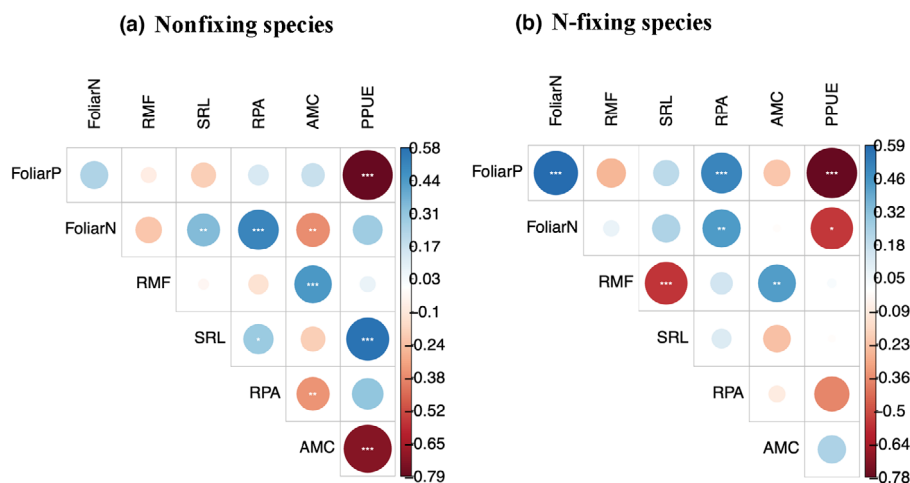
Overall, functional type accounted for  $c. 24\%$  of the variance across all traits, species identity  $c. 51\%$ , individual  $c. 25\%$ , and treatment  $c. 0.03\%$ . However, the importance of each source of variation differed among traits. Functional type differences were most important for TDB (45%), RPA (42%), foliar N (76%), and foliar N : P (53%). By contrast, species identity was a dominant source of variation for RGR (78%),  $\delta^{13}\text{C}$  (76%), AMC (61%), SRL (48%), RMF (75%), foliar P (66%), and PPUE (80%). Individual differences contributed to most of the variation for  $A_{\text{max}}$  (48%; Fig. 4).

### Correlations among nutrient acquisition traits, PPUE, and foliar nutrients

Nonfixers showed no correlation between foliar P and N ( $P > 0.05$ ). Foliar P was only negatively correlated with PPUE ( $r = -0.77$ ,  $P < 0.001$ ). None of the P acquisition traits were correlated with foliar P ( $P > 0.05$ ). Foliar N was positively correlated



**Fig. 4** Stacked bar plot of variance partitioning for each trait across four nested sources of variation. To determine variance partitioning, we used a nested linear model for each trait (Messier *et al.*, 2010, 2016). For this approach, we used the same trait sampling scheme as our classification of variation sources: treatment, functional type, species, and individual. The within factor explains the variation that cannot be explained by the model. Plant functional traits defined as: %AMC, arbuscular mycorrhizal colonization;  $A_{\text{max}}$ , maximum photosynthetic rate; Foliar P, Foliar N, Foliar N : P; PPUE, photosynthetic phosphorus-use efficiency; RGR, relative growth rate; RMF, root mass fraction; RPA, root phosphatase activity; SRL, specific root length; TDB, total dry biomass; and  $\delta^{13}\text{C}$ , proxy for water use efficiency.



**Fig. 5** Pearson correlation coefficients for pairwise relationships among foliar nutrient content (a metric of nutrient demand) and nutrient acquisition traits for (a) nonfixing species and (b) nitrogen (N)-fixing species. Significant relationships are denoted with an asterisk (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). Only significant correlations are represented. Nutrient acquisition traits defined as: AMC, arbuscular mycorrhizal colonization; RMF, root mass fraction; RPA, root phosphatase activity; SRL, specific root length.

with RPA ( $r = 0.52$ ,  $P < 0.001$ ) and SRL ( $r = 0.34$ ,  $P < 0.001$ ). Arbuscular mycorrhizal colonization ( $r = -0.37$ ,  $P < 0.001$ ) was negatively correlated with foliar N. Finally, RMF–AMC ( $r = 0.53$ ,  $P < 0.001$ ) and SRL–RPA were positively correlated ( $r = 0.49$ ,  $P < 0.01$ ), while RPA–AMC ( $r = -0.22$ ,  $P < 0.001$ ) was negatively correlated. Photosynthetic P-use efficiency was positively correlated with SRL ( $r = 0.58$ ,  $P < 0.001$ ) and negatively correlated with AMC ( $r = -0.73$ ,  $P < 0.001$ ; Fig. 5a).

Nitrogen fixers showed a positive correlation between foliar N and P ( $r = 0.59$ ,  $P < 0.001$ ). In N fixers, RPA ( $r = 0.51$ ,  $P < 0.001$ ) was the only nutrient acquisition trait positively correlated with foliar P, while PPUE ( $r = -0.78$ ,  $P < 0.001$ ) was negatively correlated with foliar P. The other traits were not correlated with foliar P ( $P > 0.05$ ). Foliar N, on the contrary, was positively correlated with RPA ( $r = 0.44$ ,  $P < 0.001$ ) and negatively correlated with PPUE ( $r = -0.54$ ,  $P < 0.001$ ). Specific root length–RMF ( $r = -0.55$ ,  $P < 0.001$ ) were the only P acquisition traits negatively correlated. By contrast, RMF–AMC were positively correlated ( $r = 0.43$ ,  $P < 0.001$ ). The other P acquisition traits evaluated were not correlated ( $P > 0.05$ ; Fig. 5b).

## Discussion

Efforts to identify which resource limits productivity in lowland tropical forests have produced mixed results. Some studies have found that P is the only nutrient limiting plant performance (Vitousek, 1984; Campo & Vázquez-Yanes, 2004; Condit *et al.*, 2013), while other fertilization studies and meta-analyses suggest that at the ecosystem scale, there is colimitation by N, P, and other macronutrients (Santiago *et al.*, 2012; Wright, 2019). Our study is the first to compare multiple above- and below-ground responses to added P for a diversity of TDF species spanning an important functional type distinction (N fixers vs nonfixers). We found that the growth of N-fixing seedlings (Papilionoideae subfamily) appeared to be P-limited, while nonfixers showed little evidence of P limitation suggesting that seedlings and mature trees respond in similar ways to P addition in TDFs (Waring *et al.*, 2019). We also found that N fixers and nonfixers invested differentially in specific sets of P acquisition

traits. Nitrogen fixers invested more in a key physiological trait (RPA), while nonfixers invested more in allocation (greater RMF) and had distinct morphological traits (higher SRL). Finally, contrary to what previous studies conducted in wet tropical forests have suggested (Batterman *et al.*, 2013; Zalamea *et al.*, 2016; Nasto *et al.*, 2019), we found that P acquisition traits were not downregulated even with significant P addition. These differences could be the product of seedlings' high nutrient demand in TDFs. However, PPUE was reduced suggesting that plants might be experiencing P luxury consumption (Lawrence, 2003). Although we used the functional type framework to analyze our dataset, the variance partitioning analysis underscored the strong role of individual species identity in determining many of the patterns observed (Batterman *et al.*, 2018; Soper *et al.*, 2019), suggesting that broad generalizations about functional type responses regarding some traits should be taken with caution.

## Differential P limitation

We found strong evidence that N fixers responded to P addition, while nonfixers showed little to no response. Nitrogen fixers had increased height and biomass gain when P was added, and high doses of P magnified these responses (Fig. 1). Such responses are evidence of P limitation (Vitousek & Howarth, 1991; Batterman *et al.*, 2013). Differential limitation between N fixers and nonfixers was also reflected in their foliar N : P ratios ( $> 16$  for N fixers,  $< 14$  for nonfixers; Fig. 2c); N : P ratios above 16 have been suggested as evidence of P limitation, and values below 14 are indicative of N limitation (Koerselman & Meuleman, 1996). While a previous shade house experiment (Huante *et al.*, 1995) and an ecosystem-scale fertilization study (Waring *et al.*, 2019) have suggested this pattern in both seedlings and trees in TDFs, our experiment is the first to demonstrate it for a larger number of tropical species seedlings growing across a P gradient.

Additionally, RGR and WUE did not change for our taxonomically diverse group of seven nonfixing species when P was added, and TDB only slightly compared with the changes N fixers experienced (Fig. 1). This suggests that nonfixers might be

limited by other resources (Santiago *et al.*, 2012; Chou *et al.*, 2018; Waring *et al.*, 2019). Our experimental design does not allow us to determine whether N, some other nutrients, or another resource such as water ultimately limits nonfixers. An obvious follow-up to our experiment would be to test the hypothesis that nonfixing seedlings are limited by N or colimited by N and P. Overall, our experiment corroborates what Waring *et al.* (2019) suggested at the stand level, that is that species-specific responses to nutrient addition depend in part on plant functional type. However, that study lacked the replication to definitively resolve differential P limitation between N fixers and nonfixers.

### Photosynthetic P-use efficiency

We found that overall N fixers had a higher PPUE across P treatments compared with nonfixers, suggesting that N fixers may be more competitive than nonfixers under low resource conditions (Fig. 2d; Veneklaas *et al.*, 2012). We also observed that PPUE values tended to decrease with P addition, suggesting that seedlings are less efficient at conserving and using P when it is in excess (Lovelock *et al.*, 2004). In fact, the observed increases in foliar P with P addition (Fig. 2a) imply that seedlings might be experiencing P luxury consumption (Cordell *et al.*, 2011). To our surprise, N fixers in the control and high P treatment had similar PPUE values. This finding could indicate PPUE is a trait with low variability. Photosynthetic P-use efficiency seems to be an important functional trait to measure in trees growing on P-poor soils because it shows how species adapt to low-P soils (Hidaka & Kitayama, 2011). Therefore, including traits like PPUE in earth system models may increase our capability to predict future conditions in P-limited tropical forests (Reed *et al.*, 2015; Norby *et al.*, 2016).

### Relative investment and downregulation of P acquisition traits

In theory, once a limiting resource becomes available, plants should downregulate investment in nutrient acquisition traits and shift biomass allocation patterns to optimize expenditure of other key resources (Bloom *et al.*, 1985). Although we found no evidence of trait downregulation with P addition, seedlings from different functional types invested differentially in P acquisition traits. Nonfixers invested more in less C-costly root traits, such as exploring more soil volume with high SRL values. Nonfixers had higher biomass allocation to roots overall (Fig. 2). By contrast, N fixers invested in more N-costly traits such as the production of root phosphatases (Yadav & Tarafdar, 2001; Houlton *et al.*, 2008; Guilbeault-Mayers *et al.*, 2020).

Contrary to findings from both tropical and temperate systems, investment in root phosphatase did not decrease with P addition (Marklein & Houlton, 2012; Cabugao *et al.*, 2017, 2021; Lugli *et al.*, 2021). This lack of downregulation response could be related to phylogenetic trait conservation, that is that certain trait expressions are conserved across lineages, rather than being plastic in response to environmental conditions (Valverde-

Barrantes *et al.*, 2015, 2017; Wurzbürger & Hedin, 2016; Png *et al.*, 2017). Similarly, the percentage of AMC did not differ across treatments (Treseder & Allen, 2002). Seedlings from both functional types had equivalent colonization percentages, suggesting that AMC could be decoupled from soil P availability as Waring *et al.* (2019) reported in a factorial N and P fertilization experiment at the same site and Powers *et al.* (2004) observed across neotropical rain forests. Even though the major benefit of AM has been considered translocation of inorganic P (Smith & Read, 2010), AM could provide other benefits to TDF seedlings. For example, AM might help seedlings acquire other nutrients (Siqueira & Saggin-Júnior, 2001; Zangaro *et al.*, 2005; Lambers *et al.*, 2008), facilitate water acquisition, or protect them from pathogens (Herre *et al.*, 2007; Laliberté *et al.*, 2015). It is also possible that tree species in TDF lack efficient mechanisms to actively regulate AM colonization (Valverde-Barrantes *et al.*, 2016) or that seedlings growing in pots have to invest more C in mycorrhizal associations because they cannot access existing networks.

The other two P acquisition traits evaluated, SRL and RMF, were higher for nonfixers than N fixers (Fig. 3). Root mean fraction was the only trait that increased with P addition for nonfixers, but SRL did not vary (Ostertag, 2001; Wurzbürger & Wright, 2015). When plants experience N or P deficiencies, allocation to roots generally increases. However, the effect is stronger for N deficiencies, which is further evidence that nonfixers might be experiencing N limitation (Andrews *et al.*, 1999; Groot *et al.*, 2003). It is also possible that the species used in this experiment were experiencing incomplete downregulation of P acquisition traits when P was added. Tropical N fixers have shown signs of incomplete downregulation of N fixation when nutrients were added (Taylor & Menge, 2018, 2021). To our knowledge, incomplete downregulation of P acquisition traits has not been reported yet. Finally, the absence of change in P acquisition traits with P addition could also be the product of seedlings' development stage and high nutrient demand, or an indication of the low plasticity of nutrient acquisition traits in TDF tree species (Valverde-Barrantes *et al.*, 2015, 2017).

### Evidence for trait integration

We found a positive correlation only between foliar N and P of N fixers (Townsend *et al.*, 2007; Soper *et al.*, 2019). There was varying evidence for trade-offs (i.e. negative correlations) between pairs of P acquisition traits (McCormack *et al.*, 2015). Root mean fraction and AM colonization were positively correlated for both functional types. This suggests that species with higher root biomass could allocate more C to AM fungi and therefore rely more on this symbiotic association for acquiring P (Kong *et al.*, 2016; Bergmann *et al.*, 2020). Similar to that reported by other studies, AMC (specialized in inorganic P uptake) decreased with increases in root phosphatase (a mechanism to access organic P) for nonfixers (Nasto *et al.*, 2017; Soper *et al.*, 2019), indicative of a trade-off among these P acquisition traits. However, we found that these two traits were not correlated for N fixers, similar to that reported in central Amazon and in a

Panamanian montane forest (Steidinger *et al.*, 2015; Lugli *et al.*, 2019). Nitrogen fixers' capacity for fast growth may let them prioritize investment in both AMC (Ficano *et al.*, 2021) and RPA, traits that allow N fixers to access both organic and inorganic P (Turner, 2008; Nasto *et al.*, 2014). Finally, RMF decreases with increases in SRL for N fixers, suggesting that possessing thinner and less C-costly roots may allow N fixers to allocate less biomass to roots while maintaining a large area for nutrient uptake (Bergmann *et al.*, 2020). Overall, seedlings seem to invest in multiple, complementary traits to acquire different forms of P (Zemunik *et al.*, 2015; Lugli *et al.*, 2019). This investment varies between functional types (Turner *et al.*, 2018; Fig. 5) and values of these traits are relatively fixed over a large gradient of soil P availability.

## Conclusion

Our study provides the first evidence for differences in responses of N-fixing and nonfixing seedlings to P availability in TDFs. These results need to be taken with the caveat that our species only included members of the Papilionoideae subfamily, and it is possible that species from other Fabaceae subfamilies would respond differently. Nevertheless, these results have implications for understanding secondary succession and for defining the behavior of plant functional types in earth system models (Medvigy *et al.*, 2019; Hanbury-Brown *et al.*, 2022). As N-fixing legumes dominate during early succession in TDFs and then give way to nonfixing species, we expect that nutrient limitation may shift over successional time from P to N limitation. Our analysis also suggests that it is necessary to evaluate multiple nutrient acquisition traits when assessing species' responses to nutrient limitation because different functional types might differentially invest in certain nutrient acquisition traits to access a limiting resource. Finally, this multispecies experiment corroborates that the responses of N fixers and nonfixers to P limitation are consistent at both the ecosystem and seedling levels (Waring *et al.*, 2019). These results suggest that nutrient limitation in TDFs is more complex and heterogeneous than previously thought (Powers *et al.*, 2015), which requires attention to how this process is implemented in simulation models.

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## Author contributions

LT and JSP developed the idea and designed the study sampling scheme. FMS provided insight into P acquisition traits selection. LT, D Pereira-Arias, D Perez-Aviles and GVG performed the sampling. LT performed data management and statistical analysis and wrote the manuscript with help from JSP and edits from all authors. JSP, GVG, JG and FMS provided support on the interpretation of results.

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## Data availability

The data that support the findings of this study are available from doi: [10.5061/dryad.9kd51c5n0](https://doi.org/10.5061/dryad.9kd51c5n0).

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Distribution of the most common 82 tree species found in tropical dry forests in Costa Rica across a fertility gradient.

**Fig. S2** Distribution of the most common 82 tree species found in tropical dry forests in Costa Rica across a successional gradient.

**Fig. S3** Specific leaf area of four N-fixing and seven non-N-fixing species of seedlings across phosphorus treatments.

**Fig. S4** Nodule mass fraction across phosphorus treatments of *Dalbergia retusa*, *Gliciridia sepium*, *Lonchocarpus rugosus*, and *Lonchocarpus felipei*.

**Fig. S5** Relative growth rate of species across phosphorus treatments.

**Fig. S6** Total dry biomass of species across phosphorus treatments.

**Fig. S7** Leaf mass fraction and stem mass fraction of four N-fixing and seven non-N-fixing species of seedlings across phosphorus treatments.

**Fig. S8** Maximum photosynthetic rate ( $A_{\max}$ ) of species across phosphorus treatments.

**Fig. S9** Water use efficiency as  $\delta^{13}\text{C}$  of species across phosphorus treatments.

**Fig. S10** Instantaneous water use efficiency ( $\text{WUE}_i$ ) of four N-fixing and seven non-N-fixing species across phosphorus treatments.

**Fig. S11** Foliar P of species across phosphorus treatments.

**Fig. S12** Foliar N of species across phosphorus treatments.

**Fig. S13** Foliar N to P ratios of species across phosphorus treatments.

**Fig. S14** Root mass fraction of species across phosphorus treatments.

**Fig. S15** Root phosphatase activity of species across phosphorus treatments.

**Fig. S16** Boxplots showing the specific root length of species across phosphorus treatments.

**Fig. S17** Arbuscular mycorrhizal colonization of species across phosphorus treatments.

**Table S1** *P*-values from *t*-test comparing the distribution across successional and fertility gradients of the 82 most common tree species found in tropical dry forests in Costa Rica.

**Table S2** List of species planted with functional trait information.

**Table S3** Chi-squared *P*-values from a one-way analysis of variance of biomass fraction traits and arbuscular mycorrhizal colonization.

**Table S4** *F*-values from a one-way analysis of variance of nodule mass fraction and nodule dry mass.

**Table S5** Mean soil pH and P fraction values.

**Table S6** *P*-values from a one-way analysis of variance of plant traits.

**Table S7** *F*-values from a one-way analysis of variance of foliar nutrient.

**Methods S1** Focal and regional species distribution.

**Methods S2** Root phosphatase activity.

**Methods S3** Arbuscular mycorrhizal colonization.

**Methods S4** Data analysis of nitrogen fixation proxies.

**Methods S5** Equations used for the data analysis.

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