Contrasting species responses to continued nitrogen and phosphorus addition in tropical montane forest tree seedlings

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ABSTRACT

Global changes in nutrient deposition rates are likely to have profound effects on plant communities, particularly in the nutrient-limited systems of the tropics. We studied the effects of increased nutrient availability on the seedlings of six tree species in montane forests of southern Ecuador *in situ*. After five years of continued N, P, or N+P addition, naturally grown seedlings of each of the two most common species at each elevation (1000, 2000, and 3000 m asl) were harvested for analyses of leaf morphology, nutrient content, herbivory, and tissue biomass allocation. Most species showed increased foliar N and P concentrations after addition of each respective element. Leaf tissue N:P ratios of >20 in the control plants of all species suggest that P is more growth-limiting in these forests than N. Leaf morphological responses to nutrient addition were species and nutrient specific, with some species (*Hedyosmum purparescens, Graffenrieda emarginata*) exhibiting increased specific leaf area (SLA), and others (*Graffenrieda harlingii*) increased leaf area ratios (LAR). *Pouteria torta* (1000 m) had lower SLA and LAR after P addition. Increased herbivory was only evident in *G. emarginata* (after N and N+P addition). Only the species from 3000 m asl modified biomass allocation after nutrient addition. In general, N and N+P addition more strongly affected the species studied at the upper elevations, whereas P addition had a similar range of effects on the species at all elevations. We conclude that the responses of the studied tropical montane forest tree seedlings to chronic N and P addition are highly species-specific and that successful adaptation to increased nutrient availability will depend on species-specific morphological and physiological plasticity.

Abstract in Spanish is available with online material.

Key words: arbuscular mycorrhizal fungi; biomass allocation; Ecuador; foliar nutrients; nutrient manipulation; nutrient manipulation experiment; root-shoot ratios; specific leaf area.

HUMAN ALTERATION OF NUTRIENT AVAILABILITY IN ECOSYSTEMS IS ONE OF THE MOST SEVERE ENVIRONMENTAL PROBLEMS OF OUR TIME. Changes in the availability of phosphorus (P) and nitrogen (N) are supposed to affect ecosystem dynamics in tropical forests (Tanner *et al.* 1998, Lewis & Tanner 2000, Elser *et al.* 2007, Vitousek *et al.* 2010, Harpole *et al.* 2011, Homeier *et al.* 2012, 2017, Fisher *et al.* 2013, Peñuelas *et al.* 2013). Sources of N inputs include N compounds emitted from farming, livestock, and combustion of fossil fuel and biomass burning (Galloway *et al.* 2008, Peñuelas *et al.* 2013), whereas the only sources of atmospheric P deposition are mineral aerosols (Mahowald *et al.* 2005, 2008, Peñuelas *et al.* 2013). The deposition of P is always small compared to N deposition.

Previous studies have concluded that primary production in tropical lowland forests on old soils is likely to be limited by P, whereas in tropical montane forests on younger soils, N is more likely to be limiting (Tanner *et al.* 1998, Unger *et al.* 2010, Vitousek *et al.* 2010, Wolf *et al.* 2011, Fisher *et al.* 2013). Apart from this general elevation-dependent pattern, the specific local

conditions (temperature, precipitation, soil type, and local biota) determine whether an element limits plant growth or not.

Several fertilization experiments in tropical forests have studied how juvenile trees respond to the addition of limiting nutrients in different forest types (tropical dry forest: Ceccon *et al.* 2003, subtropical moist forest: Lu *et al.* 2010, tropical rain forest: Santiago *et al.* 2012). According to these studies, seedlings showed varying responses to nutrient addition (Palmiotto *et al.* 2004, Yavitt & Wright 2008, Eichhorn *et al.* 2010, Santiago *et al.* 2012, Alvarez-Clare *et al.* 2013). Some studies demonstrated that juvenile trees respond to N and/or P addition by increased height growth or producing additional leaves (Bungard *et al.* 2000, Yavitt & Wright 2008, Zalamea *et al.* 2016). However, from the few studies carried out so far with different tree species across a wide range of tropical forest ecosystems, it is not possible to deduce general patterns of seedling growth responses to nutrient addition.

Foliar nutrient concentrations after fertilization can represent a suitable means for detecting nutrient limitation of ecosystems (Ostertag & DiManno 2016). However, for some elements, a change in nutrient concentration may not result in a change in physiological function, and vice versa, a change in function may

not be accompanied by a change in concentration. For example, Santiago *et al.* (2012) showed that K addition could stimulate juvenile tree growth but that foliar K concentrations were not affected. Likewise, Cárate-Tandalla *et al.* (2015) observed increased diameter growth for *Pouteria torta* seedlings after N or P addition but no change in foliar concentrations of these elements. However, some studies have reported luxury consumption of P following P additions in tropical plant species, which were not accompanied by observed functional responses (Ostertag 2010, Mayor *et al.* 2013).

In earlier studies, tropical tree seedlings responded to an increase in N and/or P availability with higher specific leaf area (SLA) (*e.g.*, Burslem *et al.* 1995, Cárate-Tandalla *et al.* 2015, Wan Juliana *et al.* 2009), higher foliar nutrient concentrations (*e.g.*, Palmiotto *et al.* 2004, Santiago *et al.* 2012, Ostertag & DiManno 2016), and altered N:P ratios (*e.g.*, Schreeg *et al.* 2014, Ostertag & DiManno 2016). In several fertilization experiments, fertilized plants were more attractive to herbivores than control plants due to thinner leaves and higher nutrient contents (*e.g.*, Campo & Dirzo 2003, Andersen *et al.* 2010, Santiago *et al.* 2012, Cárate-Tandalla *et al.* 2015). The availability of nutrients is, apart from light, one of the determinants of C allocation patterns in tree seedlings. Increased nutrient availability is predicted to result in a higher leaf mass fraction and a reduced root mass fraction (Paz 2003, Poorter *et al.* 2012).

Few studies have examined how tropical rain forest tree seedlings perform under experimentally modified N and P levels *in situ* and whether changes in nutrient availability result in morphological adaptations (Kobe 1999, Baraloto *et al.* 2006, Dent & Burslem 2009, Wan Juliana *et al.* 2009). Although tropical plot-level fertilization studies began in montane forests (see compilation in Tanner *et al.* 1998), most of our current knowledge on the effects of increasing nutrient availability in the tropics comes from lowland forests. Many studies have focused on ecosystem processes, such as net primary productivity, nutrient cycling, or growth of canopy trees, but the response of the seedling community in tropical montane forests has thus far been neglected (Andersen *et al.* 2014, Cárate-Tandalla *et al.* 2015).

The Ecuadorian Nutrient Manipulation Experiment (NUMEX) was started in 2008 to study the role of N and P limitation in controlling tropical montane forest functioning, with the goal of simulating the effects of future increases in atmospheric nutrient deposition on ecosystem processes (Homeier *et al.* 2012, 2013). Along the experiment's elevation gradient (1000–3000 m asl), forest structure and species composition change predictably related to environmental conditions; with increasing elevation, temperature decreases, precipitation increases, and forest productivity and soil N mineralization decrease (Leuschner *et al.* 2007, Bendix *et al.* 2008, Homeier *et al.* 2010, Wolf *et al.* 2011). Leuschner *et al.* (2007) showed that the root:shoot ratios of trees increased from 1000 to 3000 m asl, likely due to less favorable soil conditions at upper elevations, namely low N availability. Levels of herbivory have also been linked to soil nutrient availability, as reflected in foliar N and P concentrations (Werner & Homeier 2015). High average foliar N:P ratios ($\geq$22) measured in non-fertilized trees at all three study sites (average of $\geq$65 trees from $\geq$16 plant families per site, Homeier *et al.* unpubl. data) suggest that P is the prevailing limiting nutrient for plant growth in this study region (Townsend *et al.* 2007).

Continued nutrient addition in NUMEX has altered several ecosystem state variables after the first years and is increasingly affecting biological and biogeochemical processes that may influence seedling vitality, growth, and survival. For mature trees of common species at 2000 m asl, Homeier *et al.* (2012) reported a positive growth response after N (two of four studied species), P (two of four species), and N+P addition (three out of four species). They also observed increased foliar N and P concentrations in three of the four study species after N+P addition, suggesting that N and P might be co-limiting in the studied montane forest. At the same site, Camenzind *et al.* (2014) detected reductions in the abundance and species richness of arbuscular mycorrhizal fungi (AMF) after N and P addition, and Krashevska *et al.* (2013) found that soil microorganisms were generally limited by N at all elevations and saprophytic fungi were limited also by P. Moreover, at 1000 m asl, P (but not N) addition increased the amount of water-stable soil macro-aggregates, which play an important role in soil stability and nutrient cycling (Camenzind *et al.* 2016b). Four years of N and N+P addition (but not of P addition alone) resulted in increased mineral N production and decreased microbial N retention (Baldos *et al.* 2015). In addition, the topsoil NO_3^- concentration increased in the N- and N+P-treated plots but declined when only P was added (Müller *et al.* 2015, Table S1). Separate N addition led to an increased NO_3^- loss, while separate P and combined N+P amendments reduced leaching of both NO_3^- and NH_4^+ from the organic layer (Velescu *et al.* 2016). Dietrich *et al.* (2016) found that P and N+P addition increased the bioavailable inorganic P concentration and decreased the phosphatase activity in the organic layer (Table S1). Even canopy soils were affected by ground-level nutrient additions and showed increased N cycling after combined N and P addition (Matson *et al.* 2014).

Building on previous work at NUMEX, we sought to understand seedling responses to continued N and P addition for several tree species common in old-growth montane forests. We were especially interested in adaptive modifications of leaf morphology and chemistry, and altered C allocation patterns toward roots, whereby nutrient uptake capacity would be increased. Such modifications in response to nutrient addition could give insights into prevailing growth limitation at different elevations by N, P, or both elements. With reference to earlier results from the study region and published data from other tropical montane forests, we assumed that the tree seedlings were limited by both N and P at the NUMEX control sites; thus, N and P addition should partly release seedlings from nutrient limitation. We hypothesized that N and P addition would lead to (1) higher SLA, a greater leaf area fraction, and increased foliar N and P concentrations; (2) higher herbivory levels due to herbivore preference for higher quality food; and (3) reduced carbohydrate allocation to the root system, a lowered root:shoot ratio, and a reduced mycorrhizal infection rate of the roots.

METHODS

STUDY SITES.—The study was conducted at the NUMEX sites, in the tropical montane forests of the southern Andes of Ecuador within Podocarpus National Park (PNP) and the San Francisco Reserve (SFR), east of the city of Loja (Fig. S1). Three study sites were selected along an elevation gradient at ca. 1000 m asl. (Bombuscaro), ca. 2000 m asl (San Francisco), and ca. 3000 m asl (Cajanuma). All study sites comprise evergreen old-growth forest (Homeier *et al.* 2013) with noticeable differences in climate, soil characteristics, and forest structure (Tables 1 and S1).

The climate of the study area is tropical humid with a wet season from April to July and a lesshumid period from September to December (Emck 2007). Mean annual temperature decreases with elevation at a lapse rate of ~5°C/km from 19.4°C at 1000 m asl to 9.4°C at 3000 m asl. Annual precipitation increases from about 2000 mm at 1000 and 2000 m asl to 4500 mm at 3000 m asl (Bendix *et al.* 2008, Wittich *et al.* 2014, Wolf *et al.* 2011).

Soils are Dystric Cambisols at the 1000 m asl site, Stagnic Cambisols at 2000 m and Stagnic Histosols at 3000 m asl (Wolf *et al.* 2011, Homeier *et al.* 2013). At the two upper sites, soils are periodically influenced by surface water and show in some parts chemically reducing conditions.

The forests at the three study sites differ in structure and species composition (Homeier *et al.* 2008). Evergreen premontane rain forest (PMF) that reaches up to 40 m height occurs at the lowest site. Evergreen lower montane rain forest (LMF) is present at the second site at 2000 m asl with a canopy height of 18 to 22 m. Evergreen upper montane rain forest (UMF) with a canopy height rarely exceeding 8 to 10 m occurs at the highest site. At the NUMEX sites, canopy openness increased with elevation from 9.7 ± 0.3% (mean ± SE) at 1000 m asl to 9.8 ± 0.4% at 2000 m asl, and to 16.4 ± 0.9% at 3000 m asl.

EXPERIMENTAL DESIGN.—The Ecuadorian Nutrient Manipulation Experiment was designed to examine the effects of moderate nutrient deposition on tropical montane forests at different elevations. Within a full-factorial experimental approach set up at each study site (1000, 2000, and 3000 m asl), 16 plots of 400 m^2 each (20 m × 20 m) were established. These accommodate four treatments (N, P, N + P, control) in fourfold replication. One replicate plot per treatment was assigned to four different blocks in which they were placed randomly. The plots were installed in old-growth, closed-canopy stands without visible signs of human or natural disturbance and were at least 10 m distant to each other. N and P were added to the plots at an annual rate of 50 kg N/ha (as urea, CH_4N_2O) and 10 kg P/ha (as NaH_2PO_4). The nutrients were dispersed homogeneously within the plots on two application dates per year (in February, before the start of the more humid season, and in August, before the start of the drier months). NUMEX was started in February 2008 and our sampling took place in 2013.

For this study on seedling responses, we collected naturally occurring individuals of the two most common tree species at each elevation level. We selected *Clarisia racemosa* (Moraceae) and *Pouteria torta* (Sapotaceae) at 1000 m asl, *Graffenrieda emarginata* (Melastomataceae) and *Palicourea angustifolia* (Rubiaceae) at 2000 m asl, and *Graffenrieda harlingii* (Melastomataceae) and *Hedyosmum purpurascens* (Chloranthaceae) at 3000 m asl, which represented the most abundant species observed in the tree seedling communities at the respective sites (Cárate-Tandalla *et al.* unpubl. data from repeated seedling censuses in all study plots between 2011 and 2013). We attempted to harvest 20 individual seedlings of each species equally distributed within the four treatment plots. We selected individuals aged ca. 2–3 years and did not give preference to any individuals in terms of height, stem diameter, or leaf number. We estimated the age of the seedlings from seedling monitoring data collected in tree regeneration plots in all NUMEX sites (Cárate-Tandalla *et al.* unpubl. data). We omitted plants growing in recent understory gaps. As the seedling density of the six target species varied considerably in these species-rich forests, we were not able to study 20 seedling individuals in each species and treatment. Where more than five seedlings of a species were present in one plot, we chose the five harvested individuals randomly. Details on sampled replicate numbers in the different plots and the size of the sampled seedlings are given in Table S2. All species occurred in all plots at their corresponding elevation level, except *Pouteria* (three of the four N addition plots at 1000 m asl) and *Hedyosmum* (three of four plots in the N and the N+P treatment at 3000 m asl). Total number of sampled seedlings per treatment differed between the species (*Clarisia*: 18–

TABLE 1. *Physiogeographic and stand structural characteristics of the three study sites.*

Site characteristics	1000 m asl	2000 m asl	3000 m asl
Location			
Altitudinal range[a]	990–1100 m asl	2020–2100 m asl	2900–3020 m asl
Coordinates[a]	4°7′ S, 78°58′ W	3° 58′ S, 79° 11′ W	4° 7′ S, 79° 11′ W
Temperature[b]	19.4°C	15.7°C	9.4°C
Mean precipitation[b]	2230 mm	1950 mm	4500 mm
Forest structure			
Canopy height[a]	20–25 m	10–14 m	6–8 m
Tree density[a]	748 trees/ha	1143 tree/ha	1305 trees/ha
Basal area[a]	33.4 m^2/ha	22.8 m^2/ha	25.5 m^2/ha
Vegetation type[a]	Evergreen premontane forest	Evergreen lower montane forest	Evergreen upper montane forest
Soil type[a]	Dystric Cambisol	Stagnic Cambisol	Stagnic Histosol
Organic layer depth[a]	<1 cm	10–30 cm	10–40 cm

[a]Homeier *et al.* (2013).
[b]Wittich *et al.* (2014).

20 individuals, *Pouteria*: 12–20, *G. emarginata*: 20, *Palicourea*: 19–20, *G. harlingii*: 15–20, *Hedyosmum*: 14–20).

Immediately before seedling harvest in June 2013, we measured every individual (stem diameter, stem length, number of leaves, length, and width of the largest leaf) and photographed them. We completely removed seedlings from the soil and later separated them into stem, root, and leaf fractions. We tried to keep the root systems of the target seedlings intact by digging at least to a depth similar to the aboveground height of the plant and a breadth of double its crown diameter. We scanned fresh leaves at 150 dpi (Canon LIDE 100) and determined total leaf area and leaf area loss by herbivory with WinFolia 2005b (Régent Instruments Inc., Quebec City, QC, Canada) software. We dried all fractions for two days at 65°C and then weighed them. We calculated specific leaf area (SLA) and leaf area ratio (LAR) based on total leaf area per plant per total plant dry mass. We quantified patterns of biomass partitioning through root:shoot mass ratio and the root, stem, and leaf fractions, which we calculated after Poorter and Nagel (2000). Additionally, we estimated stem slenderness as the relationship between stem length and basal diameter. We sent dried leaves to the laboratory of the Department of Plant Ecology, Göttingen, and analyzed them for foliar C, N, and P concentrations using a C/N elemental analyzer (Vario EL III, Elementar, Hanau, Germany) and Inductively Coupled Plasma Analysis (Optima 5300DV ICP-OES, Perkin Elmer, Waltham, Massachusetts, USA) in the case of total P after digesting the leaf samples with HNO_3. We derived both foliar C:N and N:P mass ratios. We sent dried root material to the laboratory of the Department of Ecology, University of Berlin, Germany, for mycorrhizal analyses.

MYCORRHIZAL INFECTION.—We determined the intra-radical abundance of mycorrhizal fungi in roots stained with Trypan Blue according to a modified staining protocol (Phillips & Hayman 1970, Camenzind & Rilling 2013). We stained roots with 0.05% Trypan Blue at 60°C after an extended clearing phase with 10% KOH at 60°C for 12 h followed by exposure to 20% H_2O_2 at room temperature for 20–30 min. Exact staining periods depended on the root characteristics of the respective tree species. We determined the percentage of arbuscular mycorrhizal and ectomycorrhizal root colonization at 200× magnification following the line-intersect method of McGonigle *et al.* (1990). In the case of *Pouteria torta*, determination of AMF abundance was not possible due to the predominance of a thick taproot and an insufficient amount of fine roots.

STATISTICAL ANALYSES.—We performed all statistical analyses using R 3.1.1 software (R Development Core Team 2014). We evaluated the seedling attributes mentioned above using linear mixed model analysis (LMM). Applying the function "lmer" of the R package "lme4" (Bates *et al.* 2014), we contrasted the treatments with the control. We included "block" as a random intercept in all cases to account for natural variability within sites. Since we found significant relationships between several of the observed parameters and plant height in some of the study species, we incorporated "stem length" as a fixed effect in an alternative model (except the model for AMF root colonization). We then compared the two main models with and without "stem length" using a likelihood-ratio test (ANOVA). Predicted values for the fitted model were evaluated by calculating mean values using restricted maximum likelihood (REML). P-values were estimated with the function "cftest" from the package "multcomp" (Hothorn *et al.* 2008). To scale the predicted values, we calculated confidence intervals at 95% with the function "confint." Where necessary, we transformed variables prior to analysis (using logarithmic or square root transformation, or logit transformation in the case of percentages) to fit assumptions of normality.

RESULTS

OVERVIEW.—The six studied species showed species-specific responses to nutrient addition in the morphological traits examined. In general, seedlings from the two upper sites (2000 m asl and 3000 m asl) responded more strongly to nutrient addition than those of the two species of the lowermost sites (1000 m asl, Table 2).

LEAF MORPHOLOGY AND HERBIVORY.—The two species at 1000 m asl (*Clarisia racemosa* and *Pouteria torta*) showed contrasting changes in leaf morphology in response to nutrient addition. The leaves of *Pouteria* had a lower SLA and LAR than the control plants, while the *Clarisia* seedlings showed no detectable effects (Fig. 1, Table S3). At 2000 m asl, *Palicourea angustifolia* showed no change in SLA or LAR after nutrient addition, but *Graffenrieda emarginata* increased in SLA after N and N+P addition, whereas its LAR was lower after N + P addition. At 3000 m asl, both species (*Graffenrieda harlingii* and *Hedyosmum purpuracens*) had higher average SLA values in all three nutrient addition treatments. Significantly higher SLA and LAR values were found only for *Hedyosmum* in the two P treatments (Fig. 1, Table S3). *G. harlingii* (3000 m asl) had higher LAR values in both N treatments.

At 1000 m asl and 3000 m asl, we found no significant nutrient addition effects on herbivory rate. However, at 1000 m asl, *P. torta* showed a trend toward higher herbivory in response mainly to P and N+P addition (Fig. 2, Table S3). *G. emarginata* at 2000 m asl was the only species that showed higher leaf area consumption after N and N+P addition.

FOLIAR NUTRIENTS.—In the control plots, the *Clarisia* (1000 m asl) seedlings had the highest foliar N concentrations of the six species (mean = 27.3 mg/g), while the two *Graffenrieda* species (at 2000 and 3000 m asl) showed the lowest means (both <12 mg/g) (Fig. 3, Table S3). All species had at least slightly increased foliar N concentrations in the N and N+P treatments. Foliar N was significantly increased in four species in the N+P treatment (*Clarisia*, 1000 m asl; *G. emarginata*, 2000 m asl; *G. harlingii* and *Hedyosmum*, 3000 m asl), and in three species in the N treatment (*Palicourea*, 1000 m asl; *G. emarginata*, 2000 m asl; and *G. harlingii*, 3000 m asl).

Among the control plants, the two species from 1000 m asl had the highest P concentrations (*Pouteria*: 0.92 mg/g, *Clarisia*:
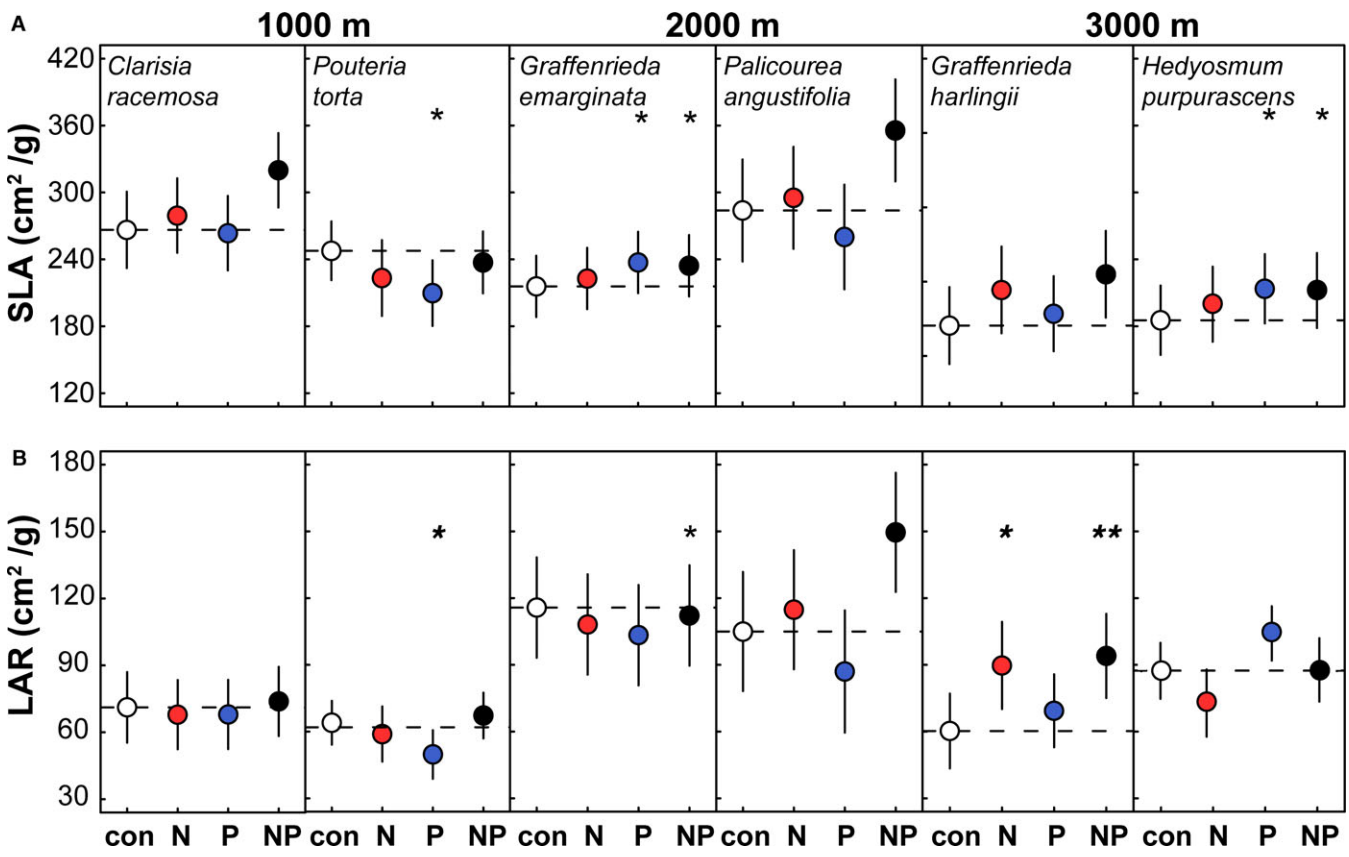
FIGURE 1. Morphological leaf attributes of the seedlings of six common tree species after nutrient addition. Addition of N (red), P (blue), or combined N and P (black). Mean values were obtained from fitted models and scaled to 95% confidence intervals. A) Specific leaf area (SLA), B) Leaf area ratio (LAR). Significance levels are as follows: * $P < 0.05$, ** $P < 0.01$. Stem length had a negative effect on both SLA (significant in *Clarisia racemosa, Graffenrieda emarginata, Palicourea angustifolia,* and *Graffenrieda harlingii*) and LAR (significant in *Clarisia racemosa, Graffenrieda emarginata, Palicourea angustifolia, Graffenrieda harlingii,* and *Hedyosmum purpurascens*).
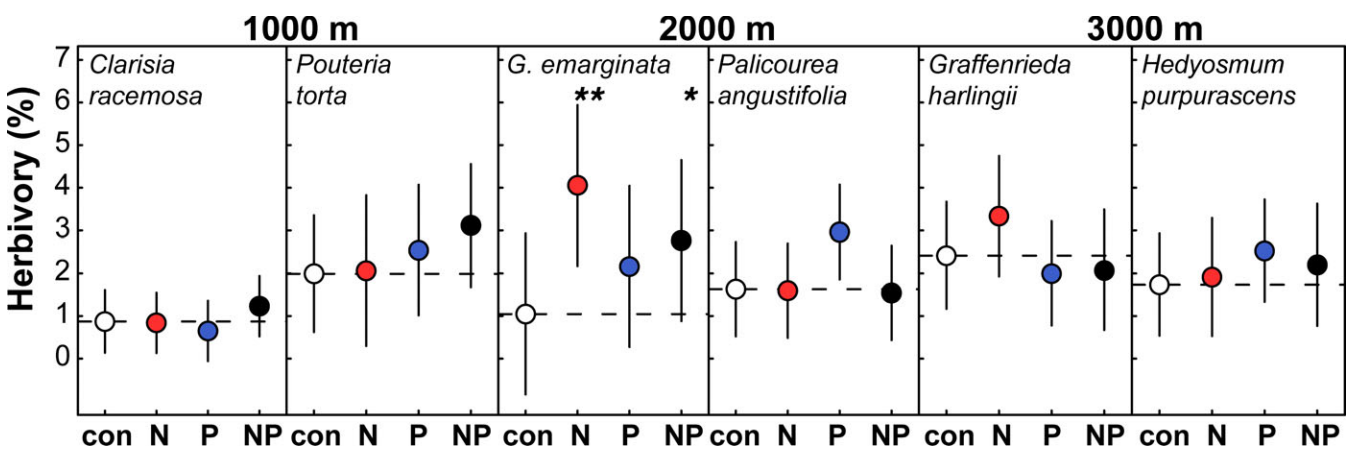

FIGURE 2. Leaf area loss due to herbivory in the seedlings of six common tree species after nutrient addition. Additions of N (red), P (blue), or combined N and P (black). Mean values were obtained from fitted models and scaled to 95% confidence intervals. Significance levels are as follows: * $P < 0.05$, ** $P < 0.01$. Stem length had a positive effect on herbivory (significant in *Palicourea angustifolia, Graffenrieda harlingii,* and *Hedyosmum purpurascens*).

1.11 mg/g), while the four species from the 2000 and 3000 m asl sites all had lower means, in the range of 0.49–0.69 mg/g (Fig. 3, Table S3). Higher foliar P concentrations were found in five species (all except *Clarisia*) after P addition, in four species (*Pouteria*, 1000 m asl; *G. emarginata*, 2000 m asl; *G. harlingii* and *Hedyosmum*, 3000 m asl) after combined N and P addition, and in *Clarisia*
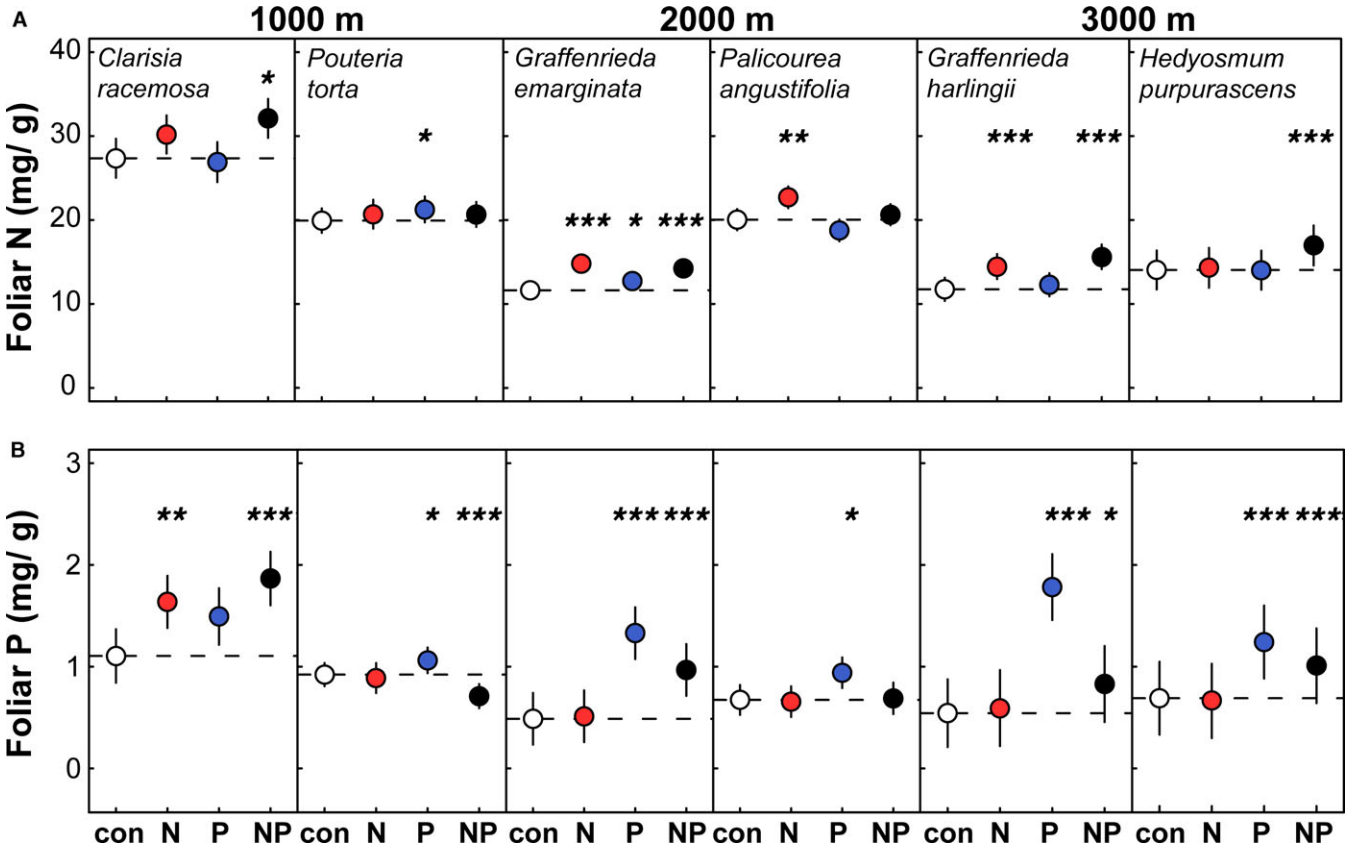
FIGURE 3. Foliar N and P concentrations in the seedlings of six common tree species after nutrient addition. Additions of N (red), P (blue), or combined N and P (black). Mean values were obtained from fitted models and scaled to 95% confidence intervals. A) Foliar N concentration; B) Foliar P concentration. Significance levels are as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Stem length had a negative effect on both foliar N (significant in *Clarisia racemosa* and *Pouteria torta*) and foliar P (significant only in *Pouteria torta*).
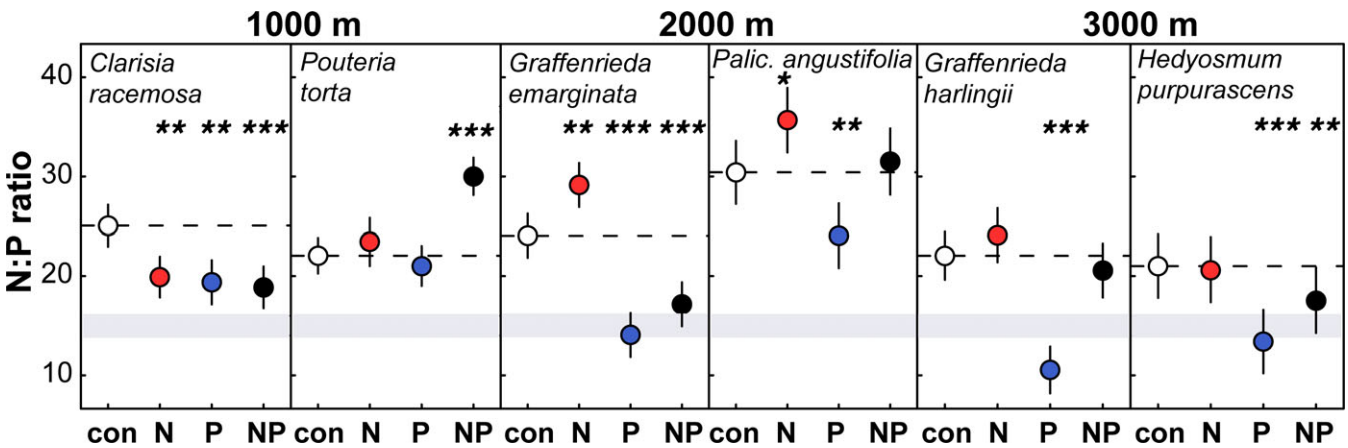

FIGURE 4. Foliar N:P ratios in the seedlings of six common tree species after nutrient additions. Additions of N (red), P (blue), or combined N and P (black). Following Townsend *et al.* (2007) and Aerts and Chapin (2000), values of <14 suggest N limitation, while P limitation is given at values of >16; the range between these two thresholds is highlighted by the light gray area. Mean values were obtained from fitted models and scaled to 95% confidence intervals. Significance levels are as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Stem length had a positive effect on leaf N:P ratio (significant only in *Pouteria torta*).
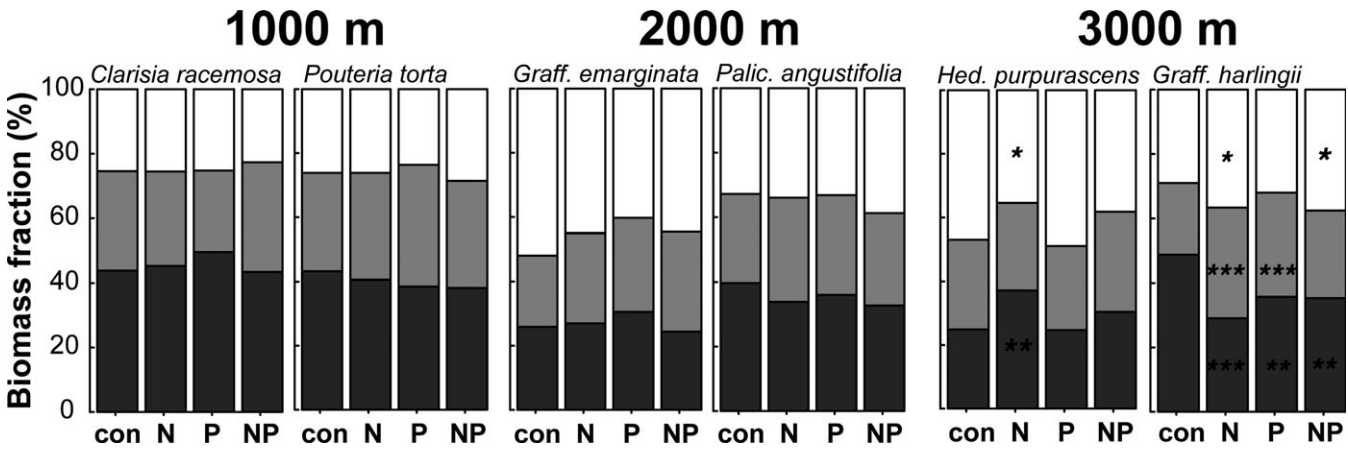
FIGURE 5. Comparison of the proportions of tissue biomass in six common tree seedling species after addition of N or/and P. Biomass proportions assigned to roots (black), shoots (gray), and leaves (white). Significance levels are as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Stem length had a significant negative effect on the root fraction *in Pouteria torta* and a positive effect on the root fraction in *Graffenrieda harlingii*, a positive effect on stem fraction (significant in *Clarisia racemosa, Graffenrieda emarginata, Palicourea angustifolia, Graffenrieda harlingii,* and *Hedyosmum purpuracens*), and a negative effect on leaf fraction (significant in *Graffenrieda emarginata* and *Graffenrieda harlingii*).
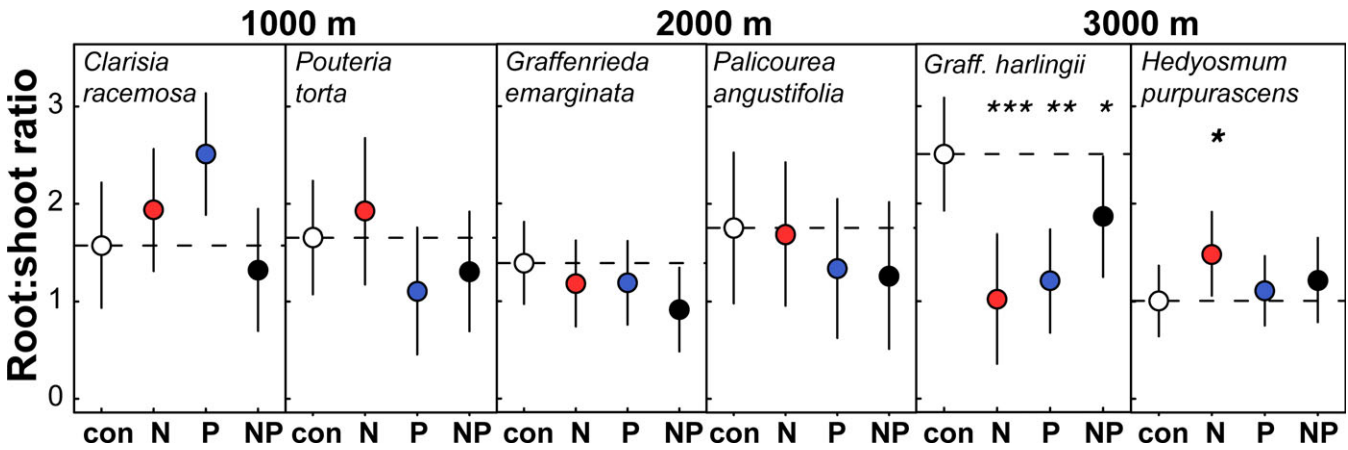

FIGURE 6. Root:shoot ratios of the seedlings of six common tree species after nutrient addition. Additions of N (red), P (blue), or combined N and P (black). Mean values were obtained from fitted models and scaled to 95% confidence intervals. Significance levels are as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Stem length had a significant negative effect on the root:shoot ratio in *Palicourea angustifolia*.

(1000 m asl), foliar P was also increased in the N treatment. In contrast to the other species, *Pouteria* (1000 m asl) showed significantly reduced foliar P concentrations in the N+P treatment.

All species had foliar N:P ratios of above 20 in the control plots; the highest ratio was found at 2000 m asl in *Palicourea* (30.4). The two species from 3000 m asl had the smallest N:P ratios (*G. harlingii*: 22.0, *Hedyosmum*: 21.0) (Fig. 4, Table S3). In all species except *Pouteria*, the N:P ratio was reduced in the P treatment. In three species (*Clarisia*, 1000 m asl; *G. emarginata*, 2000 m asl and *Hedyosmum*, 3000 m asl), combined N and P addition also resulted in a significantly decreased N:P ratio. By contrast, N addition increased the N:P ratio of both species at 2000 m asl (*G. emarginata* and *Palicourea*), while it decreased the ratio in *Clarisia* seedlings at 1000 m asl.

BIOMASS ALLOCATION.—Comparing the fraction of leaves, stem and roots in total seedling biomass, we found no effects of nutrient addition at 1000 m asl and 2000 m asl (Fig. 5, Table S3). At 3000 m asl, in *G. harlingii,* the stem fraction was larger than in the control in the N and the P treatments (Fig. 5). Leaf fractions of this species were increased after N and N+ P addition. In addition, this species decreased its root fraction in all treatments. At the same elevation, *Hedyosmum* showed a smaller leaf fraction and an increase in the root fraction in response to N addition.

We found no systematic change in the root:shoot ratio with elevation. In all species, the root fraction contributed 50% or more of total biomass (Fig. 6, Table S3). At the two lower elevations, there was no difference in root:shoot ratio between control
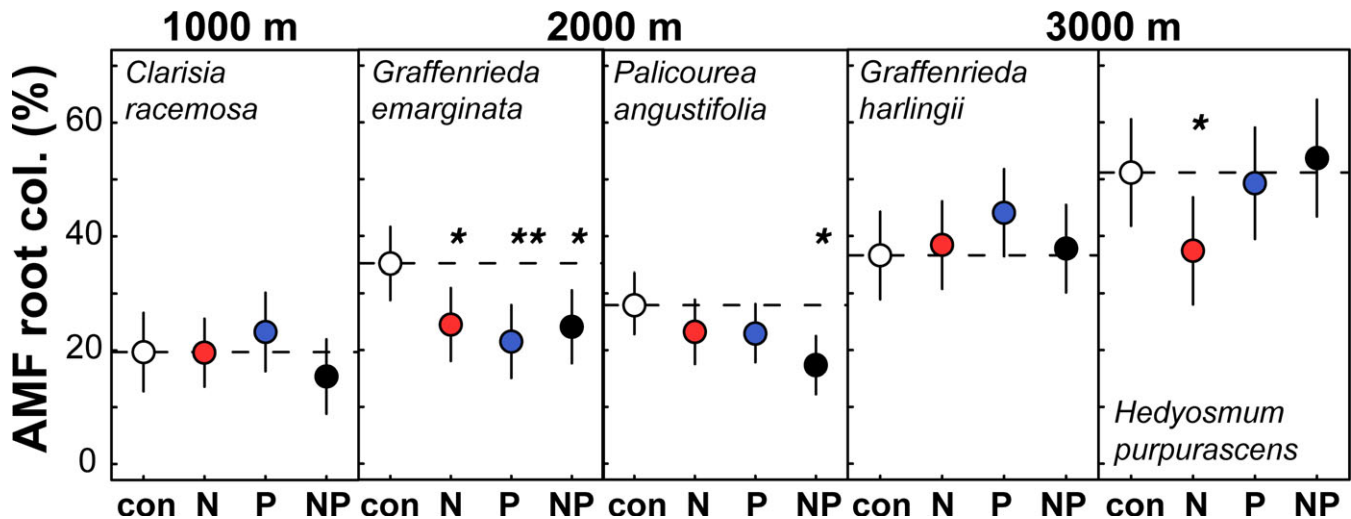
FIGURE 7. Response in the percentage of AMF root colonization in seedlings of five common tree species to nutrient addition. Additions of N (red), P (blue), or combined N and P (black). Mean values were obtained from fitted models and scaled to 95% confidence intervals. Significance levels are as follows: * $P < 0.05$, ** $P < 0.01$.

and nutrient addition for any of the species. At 3000 m asl, *G. harlingii* seedlings from all treatments had lower root:shoot ratio than control due to a reduced root fraction. By contrast, *Hedyosmum* (3000 m asl) showed an increased root:shoot ratio in N addition plots.

MYCORRHIZAL COLONIZATION.—AMF represented the dominant mycorrhizal group in all seedlings, though the two *Graffenrieda* species (2000 and 3000 m asl) were co-colonized by ectomycorrhizae, with an average colonization rate of 2% at 2000 m asl in *G. emarginata* and 3% at 3000 m asl in *G. harlingii* (data not presented; no significant treatment effects observed). The amount of

AMF root colonization generally increased with elevation in the control plots (Fig. 7). Strongest treatment effects were observed at 2000 m asl: in *G. emarginata*, all three treatments significantly decreased AMF abundance, whereas in *Palicourea*, only the combined N+P addition exhibited a negative effect. At 1000 m asl, no difference to the control was found; at 3000 m asl, a significant AMF reduction was observed only in *Hedyosmum* after N addition.

DISCUSSION

GENERAL SEEDLING RESPONSES.—Our results show that seedling responses to nutrient addition are highly species-specific at each elevation level (Table 2). These results are similar to those of Pivovaroff *et al*. (2016), who reported different functional and physiological responses of four shrub species to long-term nitrogen addition in a Californian semi-arid ecosystem. In general, strongest effects were found at 2000 and 3000 m asl, and some species were more responsive to nutrient addition than others.

FOLIAR NUTRIENT CONCENTRATION AND HERBIVORY.—Leaf traits are important for determining the establishment and survival of tree seedlings under varying site conditions. A trade-off exists between high leaf longevity, leaf toughness, and resistance to herbivory on the one hand, and high SLA, foliar nutrient concentrations, and seedling growth rates on the other (*e.g.*, Poorter & Bongers 2006, Wright *et al*. 2010, Kitajima *et al*. 2013, Philipson *et al*. 2014). The high leaf tissue N:P ratios (>20 in the control plants of all species) suggest that P might limit plant growth at our study sites more than N (Townsend *et al*. 2007). This matches information gained from foliar N:P ratios data from mature trees in the same study sites (average foliar N:P ratios of ≥ 22 were measured in non-fertilized trees at all three study sites,

TABLE 2. *Summary of seedling responses to continued nutrient addition. Symbols represent significant (P < 0.05) positive (+) or negative (−) effects in one species, or contrasting (+/−) effects in the two species per elevation. Double symbols indicate that both species responded in the same way.*

Seedling parameters	1000 m asl			2000 m asl			3000 m asl		
	N	P	N+P	N	P	N+P	N	P	N+P
SLA (cm²/g)		−			+	+		+	+
LAR (cm²/g)		−				+	+		+
Herbivory (%)				+		+			
Foliar N (mg/g)		+	+	++	+	+	+		++
Foliar P (mg/g)	+	+	+/−		++	+		++	++
Foliar N:P ratio	−	−	+/−	++	−	−		−	−
Leaf fraction (%)							+/−		+
Stem fraction (%)							+	+	
Root fraction (%)							+/−	−	−
Root:shoot ratio							+/−	−	−
AMF colonization (%)				−	−	−	−		

Homeier *et al.* unpubl. data). The strong effect of P addition on foliar P concentration and N:P ratios (significant in five species) points at the limiting role of P. However, this effect must be interpreted with caution, as it could also be at least partly due to luxury consumption. Except for *Pouteria* (which showed a reduced foliar P concentration after combined N + P addition), all species responded positively to both added nutrients. Our results fit with those reported by Homeier *et al.* (2012), who found a reduction of foliar N:P ratios after P addition in mature trees of *Graffenrieda emarginata* and two other common tree species (three of the four studied species) after the experiment's first year at the 2000 m asl site. Several studies have found an increase in foliar nutrient concentrations in shade-tolerant tree seedlings after fertilization (Burslem *et al.* 1995, Lawrence 2001, Andersen *et al.* 2010, Santiago *et al.* 2012). Higher leaf tissue P concentrations after N addition as observed in *Clarisia* might be caused by increased P acquisition due to higher external phosphatase activity. Such an effect has been observed by Treseder and Vitousek (2001) in a similar experiment conducted in Hawaiian rain forests. The higher accumulation of foliar P compared to foliar N suggests that there is some P luxury consumption in all species. Luxury consumption of P was reported for several tropical plant species after P addition (Ostertag 2010, Mayor *et al.* 2013), but other species did not respond (*e.g.*, six tropical lowland tree species in Costa Rica; Alvarez-Clare & Mack 2015) or even exhibited lower P concentrations, similar to *Pouteria* in our study after combined N + P addition (*e.g.*, the palm *Oenocarpus mapora*; Mayor *et al.* 2013). Studies on tropical tree seedlings (Schreeg *et al.* 2014) and trees (Mo *et al.* 2015) suggest that changes in nutrient concentrations after nutrient addition may become more visible in older leaves, stem tissue, or roots because these tissues can be used as nutrient reservoirs and the foliar N:P ratios of young leaves need to be maintained in the range of optimal performance.

We only found significant effects of nutrient addition on the herbivory rate in *G. emarginata* at 2000 m asl, for which leaf area loss was higher after N and N+P addition, related to higher foliar N concentrations in both treatments. At this site, an earlier study reported increased herbivory on the stand level with higher foliar N and P concentrations (Werner & Homeier 2015). For seedlings of *Pouteria torta* that were transplanted for one year to our study plots at 1000 m asl (2011–2012), Cárate-Tandalla *et al.* (2015) reported an increase in herbivory in the P- and N+P-treated plots. By contrast, our results show only slightly (but not significantly) higher leaf area loss in this species in the same treatments. These contradicting observations may result from inter-annual variation of herbivore pressure. Herbivory in response to elevated foliar N levels is a well-studied phenomenon in tropical forests (Campo & Dirzo 2003, Eichhorn *et al.* 2010), which is associated with a preferential choice by predators searching for high-quality food (Andersen *et al.* 2010). Increased leaf damage by herbivory after P addition was also reported by Santiago *et al.* (2012) from a fertilization experiment in Panama where the consumption by herbivores in seedlings was higher after P or K addition. These studies show that plant selection by herbivores is strongly driven by resource quality (*i.e.*, nutrient content and palatability of leaves). Damage by herbivores may result from an overall reduction of photosynthetic leaf area but may also be caused by a lowering of photosynthesis rates in partially damaged leaves (Zangerl *et al.* 2002, Eichhorn *et al.* 2010). Thus, herbivory can be an important factor structuring seedling communities by negatively affecting the growth and survival of plants or, at least, mediating the responses to increased nutrient availability (*e.g.*, Andersen *et al.* 2010, Eichhorn *et al.* 2010, Barton & Hanley, 2013). Many herbivore species are adapted to specific host taxa, and changes in herbivory rate after nutrient addition may depend on the ability of the herbivores to locate and detect fertilized plants. In this way, our experiment may also be seen as a test of the responsiveness of herbivores to changed resource availability.

BIOMASS PARTITIONING AND MYCORRHIZAL COLONIZATION.—The carbohydrate allocation patterns observed in the six studied species indicate different strategies with respect to nutrient uptake and accumulation. In accordance with the concept of functional equilibrium discussed by Poorter *et al.* (2012) and Johnson (2010), seedlings allocate more resources to roots and mycorrhizal structures if nutrients or water are limiting growth, while light limitation should promote shoot growth over root growth. Markesteijn and Poorter (2009) reported an average root mass fraction (RMF) of 0.3 in seedlings of 37 tropical moist forest tree species; Paz (2003) found RMF site means of between 0.12 and 0.31 for seedlings of 55 tree species collected in four neotropical moist lowland forests and Poorter *et al.* (2012) reported an average RMF of 0.16 in a meta-analysis using ca. 850 tropical forest species. In our study, four of the six tropical montane forest species partitioned 40% or more of their biomass to the root system, indicating that these species prioritize belowground resource acquisition. Such a strategy may be interpreted as an adaptation to the generally low nutrient availability at our study sites, as is indicated by the observed stimulation of tree root growth after nutrient addition in the ingrowth core experiment of Graefe *et al.* (2010). Relatively low root:shoot ratios were observed in our experiment in *G. emarginata* (at 2000 m asl) and *Hedyosmum* (at 3000 m asl). A possible explanation is that these species are relatively efficient in nutrient acquisition in unfertilized soil due to their mycorrhizal status: *G. emarginata* shows dual colonization by AMF and ectomycorrhizae (Haug *et al.* 2004, Kottke *et al.* 2004) and *Hedyosmum* had the highest colonization rate by AMF.

In response to N and P addition, we expected a change in biomass partitioning toward aboveground organs in the seedlings (*e.g.*, Marschner 1995, Poorter *et al.* 2012). However, *G. harlingii* (3000 m asl) was the only species that reduced its root mass fraction while increasing leaf and/or stem biomass after N, P and N+P addition. This suggests that seedling height growth is promoted in *G. harlingii* by both N and P addition, probably in part driven by a higher photosynthetic capacity due to increased foliar N concentrations. The percentage of AMF root colonization was reduced in *G. emarginata* after N and P addition, whereas there were no changes in *G. harlingii*. This fact points to different strategies of these congeners with respect to carbohydrate

investment in nutrient uptake structures in response to increased N and P availability (Ericsson 1995, Johnson 2010). The fact that strongest mycorrhization responses to nutrient addition were found at 2000 m asl is in line with results obtained from the investigation of mixed tree root samples from the same three study sites (Camenzind *et al.* 2016a). However, species differences are likely important, since the direction of mycorrhization responses differed in this study.

Both N and P caused changes in seedling morphology, chemistry, and allocation patterns, and we assume that both elements are limiting tree seedlings in our stands as has been suggested for other tropical species (Lawrence 2001, Elser *et al.* 2007, Bracken *et al.* 2014). However, as the foliar N:P ratios decreased and the number of affected traits was higher in response to P addition, it is likely that P is more limiting than N in most sites in our study region.

Although seedlings may have different nutrient requirements than adult trees, survival in the seedling community is a critical bottleneck for tree populations. Higher seedling foliar nutrient concentrations after fertilization indicate that most species take up additional N or P. However, successful utilization of improved nutrient availability depends primarily on species-specific morphological and physiological plasticity. Complementary investigations on seedling growth rates and photosynthetic nutrient use efficiency are needed to reach a more mechanistic understanding of the response of tropical tree seedlings to chronic nutrient addition via deposition. Ideally, such studies should also incorporate measures of light conditions, as seedling trait variation is known to depend on the interaction of nutrient availability and local light environment. The observed shifts in seedling traits after continued nutrient addition will likely have consequences for the competitive interactions among the studied species. The ongoing increase in nutrient deposition to tropical montane forests in the Andes and elsewhere will most likely result in shifts in tree community composition, favoring uncommon or newly invading species able to exploit higher soil fertility over traditionally common species adapted to less fertile soil conditions. The responsiveness of the studied tree seedlings to nutrient addition varied widely among the six species, which emphasizes the need to investigate not only single selected model species, but to examine a larger species sample. Data from single or very few tropical montane forest tree species seedlings are rarely suitable indicators for assessing the effects of N and P addition on plant properties.

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DATA AVAILABILITY

Data available from the Tropical Mountain Forest Database https://doi.org/10.5678/lcrs/pak823-825.dat.1478

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

FIGURE S1. *Location of the three study sites in Podocarpus National Park and San Francisco Reserve. Every study site is marked with a star.*
TABLE S1. *Study site soil characteristics after continued nutrient addition.*
TABLE S2. *Summary of the attributes of harvested seedlings of the six most common species in the three study sites. Given are means and ranges.*
TABLE S3. *Summary of mean values of the attributes analyzed in the six most common species.*

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