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Stem, root, and older leaf N:P ratios are more responsive indicators of soil nutrient availability than new foliage

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Abstract. Foliar nitrogen to phosphorus (N:P) ratios are widely used to indicate soil nutrient availability and limitation, but the foliar ratios of woody plants have proven more complicated to interpret than ratios from whole biomass of herbaceous species. This may be related to tissues in woody species acting as nutrient reservoirs during active growth, allowing maintenance of optimal N:P ratios in recently produced, fully expanded leaves (i.e., "new" leaves, the most commonly sampled tissue). Here we address the hypothesis that N:P ratios of newly expanded leaves are less sensitive indicators of soil nutrient availability than are other tissue types in woody plants. Seedlings of five naturally established tree species were harvested from plots receiving two years of fertilizer treatments in a lowland tropical forest in the Republic of Panama. Nutrient concentrations were determined in new leaves, old leaves, stems, and roots. For stems and roots, N:P ratios increased after N addition and decreased after P addition, and trends were consistent across all five species. Older leaves also showed strong responses to N and P addition, and trends were consistent for four of five species. In comparison, overall N:P ratio responses in new leaves were more variable across species. These results indicate that the N:P ratios of stems, roots, and older leaves are more responsive indicators of soil nutrient availability than are those of new leaves. Testing the generality of this result could improve the use of tissue nutrient ratios as indices of soil nutrient availability in woody plants.

Key words: Barro Colorado Nature Monument, Panama; forests; nitrogen; N:P ratios; phosphorus; soil nutrient availability; stoichiometry; woody plants.

INTRODUCTION

Tissue nitrogen to phosphorus (N:P) ratios are widely used to indicate relative nutrient availability to plants (Koerselman and Meuleman 1996, McGroddy et al. 2004, Reich and Oleksyn 2004). The use of tissue N:P ratios as an indicator of nutrient availability and limitation was developed with algae and phytoplankton in aquatic systems (Redfield 1958, Downing and McCauley 1992). After being tested with herbaceous species in wetlands (Koerselman and Meuleman 1996), the index gained popularity with terrestrial ecologists (Fenn et al. 1999, Covelo and Gallardo 2002, Tessier and Raynal 2003) and built on a history of using regressions of foliar N and P concentrations (Vitousek and Sanford 1986). Although patterns in N:P ratios of tree foliage coincide with expectations of plant-available nutrients at large spatial scales (McGroddy et al. 2004, Reich and Oleksyn 2004), interpreting trends through time and across smaller spatial scales has proven to be less straightforward (Townsend et al. 2007, Ostertag 2010).

The use of N:P ratios to indicate plant nutrient availability is based on the premise that plants grow best near their optimal element ratios (Ingestad 1979, Sterner and Elser 2002), but the maintenance of optimal ratios can be influenced by the surrounding environment. Variation in the availability of one nutrient relative to the other can alter plant nutrient ratios (Shaver and Melillo 1984); for example, the N:P ratio can increase as a consequence of either an increase in N availability or a decrease in P availability. Deviation from optimal ratios in response to soil availability of one nutrient relative to another underpins the use of tissue nutrient ratios as indicators of nutrient availability and limitation to plants. Experiments with herbaceous species provide support for this index (Koerselman and Meuleman 1996, Güsewell and Koerselman 2004).

Based on the convincing evidence from studies focused on herbaceous plants, forest ecologists have extended the use of N:P ratios to tree foliage as an indicator of soil nutrient availability. Yet potential complications in applying the index to large woody plants have received surprisingly little attention. In herbaceous plants, N:P ratios are determined on samples that represent all aboveground biomass. In contrast, in woody species, recently produced, fully expanded leaves,

which we will refer to as "new" leaves, are targeted. However, if optimal performance is indeed achieved at a given foliar N:P ratio, it would be beneficial for a plant to constrain the N:P ratio of new leaves to the greatest extent possible. This could be accomplished by using other tissues to regulate nutrient concentration, as has been demonstrated by the transport of N from shaded leaves to new sun-exposed leaves (Field 1983). The N:P ratios of other plant tissues might therefore be stronger indicators of soil nutrient availability than freshly expanded foliage because, unlike new leaves, the function of other tissues is less dependent on maintaining an optimal ratio. This expectation is supported by greenhouse studies on seedlings (Olsen and Bell 1990, Garrish et al. 2010), but has received little attention in field studies and has not been thoroughly considered as a caveat to current sampling practices.

Here we address the hypothesis that N:P ratios in stems, older leaves, and roots are more responsive indicators of nutrient availability than N:P ratios of new leaves, even though new leaves are most commonly used to infer nutrient availability. We further hypothesize that, due to the role of N:P ratio in carbon gain, new leaf N:P ratios will be more constrained than those of stems and older leaves, in which N:P ratio may be less critical for optimal function. We use data from five species of tropical tree seedlings: *Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*. Seedlings were naturally established and harvested after two years of plot-level fertilizer additions (Santiago et al. 2012).

Thus, this study tests the validity of using N:P ratios in young leaves to indicate soil nutrient availability compared to N:P ratios of other plant tissues by considering seedlings of five woody species in the field.

Methods

The study was carried out on Gigante Peninsula, within the Barro Colorado Nature Monument, Republic of Panama. The site supports mature secondary forest, with mean annual precipitation of 2600 mm and a four-month dry season. The fertilization experiment, along with responses of tree productivity to fertilizer additions, is described in detail elsewhere (Wright et al. 2011). Briefly, starting in 1998, factorial fertilizer treatments of N, P, and K were applied to 40×40 m plots at rates of 125 kg $N \cdot ha^{-1} \cdot yr^{-1}$, 50 kg $P \cdot ha^{-1} \cdot yr^{-1}$, and 50 kg $K \cdot ha^{-1} \cdot yr^{-1}$, respectively. To put the nutrient additions in context, the P fertilizer rate was five times that of P in annual litterfall, whereas N and K fertilizer rates were similar to litterfall (Sayer et al. 2012). Phosphorus was added at a higher relative rate to overcome the strong P sorption capacity of the soil (e.g., Schreeg et al. 2013). Nitrogen was added as coated urea ($(NH_2)_2CO$), P as triple superphosphate ($Ca(H_2PO_4)_2 \cdot H_2O$), and K as potassium chloride (KCl) in four equal doses during the wet season (Wright et al. 2011). Four additional plots received dolomitic limestone and micronutrients (San-

tiago et al. 2012). Each factorial NPK treatment was replicated four times (32 plots total), using an incomplete block design with N, P, K, and NPK treatments in one block and NP, NK, PK, and control (CON) treatments in a second block nested within each of the four replicates. The soils are developed on Miocene basalt and are Oxisols in the upper part of the landscape and Inceptisols in the lower part (Corre et al. 2010, Turner et al. 2013). The study area ranges from 25 to 61 m above sea level, and this variation is accounted for by the experimental blocks (Wright et al. 2011, visually outlined in Ecological Archives E092-136-A1). Topsoil texture is clay, and texture is similar across the study area (73% clay, 13% silt, 14% sand); see Yavitt et al. (2009) and Turner et al. (2013) for details on soil data across the experiment.

Four seedlings of five of the most common species in the community were harvested from each plot. The species were *Alseis blackiana* Hemsl. (Rubiaceae), *Desmopsis panamensis* (B.L. Rob.) Saff. (Annonaceae), *Heisteria concinna* Standl. (Olacaceae), *Sorocea affinis* Hemsl. (Moraceae), and *Tetragastris panamensis* (Engl.) Kunze. (Burseraceae) (Santiago et al. 2012). All species are relatively shade tolerant as seedlings, and range from shrubs to canopy trees as adults (see Santiago et al. 2012). Harvested seedlings were 10–15 cm tall, at least 2 m apart, and growing away from gaps in the understory (canopy openness was 4.9% ± 0.7%, mean ± SE; Santiago et al. 2012). New leaves (<1 yr old), which were fully expanded, were assessed based on position, color, and texture (Santiago et al. 2012). All below-ground biomass is included in the root category. Leaves were brushed clean and roots were rinsed with deionized water before drying and grinding to a fine powder. Phosphorus content was determined by nitric acid digestion and detection by induced coupled plasma optical emission spectroscopy (Optima 7300 DV, Perkin Elmer, Shelton, Connecticut, USA). Tissue N content was determined on an elemental analyzer (ECS 4010; Costech, Valencia, California, USA). Values are expressed on a 60°C dry-mass basis.

Three analyses were included in this study: (1) linear mixed models to evaluate the response of tissue N:P ratios to soil nutrient additions; (2) Brown-Forsythe tests to determine significant differences in the variance of N:P ratios among the tissue types; and (3) linear regressions to investigate relationships between N:P ratios in stems, old leaves, new leaves, and roots and to determine the extent to which N:P ratios are constrained among tissues. For the linear mixed models, we used log likelihood ratio tests to select among nested models. Fixed effects included N, P, K, and their two-way interactions. Random effects included species, plot, and replicate, and N and P coefficients associated with the species random effect. An initial model included all fixed effects. Significant random effects were added to this initial model using log likelihood ratio tests based on restricted maximum likelihood fits (Zuur et al. 2009).

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TABLE 1. Linear mixed-model results presenting significant fixed and random effects; entries are χ^2 values based on log likelihood ratios.

Tissue type	Fixed effects		Random species intercept and coefficients			
	Nitrogen (df = 1)	Phosphorus (df = 1)	Intercept (df = 1)	Nitrogen (df = 2)	Phosphorus (df = 2)	Random plot intercept (df = 1)
New leaves	2.71	3.04	65.9***	5.42	16.4***	3.42■
Old leaves	4.83*	4.90*	150.9***	6.62*	22.3***	6.62*
Stems	6.16*	7.67**	164.5***	6.64*	18.5***	3.41■
Roots	15.2***	9.92**	67.2***	4.74	7.50*	6.72**

Notes: The fixed effects of K and N × K, P × K, and N × P interactions were also evaluated for each tissue type and were never significant. The random effect of replicate was also never significant.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; † $P < 0.10$.

Insignificant fixed effects were then removed from the model with all significant random effects using likelihood ratio tests based on maximum likelihood fits (Zuur et al. 2009). We repeated this procedure separately for each of the four tissue types because we were concerned with the responses of different tissue types to nutrient treatments in woody plants. Differences in variance among the four tissue types were evaluated using a Brown-Forsythe test. Three *F* tests, which compared two groups at a time, were then used to determine if the variance in new leaves differed from the variance of each of the other tissue types. For linear regression analyses, plot-level species average N:P ratios for each fertilizer treatment (N, P, K, NK, PK, NP, NPK, micronutrient, and no nutrient addition plots) were included in model II regressions, which account for error in both variables. All analyses were conducted in R (R Development Core Team 2012).

RESULTS

Tissue N:P ratio responses to soil nutrient additions.— In linear mixed models, the random effect of plot was significant or marginally significant for each tissue type (Table 1), whereas the random effect of replicate was never significant (not shown). Significant spatial variation in N:P ratios was present at the 40-m scale of individual plots, but not at the 200-m to 400-m scale of replicates. The random intercept for species was significant for all four tissue types (Table 1; Appendix).

For new leaves, the fixed effects of N and P addition were insignificant (Table 1). The random coefficient for species and N addition was also insignificant, but the random coefficient for species and P addition was highly significant (Table 1). Nitrogen addition had no significant effect for N:P ratios in new leaves. In contrast, P addition caused significant variation among species for N:P ratios in new leaves (Fig. 1a).

For old leaves, stems, and roots, the fixed effects of N and P addition were significant (Table 1). N:P ratios increased after N addition and decreased after P addition for all three tissue types (Fig. 1b–d). The ratio responses were due to increased tissue N (mg/g dried tissue) with +N addition, and increased tissue P with +P (reported previously in Santiago et al. 2012). The random coefficients for species and N and P addition
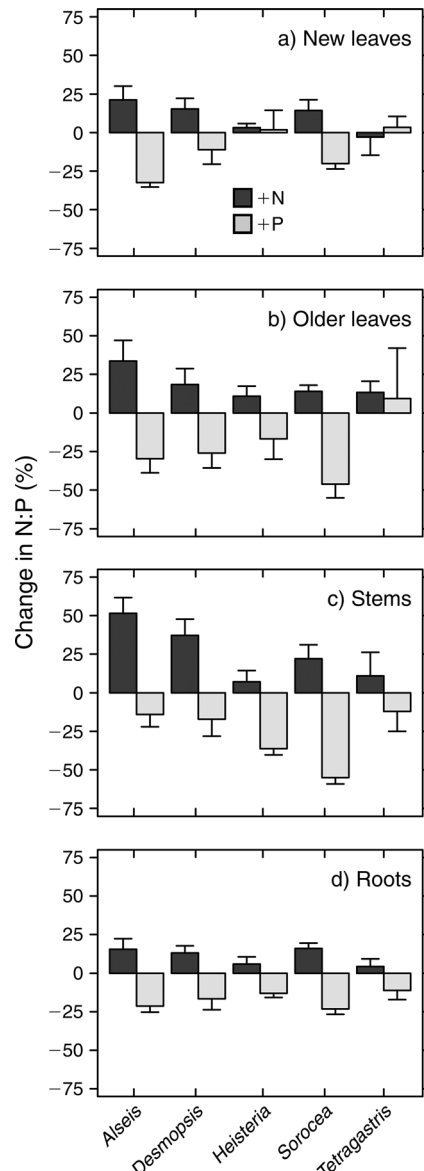

FIG. 1. Percentage change (mean + SE) in nitrogen-to-phosphorus ratios in (a) newly mature leaves, (b) old leaves, (c) stems, and (d) roots for five plant species in response to nitrogen addition (dark gray) and phosphorus addition (light gray).
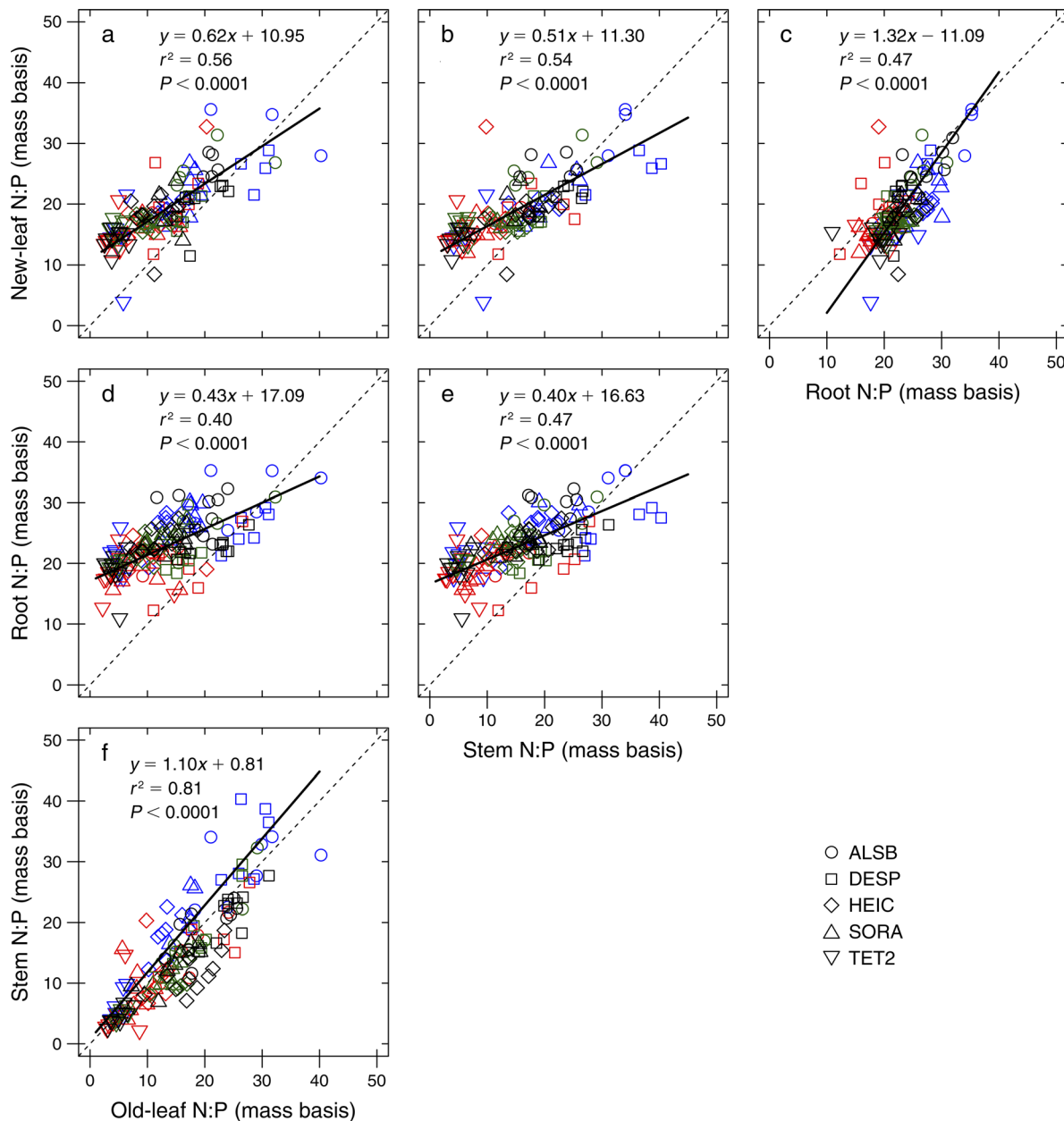
FIG. 2. Model II regressions of nitrogen (N) to phosphorus (P) ratios in new leaves, old leaves, stems, and coarse roots of naturally established seedlings of five tree species fertilized with potassium (K), micronutrients, or unfertilized controls (black); fertilized with nitrogen (N) or N and K (blue); fertilized with phosphorus (P) or P and K (red); or fertilized with N and P or N, P, and K (green). The solid black line shows the linear regression; the dashed line shows a 1:1 relationship for comparison. Each symbol represents a species–plot combination. Species are ALSB, *Alseis blackiana*; DESP, *Desmopsis panamensis*; HEIC, *Heisteria concinna*; SORA, *Sorocea affinis*; TET2, *Tetragastris panamensis*.

were also significant for five of the six combinations of tissue types and nutrient treatments (Table 1). Despite significant interspecific variation in the level of response to nutrient addition, N:P ratios increased after N addition and decreased after P addition for stems and roots of every species (Fig. 1c, d).

Potassium addition had no significant effect on N:P ratios for any tissue type. For this reason, we pooled K treatments with the appropriate N and P treatments in

figures. For Fig. 1, the pooled treatments are +N and +NK vs. +P and +PK. For Figure A1 in the Appendix, which shows N:P ratios, the pooled treatments are control and +K, +N and +NK, +P and +PK, and +NP and +NPK.

Variance of N:P ratios among the tissue types.— Variances differed significantly among the four tissue types ($P < 0.001$, $F_{3, 618} = 27.33$; Brown-Forsythe test; ranges are shown in Fig. 2). Pairwise comparisons of

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N:P ratios of new leaves with N:P ratios for each of the other tissue types demonstrated that ratios of new leaves had significantly lower variance than those of old leaves or stems (25.86 vs. 57.68 and 69.05, respectively; $P <$ 0.001 for both F tests), whereas the variance of new leaves was significantly greater than that of roots (25.86 > 18.12; $P = 0.03$).

Relationships between tissue N:P ratios.—Changes in stem and old leaf N:P ratios were characterized by relatively smaller changes in N:P ratios of new leaves, as evaluated by slopes in linear regressions (Fig. 2a, b). For the regression of new leaf N:P on old leaf N:P, the slope was 0.62 (95% CI 0.53–0.72), and the *y*-intercept was 10.95 (95% CI 9.71–12.10) (Fig. 2a). New leaf vs. stem N:P had a slope of 0.51 (95% CI 0.42–0.60) and a *y*-intercept of 11.30 (95% CI 9.99–12.53) (Fig. 2b). In contrast to stems and older leaves, changes in root N:P ratios were more constrained than those of new leaves, as evaluated through the slope (Fig. 2c; slope = 1.32, 95% CI 1.10–1.59; *y*-intercept = −11.09, 95% CI −17.33 to −6.16). Notably, the N:P ratios of older leaves and stems were similar. The regression of N:P ratios of stems on ratios of old leaves vs. stems was strong ($r^2 = 0.81$) with a slope close to 1.0 (1.10; 95% CI 1.02–1.19) and an intercept not significantly different from the origin (*y*-intercept = 0.81, 95% CI −0.43 to 1.95; Fig. 2f).

DISCUSSION

The use of tissue N:P ratios to infer soil nutrient availability and nutrient limitation gained popularity following Koerselman and Meuleman (1996), who used a two-pronged approach: (1) an experimental manipulation of nonwoody temperate herbaceous perennials from a dune slack system and (2) a literature review of European freshwater wetlands that focused on N:P ratios. Both analyses focused on samples representing all aboveground tissue (stems and leaves) and few woody species were included. A woody shrub, *Erica tetralix*, was considered in the review, but the N:P ratios were for total aboveground biomass rather than new leaves (Aerts and Berendse 1988). In forests, obtaining nutrient contents of total aboveground biomass is impractical, so studies in forested ecosystems conventionally focus on N:P in new foliage.

This study determined that N:P ratios of older leaves, stems, and roots responded more strongly to nutrient availability than did N:P ratios of new leaves, demonstrating that the conventional selection of tissues for assays of soil nutrient availability needs to be reassessed. Stem, root, and older leaf N:P ratios responded to both N and P fertilizer additions, in the expected directions, whereas new leaf N:P ratios were less responsive. New leaves showed both less consistent trends among species, as evaluated by the linear mixed models, and relatively more constrained N:P ratio responses compared to stems and older leaves (Fig. 2). This would appear to support the argument that N:P ratios of new leaves are less responsive to nutrient additions because they are relatively constrained, due to the metabolic limitations imposed by maintaining a positive carbon gain. The root results, however, challenge this generalization.

Although root N:P ratios responded strongly to N and P addition, roots also had N:P ratios that were more constrained than new leaf N:P ratios. The fact that root N:P ratios are constrained relative to stems and old leaves may make sense because, like new leaves, roots are highly metabolically active and require substantial allocation of N and P to the synthesis of carrier enzymes that actively take up nutrients from the soil solution (Treseder and Vitousek 2001). The result that root N:P ratios are also responsive is contrary to initial expectations. It appears that root N:P ratios are uniform enough that a significant response to nutrient addition can occur without broadening the overall N:P variance compared to other tissues. These results suggest that roots could be a more refined indicator of soil nutrient status than anticipated.

Our results that stem, root, and older leaf N:P ratios are stronger indicators of soil nutrient availability than are N:P ratios of new leaves are in agreement with greenhouse studies of *Eucalyptus* (Olsen and Bell 1990) and *Ficus insipida* (Garrish et al. 2010) seedlings, in which N:P ratios of older leaves and/or stems and roots were also stronger indicators of soil nutrient availability than were new leaves. The extent to which stems, older leaves, and roots from mature trees could follow similar patterns remains to be determined. It seems reasonable that saplings and mature trees could demonstrate N:P ratio patterns similar to woody seedlings, because the argument that nutrients may be shuttled to other tissue types to maintain favorable N:P and maintain metabolic activity in new leaves could apply to all life stages.

Two lines of evidence demonstrate that N and P fertilizer additions effectively increased plant-available N and P, respectively. The addition of N increased soil extractable nitrate (but not ammonium; Turner et al. [2013], but note that these data were collected after nine years of fertilizer addition) and N concentrations in new leaves, old leaves, stems, and roots of seedlings (Table 1 in Santiago et al. 2012). The addition of P increased soil-extractable phosphate (Turner et al. 2013) and P concentrations in new leaves, old leaves, stems, and roots of seedlings (Table 1 in Santiago et al. 2012). This strengthens our ability to evaluate the hypothesis that N:P ratios in stems, older leaves, and roots are more responsive indicators of nutrient availability than are N:P ratios of new leaves.

The extent to which tissue N:P ratio response to nutrient additions reflects relative soil N and P availability, vs. being restricted to conditions of limitation, has important consequences for the use of tissue nutrients to infer nutrient status and limitation (Aerts and Chapin 2000). For example, in a system limited only by N, the addition of P should not influence the N:P ratio if the ratio responds only to nutrient limitation. In contrast, if ratios respond to general nutrient availability

and are not restricted to limitation conditions, P addition is more likely to lower the N:P ratio. For this data set, relative growth rates of height were limited by N and P in combination, but not independently (Santiago et al. 2012). Limitation of N+P leads to the expectation that the N:P ratio should shift to reflect N limitation with P addition, P limitation with N addition, and no ratio change for N+P addition. Thus, the same responses to N and P additions are expected under non-limiting conditions. Stem, older leaf, and root N:P ratio responses were consistent with these expectations. Studies of systems limited by either N or P are needed to tease apart whether N:P ratio responses are restricted to conditions of limitation or serve as general indicators of relative nutrient availability.

As the search for the most appropriate woody plant tissue for linking N:P ratios to soil nutrient availability progresses, interactions with light environment, relative growth rate, and seasonality should be considered. Data in this study are from seedlings in the deeply shaded understory, whereas mature tree crowns support both sun and shade leaves. Foliar N:P ratios are known to increase with light availability both within species (Güsewell 2004) and among species that inhabit different forest strata (Specht and Specht 2010), but the sensitivities of stem, older leaf, and root N:P to light have been rarely investigated.

Similarly, changes in foliar N:P ratios with season have been considered in the context of relative growth rates, but less is known about N:P ratios of other tissues. A number of studies link relative growth rate to seasonality and show that foliar N:P ratio can be lower during periods of active growth (Méndez and Karlsson 2005, Rivas-Ubach et al. 2012), probably due to RNA demand for P (Sterner and Elser 2002). Shifts in foliar N:P with season could influence storage and thus stem N:P, but the sensitivity of this response relative to foliage, and as a function of stem age and type (twig vs. branch), remains to be determined.

We speculate that future work on N:P ratios and leaf age could provide new insight for interpreting trends in foliar N:P across biomes due to systematic bias in selecting new leaves. Temperate deciduous leaves may appear new well into the growing season compared to leaves from tropical evergreen forests, due to lower epiphyte and endophyte loads (Andrews and Harris 2000, Arnold and Lutzoni 2007). Therefore, efforts to sample leaves that appear new in tropical evergreen forests could result in samples that are systematically younger (in a physiological sense; Reich et al. 1991) in comparison to foliage sampled in deciduous temperate forests. If foliage from mature trees follows the pattern of N:P ratios decreasing with leaf age, shown here for seedling leaves, the relatively younger tropical foliage could have higher N:P ratios than temperate deciduous samples. A higher foliar N:P ratio suggests P limitation, which could bias the trend in the direction of the anticipated pattern of P limitation in the tropics (McGroddy et al. 2004, Reich and Oleksyn 2004).

We conclude that N:P ratios of stems, older leaves, and roots of woody seedlings better reflect soil nutrient availability than does the N:P ratio of new foliage. If stems, older leaves, and roots act as nutrient reservoirs that support optimal N:P ratios in new leaves, this leads to the intriguing possibility that assessment of nutrient status using tissue nutrient ratios can be improved for woody plants in general by considering N:P ratios of tissues other than recently produced, fully expanded leaves. Future research should investigate whether our results are supported (1) across soils with more subtle differences in nutrient availability than the nutrient addition plots evaluated here, and (2) across species with a larger range of functional traits.

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SUPPLEMENTAL MATERIAL

Appendix

Nitrogen-to-phosphorus (N:P) ratios in newly mature leaves, old leaves, stems, and roots for each of the fertilizer treatments (*Ecological Archives* E095-183-A1).