

LETTER

Interactions among nitrogen fixation and soil phosphorus acquisition strategies in lowland tropical rain forests

Megan K. Nasto,^{1*} Silvia Alvarez-Clare,² Ylva Lekberg,^{1,3,4} Benjamin W. Sullivan,^{5,6} Alan R. Townsend⁷ and Cory C. Cleveland⁸

Abstract

Paradoxically, symbiotic dinitrogen (N₂) fixers are abundant in nitrogen (N)-rich, phosphorus (P)-poor lowland tropical rain forests. One hypothesis to explain this pattern states that N₂ fixers have an advantage in acquiring soil P by producing more N-rich enzymes (phosphatases) that mineralise organic P than non-N₂ fixers. We assessed soil and root phosphatase activity between fixers and non-fixers in two lowland tropical rain forest sites, but also addressed the hypothesis that arbuscular mycorrhizal (AM) colonisation (another P acquisition strategy) is greater on fixers than non-fixers. Root phosphatase activity and AM colonisation were higher for fixers than non-fixers, and strong correlations between AM colonisation and N₂ fixation at both sites suggest that the N–P interactions mediated by fixers may generally apply across tropical forests. We suggest that phosphatase enzymes and AM fungi enhance the capacity of N₂ fixers to acquire soil P, thus contributing to their high abundance in tropical forests.

Keywords

Arbuscular mycorrhizal fungi, nutrient acquisition strategies, phosphatase enzymes, tropical forest.

Ecology Letters (2014) 17: 1282–1289

INTRODUCTION

Symbiotic dinitrogen (N₂) fixation is a fundamental biological process that allows some plants to overcome nitrogen (N) limitation by converting atmospheric N₂ into biologically available forms. As a result, symbiotic N₂ fixation is an important driver of ecosystem function (Vitousek & Howarth 1991; Vitousek *et al.* 2002; Gerber *et al.* 2010), especially in N-limited ecosystems where soil available N is low relative to available phosphorus (P). The N₂ fixation mutualism is energetically expensive and requires significant carbon (C) and P investment from host plants to support their microbial symbionts (Vitousek & Howarth 1991). Thus, N₂ fixers (and N₂ fixation) should theoretically have a competitive advantage in relatively low N, high P environments, but be less competitive in high N, low P environments. Yet, N₂ fixing trees are relatively rare in many N-poor temperate and high-latitude forests, but abundant in the N-rich lowland tropics (ter Steege *et al.* 2006; Hedin *et al.* 2009). This paradox raises the question: why are symbiotic N₂ fixers (and N₂ fixation) so abundant in tropical rain forests? Reconciling this disparity is critical for understanding global-scale patterns of N₂ fixer abundance and for predicting how forests may respond to a range of natural disturbances and anthropogenic environmental changes (Gerber *et al.* 2010; Cleveland *et al.* 2013).

In light of both the low P status of many tropical rain forests (e.g., Cleveland *et al.* 2011) and the high P demand of N₂ fixation (Vitousek & Howarth 1991), attempts to identify the mechanisms that promote high abundances of symbiotic N₂ fixers in these ecosystems have focused on interactions between N and P. For example, Houlton *et al.* (2008) hypothesised that N₂ fixers have the capacity to acquire more soil P than non-N₂ fixers because they can invest fixed N₂ in extracellular phosphatase production, enzymes that convert phosphomonoester- and phosphodiester-bound organic P into biologically available forms (McGill & Cole 1981). Phosphatase enzymes are N rich and production has been shown to increase substantially in response to elevated N (Olander & Vitousek 2000; Treseder & Vitousek 2001; Marklein & Houlton 2011). Thus, N₂ fixers may have a competitive advantage over non-N₂ fixers in acquiring soil P. However, while this mechanism may help to explain the abundance of N₂ fixers in lowland tropical rain forests, there have been very few attempts to assess it directly.

An additional but rarely explored mechanism that may promote high abundances of N₂ fixers in tropical rain forests involves the widespread mutualism between plants and arbuscular mycorrhizal (AM) fungi. In exchange for photosynthetically fixed C, root colonising AM fungi effectively increase the surface area of plant roots, enabling greater exploitation

¹Department of Ecosystem and Conservation Sciences, University of Montana, Missoula, MT, 59812, USA

²Department of Ecosystem and Conservation Sciences, University of Montana, Missoula, MT, 59812, USA

³MPG Ranch, Missoula, MT, 59803, USA

⁴Department of Ecosystem Conservation Sciences, University of Montana, Missoula, MT, 59802, USA

⁵Department of Natural Resources and Environmental Science, University of Nevada, Reno, NV, 89557, USA

⁶Department of Ecosystem and Conservation Sciences, University of Montana, Missoula, MT 59812, USA

⁷Nicholas School of the Environment, Duke University, Box 90328, Durham, NC, 27708, USA

⁸Department of Ecosystem and Conservation Sciences, University of Montana, Missoula, MT, 59812, USA

*Correspondence: E-mail: megan.nasto@umontana.edu

and uptake of soil P (Smith & Read 2008). The hyphae of AM fungi may exploit either the soil inorganic P pool by 'mining' for distant pools of available P, or the soil organic P pool by producing phosphatase enzymes. Although there is very little evidence of a direct link between N₂ fixation and AM colonisation in natural systems, N₂ fixation has been shown to enhance the photosynthetic rate of some plants (e.g., Harris *et al.* 1985; Jia *et al.* 2004), possibly allowing greater investment in AM fungi via C allocation. In addition, simultaneously hosting both N₂ fixing and AM fungi symbionts could be beneficial in environments where the nature of nutrient limitation (e.g., N vs. P) changes through time or where both nutrients may be colimiting, as has been seen in some tropical rain forests (Vitousek & Farrington 1997; Davidson *et al.* 2004, 2007; Townsend *et al.* 2007; Wright *et al.* 2011). Therefore, a second, non-mutually exclusive hypothesis is that N₂ fixers have the capacity to acquire more soil P than non-N₂ fixers because N₂ fixation allows them to support greater AM colonisation, giving them a competitive advantage in acquiring this often-limiting nutrient.

We tested these two complementary hypotheses by comparing phosphatase enzyme activity and AM colonisation between N₂ fixers and non-N₂ fixers in two distinct lowland primary tropical rain forests in Costa Rica. Since phosphatase enzyme production requires substantial N investment, we hypothesised that N₂ fixers would show greater rates of root and rhizosphere soil phosphatase enzyme activity than non-N₂ fixers, and a positive relationship between rates of N₂ fixation and phosphatase enzyme activity. Similarly, we hypothesised the roots of N₂ fixers would have greater AM colonisation than non-N₂ fixers, and a positive relationship between rates of N₂ fixation and AM colonisation. Overall, our results showed that N₂ fixers have both higher rates of root phosphatase enzyme activity and AM colonisation than non-N₂ fixers. Moreover, AM colonisation was significantly and positively correlated with N₂ fixation rates and nodule biomass. Many studies have demonstrated that plants have the capacity to acquire more soil P with increased root phosphatase enzyme activity and AM colonisation (Sanders & Tinker 1971; Ortas *et al.* 1996; Khaliq & Sanders 2000; Treseder & Vitousek 2001). Thus, symbiotic N₂ fixers may indeed compete more effectively for soil P than non-N₂ fixers. Together, these two strategies of soil P acquisition may help explain why symbiotic N₂ fixers are abundant in the generally N-rich forests of the lowland tropics.

METHODS

Study sites

The study was conducted in two mature lowland tropical rain forest sites – one on the Pacific coast and the other on the Caribbean coast of Costa Rica (Table S1). The Pacific site consisted of three replicated 0.5-ha primary forest plots located near Piro Biological Station on the Osa Peninsula (8°24' N, 83°19' W). Mean annual temperature (MAT) is *c.* 26 °C and mean annual precipitation (MAP) is *c.* 3500 mm (Keller *et al.* 2013), and the region experiences a dry season from December through April with heavy rains common

throughout the rest of the year. Soils in the region are classified as highly weathered, nutrient-poor Ultisols (Berrange & Thorpe 1988). Putative N₂ fixing species (e.g., *Inga* and *Zygia spp.*) are common in the primary forest plots (Table 2) and represent 5–13% of the total tree abundance (Sullivan *et al.* 2014).

The Caribbean site included four replicated 0.09-ha (30 × 30 m) primary forest plots established as part of a nutrient fertilisation experiment in 2007 (Alvarez-Clare *et al.* 2013). The plots are located in a forest reserve at EARTH University (Escuela de Agricultura de la Región del Trópico Húmedo) in Guácimo, Limón (10°11' N, 84°40' W) where the MAT is *c.* 25 °C and the MAP is *c.* 3500 mm (Alvarez-Clare *et al.* 2013). Annual precipitation at EARTH is distributed in a bimodal pattern with no months receiving < 100 mm (Alvarez-Clare 2012), and the plots are located on volcanic soils classified as Ultisols and Inceptisols. The N₂ fixing species *Pentaclethra macroloba* is abundant at EARTH (comprising *c.* 30% of the total basal area within the primary forest plots), but several other putative N₂ fixing species are also present (Table 3; Alvarez-Clare *et al.* 2013).

Sampling strategy

Previous research has shown that some species of N₂ fixers accelerate soil P cycling via increased phosphatase enzyme activity (e.g., Houlton *et al.* 2008; Keller *et al.* 2013), but other species do not (e.g., Batterman *et al.* 2013). Thus, to overcome potential variation driven by species-specific traits (e.g., shoot : root ratio, foliar stoichiometry, nutrient use efficiency, host-specific interactions) that could affect a given species' ability to directly or indirectly alter soil P cycling, our sampling scheme focused not on specific N₂ fixing and non-N₂ fixing species, but rather on roots that either did or did not contain N₂ fixing nodules. In other words, our sampling strategy was designed to assess general (rather than species-specific) relationships between nodulated/actively fixing vs. non-nodulated/non-fixing roots and P acquisition. Furthermore, we measured nutrient acquisition strategies at a small spatial scale in an attempt to differentiate between phosphatase enzymes produced by plants and microbes in the rhizosphere soil from enzymes produced by microbes in the bulk soil, as well as to link soil P acquisition strategies with N₂ fixation at the locus of acquisition – the soil–root interface. For example, we measured root phosphatase enzyme activity and AM colonisation on both nodulated and non-nodulated root segments of similar size and sampled from similar depths in the soil. Next, we measured soil phosphatase enzyme activity within the rhizosphere soil directly surrounding sampled root segments. Finally, we confirmed that the nodulated roots were actively fixing N₂ using the acetylene reduction assay (ARA; see below).

Field sampling

We collected roots, rhizosphere soil, nodules and bulk soil in October 2012, and January and June 2013 to capture rainfall seasonality (i.e. wet, dry, dry–wet transition respectively) at the Pacific site (Keller *et al.* 2013), and in January and July

2013 to capture the bimodal rainfall seasonality at the Caribbean site (Alvarez-Clare *et al.* 2013). We quantified root and rhizosphere soil phosphatase enzyme activity, N₂ fixation, and soil nutrient concentrations at each sampling period. However, we measured AM colonisation only once (after the January 2013 sampling period at both sites) as AM colonisation shows minimal seasonal variation in tropical rain forests (*e.g.* Treseder & Vitousek 2001) and is closely associated with plant phenology (Allen 1998), which does not differ between the N₂ fixers and non-N₂ fixers at the two sites.

At both sites, we used a 5.5-cm-diameter bulb corer to collect five nodulated and non-nodulated root samples from the top 10 cm of the mineral soil from each plot. From nodulated root segments, we excised 2–3 nodules from each of the five samples and placed them in acrylic tubes for the ARA (see below). Rhizosphere soil directly surrounding root segments was gently removed, bagged and stored at –20 °C for future phosphatase enzyme analyses. Root segments were stored in 20-mL scintillation vials containing a 50 mM calcium sulphate (CaSO₄) solution (to maintain structural integrity and reduce hypo-osmotic conditions) at 5 °C for future phosphatase enzyme and AM analyses. Finally, we collected six 10 cm soil cores from each of the Pacific site plots, and two 10 cm cores from each of the Caribbean site plots for soil inorganic N analyses. Soil cores were hand-homogenised *in situ* and coarse woody and mineral fragments were removed prior to analyses. Soil nutrient and enzyme data from separate seasons were averaged to generate single, site-specific estimates for the two plant functional groups ($n = 5$ per plot).

Soil nutrient analyses

Within 12 h of collection, we extracted ammonium (NH₄⁺) and nitrate (NO₃[–]) from the fresh 0–10 cm soil core samples by shaking 8 g of soil in 30 mL of a 2 M KCl solution for 1 min every 4 h. Extracts were filtered using Whatman glass microfiber filters (Grade GF/B, 47 mm) and frozen at –20 °C until further analysis. Extracts were analysed colorimetrically (Solorzano 1969; Doane & Horwath 2003) using a Synergy 2 Microplate Reader (Biotek, Winooski, VT, USA), and soil inorganic N was calculated as the sum of NH₄⁺ and NO₃[–]. We extracted available (Bray) P from air-dried subsamples of the rhizosphere soil by shaking 8 g of soil in 40 mL of a dilute ammonium fluoride solution for 1 min (Bray & Kurtz 1945). Extracts were filtered using Whatman glass microfiber filters and analysed colorimetrically on the plate reader (D'Angelo *et al.* 2001). Soil dry weight and per cent moisture were determined gravimetrically by oven drying soils for 48 h at 105 °C.

Phosphatase enzyme activity

We measured phosphatase enzyme activity from subsamples of the rhizosphere soil using a 4-methylumbelliferone (MUB)-linked substrate following the methodology of Saiya-Cork *et al.* 2002). Briefly, 2 g of fresh soil was homogenised with 125 mL of 50 mM sodium acetate buffer (SAB; pH 5) and 200 µL of the soil-buffer slurry was combined with 50 µL buffer and 50 µL of 200 µM 4-MUB phosphate in a black 96-well microplate. Each sample included eight analytical repli-

cates plus negative controls for sample and substrate fluorescence (*i.e.* quenching). Microplates were incubated at room temperature in the dark for 24 h, and read at 365-nm excitation and 450-nm emission and enzyme activities were calculated as µmol 4-MUB-P g^{–1} soil h^{–1}.

We measured phosphatase activity from excised subsamples of roots using a 4-MUB-linked substrate adopted from Sin-sabaugh *et al.* (2003). Briefly, roots were rinsed with 50 mM CaSO₄ to remove any attached soil particles. Subsequently, 20–30 mg of roots was immersed in 1 mL of 50 mM SAB (pH 5), 1 mL of 50 mM SAB (800 µL)/100 mM MUB solution (200 µL) and 1 mL of 50 mM SAB (800 µL)/100 mM MUB (200 µL)/200 mM 4-MUB phosphate (200 µL) solution in clear 12-well plates. The plates were shaken at room temperature for 1 h (110 rpm) and 200-µL subsamples from all wells were pipetted into a black 96-well microplate. Each sample included four analytical replicates and negative controls for sample and substrate fluorescence. Microplates were read at 365-nm excitation and 450-nm emission and enzyme activities were calculated as µmol 4-MUB-P g^{–1} root h^{–1}. All enzyme assays were performed within 1 week of sampling.

Arbuscular mycorrhizal colonisation

We quantified AM colonisation on air-dried subsamples of roots by rehydrating fine roots (< 1 mm diameter and 15 mm long) with tap water and staining with trypan blue as described in Koske & Gemma (1989). Briefly, roots were cleared in a 10% potassium hydroxide solution for 3 days, rinsed with tap water and placed in a 3% hydrogen chloride (HCl) solution for 12 h for acidification. The HCl solution was replaced with trypan blue for 24 h and then de-stained in water for 12 h. Ten cleared and uniformly stained root pieces per sample were mounted on slides and root colonisation was quantified using the magnified intersection method (McGonigle *et al.* 1990) on *c.* 50 intersections. Colonisation is reported as per cent of root length colonised by AM fungi.

Symbiotic N₂ fixation

We confirmed N₂ fixation in each of the five nodulated root samples using the ARA method (Hardy *et al.* 1968) by excavating nodules from N₂ fixer roots and incubating them in a 50 mL clear acrylic tube for 1 h with a 10% acetylene atmosphere. After incubation, 14 mL headspace samples were removed from tubes with a syringe, injected into 10 mL Vacutainers (Becton-Dickinson, Inc., Franklin Lakes, NJ, USA) and returned to the laboratory for analysis by gas chromatography using a Shimadzu GC-2014 equipped with a flame ionisation detector (Shimadzu Inc., Kyoto, Japan). We accounted for the ethylene produced from nodules without acetylene exposure, ethylene produced from tubes and Vacutainers, ethylene within our acetylene and ethylene lost due to photo-degradation during transport. To convert acetylene reduction rates to N₂ fixation rates, we measured the uptake of ¹⁵N-labelled N₂ in nodules to generate an ethylene : N conversion ratio of 2.8 : 1. We note that we do not explicitly present data on rates of N₂ fixation here, but instead focus on the relationship between the N₂ fixation data and P acquisition strategies.

Statistical analyses

Prior to statistical analyses, all data were tested for normality using the Shapiro–Wilk test and homoscedasticity using the Levene test. Welch two sample *t*-tests were used to determine differences in soil total P, extractable P, total N, inorganic N and total C between the two sites, and two-way analysis of variance (ANOVA) was used to test differences in phosphatase enzyme activity and AM colonisation using plant functional group and site as grouping variables. To determine relationships between N₂ fixation rates and both phosphatase enzyme activity and AM colonisation by each site, we calculated Pearson correlation coefficients and used simple linear regressions. If we detected significant relationships, analysis of covariance (ANCOVA) was used to assess the differences in the slopes of the relationships between N₂ fixation and both phosphatase enzyme activity and AM colonisation. The goal of this analysis was to assess whether the relationships were consistent between the two sites. For this analysis, root and rhizosphere soil phosphatase enzyme activity were summed, as N₂ fixation may affect both sources of phosphatase enzymes. All statistical analyses were performed using the open-source R software v. 2.13.0 (R Development Core Team). For all data, significance was determined when $P < 0.05$.

RESULTS

Soil nutrients and phosphatase enzyme activity

Both soil extractable ($P < 0.01$) and total P ($P < 0.001$) concentrations were higher at the Caribbean site than at the Pacific site (Table 1), yet inorganic ($P = 0.93$) and total soil N ($P = 0.17$) concentrations were not different between the two sites. Phosphatase enzyme activity was significantly greater on the roots of N₂ fixers than non-N₂ fixers ($P = 0.03$), and the Pacific site, in general, had greater root enzyme activity than the Caribbean site ($P = 0.03$; Fig. 1a). By contrast, there were no differences in rhizosphere soil phosphatase enzyme activity between N₂ fixers and non-N₂ fixers at either site ($P = 0.32$), but the Caribbean site had greater rhizosphere enzyme activity rates than the Pacific site ($P < 0.01$; Fig. 1b). Finally, among

Table 1 Soil carbon (C), nitrogen (N) and phosphorus (P) content of surface soils (0–10 cm) at the Pacific and Caribbean rain forest sites, Costa Rica. Values represent means with standard error in parentheses

Soil parameter	Pacific rain forest	Caribbean rain forest
Extractable P (mg kg ⁻¹)**	1.2 (0.10) [†]	7.65 (1.22) [†]
Total P (mg kg ⁻¹ ***)	665 (63.79) [‡]	1601.25 (104.57) [§]
Inorganic N (NH ₄ ⁺ + NO ₃ ; mg kg ⁻¹)	5.5 (1.07) [†]	4.39 (0.36) [†]
Total N (%)	0.39 (0.03) [‡]	0.49 (0.01) [§]
Total C (%)*	3.82 (0.24) [‡]	4.83 (0.14) [§]

[†]Mean of three sampling periods at the Pacific rain forest site and the two sampling periods at the Caribbean rain forest site.

[‡]Data from Cole, unpublished.

[§]Data from Alvarez-Clare *et al.* (2013).

* $P < 0.1$, ** $P < 0.01$, *** $P < 0.001$.

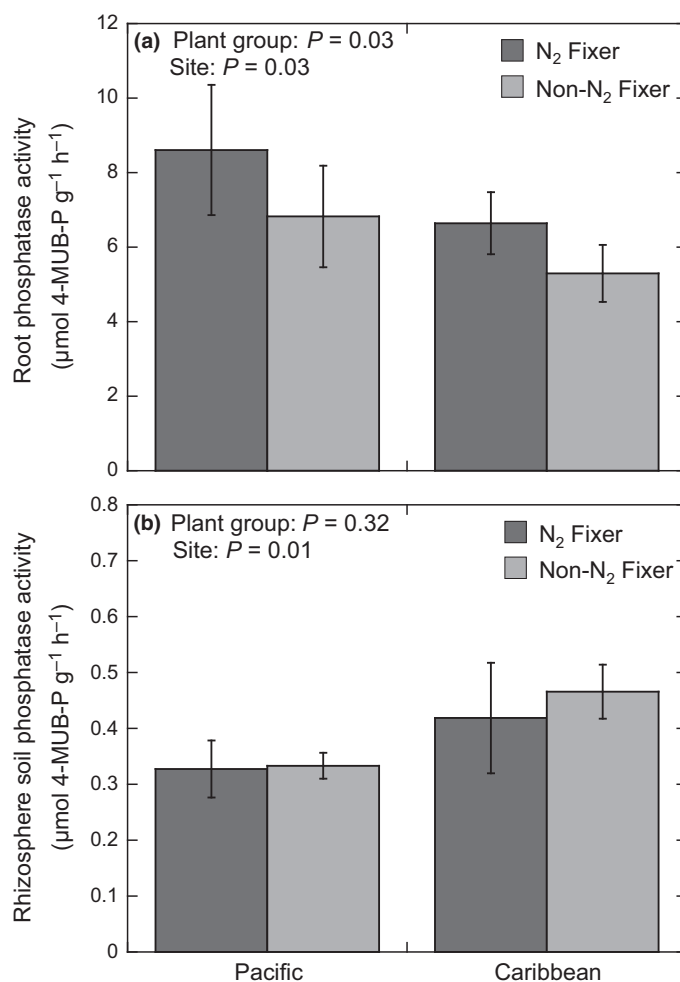


Figure 1 Potential rates of (a) root and (b) rhizosphere soil phosphatase enzyme activity of N₂ fixers and non-N₂ fixers between the Pacific and Caribbean sites, where *P*-values represent differences between plant functional groups and sites. Data are means \pm 1 SD.

N₂ fixers, there were no significant relationships between total phosphatase enzyme activity and N₂ fixation at the Pacific or Caribbean sites ($P = 0.46$ and $P = 0.57$, respectively; Fig. 2a), nor between total phosphatase enzyme activity and nodule weight ($P = 0.19$ and $P = 0.87$ respectively; Fig. 2b).

Arbuscular mycorrhizal colonisation

Arbuscular mycorrhizal colonisation was significantly greater in the roots of N₂ fixers than non-N₂ fixers ($P < 0.01$) but did not differ overall between the two sites ($P = 0.17$; Fig. 3a). In addition, AM colonisation was significantly and positively related to N₂ fixation at the Pacific and Caribbean sites ($P = 0.03$ and $P = 0.05$ respectively; Fig. 4a) and nodule weight ($P = 0.01$ and $P < 0.01$ at the Pacific and Caribbean sites respectively; Fig. 4b). Finally, the ANCOVA indicated that the positive relationships between AM colonisation and both N₂ fixation ($P < 0.01$) and nodule weight ($P < 0.01$) were consistent between the Pacific and Caribbean sites.

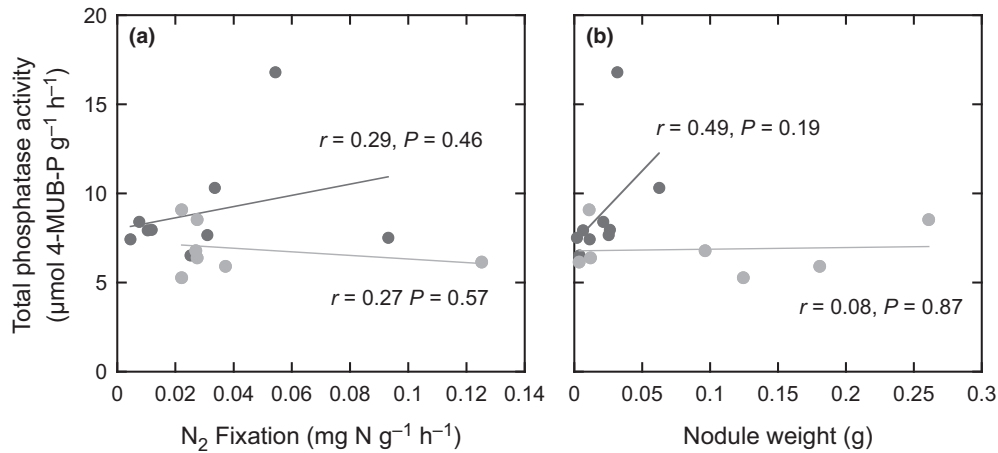


Figure 2 Relationships between potential rates of total phosphatase enzyme activity (roots + rhizosphere soil) and (a) N_2 fixation, and (b) nodule weight between the Pacific (dark grey) and Caribbean (light grey) sites. Significance and correlation coefficients are shown for each relationship at the two sites. Each point represents N_2 fixation, nodule and enzyme data from an individual nodulated root.

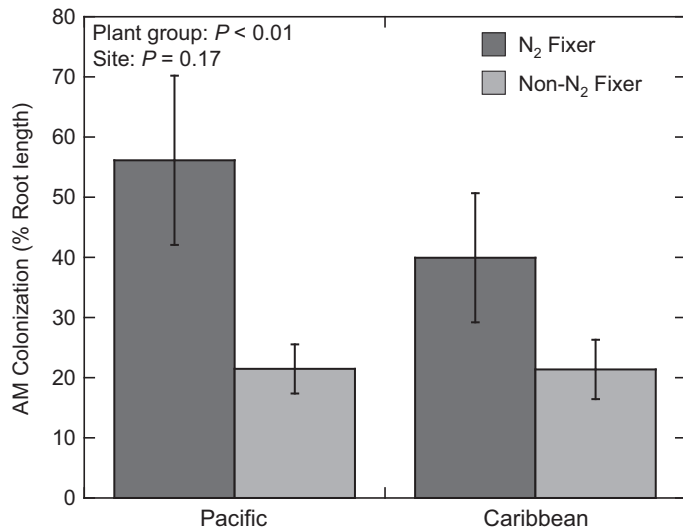


Figure 3 (a) arbuscular mycorrhizal (AM) colonisation of N_2 fixers and non- N_2 fixers between the Pacific and Caribbean sites, where P -values represent differences between plant functional groups and sites. Data are means \pm 1 SD.

DISCUSSION

Overall, our results provide compelling evidence that both phosphatase enzyme activity and AM colonisation – two common P acquisition strategies – are greater in N_2 fixers than non- N_2 fixers in tropical rain forests. While we found some evidence to support the mechanism that has most often been hypothesised to explain the abundance of N_2 fixers in the tropics (i.e. higher rates of phosphatase enzyme activity in N_2 fixers vs. non- N_2 fixers), our data clearly demonstrate the importance of an alternative, possibly synergistic and relatively unexplored mechanism: N_2 fixers have much greater AM colonisation than non- N_2 fixers. While the relationships between N_2 fixation and soil P acquisition we observed are correlations, they suggest that N_2 fixers may have a greater

capacity to acquire soil P than non- N_2 fixers via phosphatase enzymes and AM fungi, thus contributing to the high abundance of N_2 fixers in tropical rain forests.

There could be several possible explanations for the greater AM colonisation on N_2 fixer vs. non- N_2 fixer roots. First, greater AM colonisation of N_2 fixer roots may reflect a greater P demand of N_2 fixers than non- N_2 fixers because N_2 fixation requires large amounts of P (Sprent & Raven 1985). However, this fails to reconcile the abundance of N_2 fixers in tropical rain forests, where soil P is generally scarce compared to P-rich temperate and high-latitude forests. Second, many of the genes that encode for signal transduction and regulate the establishment of the N_2 fixation symbiosis in plant roots are the same genes that encode for and regulate the AM fungi symbiosis, which may make the AM fungi symbiosis inherently more common in N_2 fixers (Antunes *et al.* 2006; Herder & Parniske 2009; Javaid 2010). Therefore, N_2 fixing plants may be genetically more predisposed to colonisation by another symbiont – AM fungi. Indeed, the consistent patterns of greater AM colonisation on N_2 fixing roots relative to non- N_2 fixing roots – as well as the consistent relationships between AM colonisation and both N_2 fixation rates and nodule weight between the two sites – may reflect an evolutionary relationship between N_2 fixing plants and AM fungi (Herder & Parniske 2009).

Finally, N_2 fixation has been shown to increase the photosynthetic rate of N_2 fixers relative to non- N_2 fixers via nutrient stimulation (e.g., Harris *et al.* 1985; Jia *et al.* 2004), possibly allowing greater investment in AM fungi. However, it is important to note that the N_2 fixation-AM fungi tripartite symbiosis increases the rate of photosynthesis at a greater rate than either of the symbioses alone (Kaschuk *et al.* 2009). Therefore, N_2 fixers may have a greater capacity to acquire N and P than non- N_2 fixers because N_2 fixers may invest more C into each nutrient acquisition strategy. In fact, in a re-analysis of foliar nutrient data obtained across a range of tropical forest sites (Townsend *et al.* 2007), we found that N_2 fixers had significantly higher foliar N (and P) concentrations

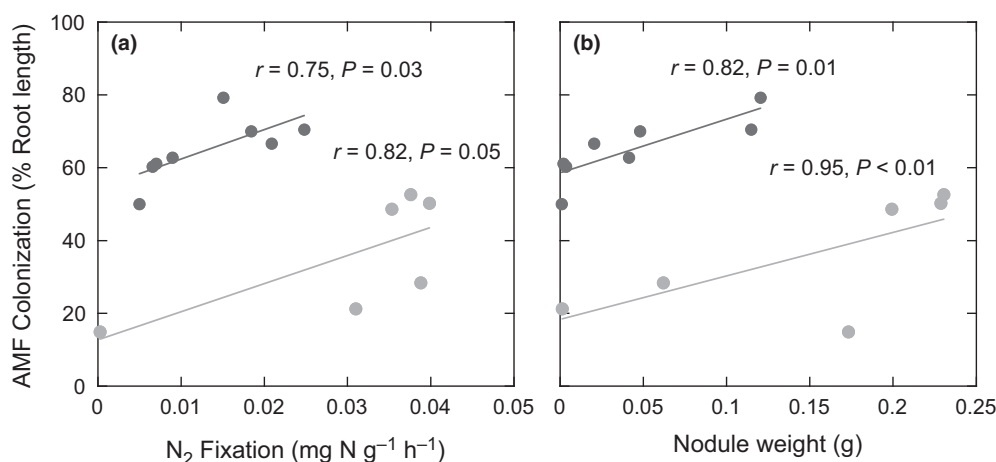


Figure 4 Relationships between arbuscular mycorrhizal (AM) colonisation and (a) N_2 fixation and (b) nodule weight between the Pacific (dark grey) and Caribbean (light grey) sites. Significance and correlation coefficients are shown for each relationship at the two sites. Analysis of covariance indicated that the positive relationships between AM colonisation and N_2 fixation ($P < 0.01$) and AM colonisation and nodule weight ($P < 0.01$) were consistent between the Pacific and Caribbean sites. Each point represents N_2 fixation, nodule and AM fungi data from an individual nodulated root.

than non- N_2 fixers (Fig. S1). Given the positive relationships between AM colonisation and both rates of N_2 fixation and nodule weight, relatively high rates of photosynthesis realised by N_2 fixers would be consistent with their high N and P content. In any case, the possible 'resource-driven' relationship between N_2 fixation and AM colonisation could also help to explain the relative abundance of N_2 fixers in tropical rain forests, where both N and P have been shown to limit ecosystem processes like net primary production (e.g., Davidson *et al.* 2004, 2007; Wright *et al.* 2011).

Our fine-scale measurements of soil phosphatase enzyme activity (i.e. in close proximity to actively fixing nodules) were intended to detect possible differences in phosphatase activity among individual plant roots and rhizosphere soil samples. As such, we expected that N_2 fixers would show greater phosphatase enzyme activity than non- N_2 fixers. However, while root phosphatase activity was higher for N_2 fixers than non- N_2 fixers, rhizosphere soil phosphatase activity was not. There are at least two possible explanations for this observation. First, the phosphatase enzyme activity in rhizosphere soil may reflect microbially produced enzymes rather than those produced by plant roots. Second, microbes in the rhizosphere soil of N_2 fixing roots may not necessarily have an advantage in producing phosphatase enzymes in a manner similar to N_2 fixing plants because they are less likely to experience the effects of fixed N in the soil. For example, increases in phosphatase enzyme activity have been most commonly observed under N_2 fixing tree canopies (Houlton *et al.* 2008; Keller *et al.* 2013), but our study was designed to analyse N_2 fixing and non- N_2 fixing roots, and associated rhizosphere soil, independent of specific tree or canopy effects. As a result, the rhizosphere soil we sampled near N_2 fixers was often in a location that was not directly underneath a canopy of a N_2 fixer.

Although we did not measure P uptake directly, the pivotal role of AM fungi and phosphatase enzymes in P acquisition has been well established (Lambers *et al.* 2008; Smith & Read 2008), and many studies have demonstrated that plants have

the capacity to acquire more P with increased AM colonisation and phosphatase enzyme activity (Sanders & Tinker 1971; Ortas *et al.* 1996; Khaliq & Sanders 2000; Treseder & Vitousek 2001). Our data also suggest that AM fungi most likely enhance P acquisition of inorganic P. For example, we saw no relationship between total (root + rhizosphere soil) phosphatase enzyme activity and AM colonisation at either the Pacific or Caribbean sites (Fig. S2). Thus, AM fungi may not simply enhance organic P mineralisation, but instead exploit other (inorganic) P pools that do not require enzyme hydrolysis or have already been hydrolysed by phosphatase enzymes. Regardless of the mechanism, however, the cumulative investment in soil P acquisition (i.e. via phosphatase enzyme and AM fungi) likely results in a greater capacity for N_2 fixers to acquire more soil P than non- N_2 fixers.

It is important to note that each of these plant nutrient acquisition strategies has significant resource costs that must be outweighed by the benefits for both to persist. For example, it is estimated that the N cost of soil P uptake via phosphatase enzymes ranges from 1 to 16 g N g⁻¹ P (Treseder & Vitousek 2001). Therefore, the benefit of soil P acquisition must exceed this N cost. However, the N_2 fixation-AM colonisation tripartite symbiosis is much more costly than the production of phosphatase enzymes. The estimated C cost of N uptake via N_2 fixation ranges from 8 to 12 g C g⁻¹ N (Fisher *et al.* 2010), and the cost of P uptake via AM colonisation ranges from 200 to 270 g C g⁻¹ P (Harris *et al.* 1985). Nonetheless, Kaschuk *et al.* (2009) found that plants with the tripartite symbiosis not only had increased photosynthetic rates but the additional C fixed exceeded the costs of both symbioses. Extending this observation further it seems plausible that tropical N_2 fixing trees with the tripartite symbiosis may not only have a greater capacity to acquire soil N and P, but may also be capable of assimilating more C than plants lacking the tripartite symbiosis.

Overall, our results provide compelling evidence that two mechanisms – AM fungi and phosphatase enzymes – may

simultaneously allow N₂ fixers to acquire P more effectively than non-N₂ fixers. Moreover, the consistent N and P patterns and interactions we observed indicate that the competitive advantage in nutrient acquisition realised by N₂ fixers as a functional group may exist across a wide range of soil N and P concentrations found in the tropics (Townsend *et al.* 2008). This may, in part, reflect their unique ability to either up- or down-regulate N₂ fixation and P acquisition strategies depending on the degree of nutrient abundance in the soil (Menge *et al.* 2009). For example, N₂ fixers at the Caribbean site, which has both greater total and extractable P concentrations than the Pacific site, had lower root phosphatase enzyme activity and AM colonisation per unit N₂ fixation. However, although robust, the correlations we observed are not capable of assessing the directionality between N₂ fixation and soil P acquisition. Therefore, experimental manipulations of N₂ fixation and/or AM colonisation (e.g., Harris *et al.* 1985), or tracer studies that track N and P within the soil-mycorrhizae-nodule-plant continuum (e.g., Frossard *et al.* 2011), are needed to determine whether N₂ fixers are fixing N to acquire soil P or whether they are investing in P acquisition to fix N. Nonetheless, the apparently superior competitive ability of N₂ fixers to acquire soil P via phosphatase enzymes and AM fungi has important ecological implications for the community assembly of lowland tropical rain forests, as well as the terrestrial distribution of N₂ fixers globally (Houlton *et al.* 2008).

ACKNOWLEDGEMENTS

We thank Osa Conservation, EARTH University, the Organización para Estudios Tropicales, and the Ministerio de Ambiente y Energía for access to field sites, assistance with research permits and logistical support in Costa Rica. We appreciate the work by R. Chazdon, B. Vilchez and E. Ortiz in establishing the plots at the Pacific rain forest site. We acknowledge field and laboratory assistance from M. Lopez Morales, J. Couvaca, S. Weintraub, S. Castle, A. Ginter, N. Boote and E. Prag. We thank R. Cole for sharing soil chemistry data. The manuscript benefitted from a discussion with B. Houlton and from the comments of three anonymous reviewers. Grants from the National Science Foundation (DEB-0919080) and the Andrew W. Mellon foundation supported this research.

AUTHORSHIP

MN, YL, BS and CC designed the study; MN, SAC and BS performed the research; MN analysed data and wrote the manuscript; all authors contributed to revisions.

REFERENCES

- Allen, E.B. (1998). Disturbance and seasonal dynamics of mycorrhizae in a tropical deciduous forest in Mexico. *Biotropica*, 30, 261–274.
- Alvarez-Clare, S. (2012). *Biological processes influencing nutrient limitation in a lowland tropical wet forest in Costa Rica*. Dissertation. University of Florida, Gainesville, Florida, USA.
- Alvarez-Clare, S., Mack, M.C. & Brooks, M. (2013). A direct test of nitrogen and phosphorus limitation to net primary productivity in a lowland tropical wet forest. *Ecology*, 94, 1540–1551.
- Antunes, P.M., de Varennes, A., Rajcanm, I. & Goss, M.J. (2006). Accumulation of specific flavonoids in soybean (*Glycine max* (L.) Merr.) as a function of the early tripartite symbiosis with arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* (Kirchner) Jordan. *Soil Biol. Biochem.*, 38, 1234–1242.
- Batterman, S.A., Wurzbarger, N. & Hedin, L.O. (2013). Nitrogen and phosphorus interact to control tropical symbiotic N₂ fixation: a test in *Inga punctata*. *J. Ecol.*, 101, 1400–1408.
- Berrange, J.P. & Thorpe, R.S. (1988). The geology, geochemistry, and emplacement of the Cretaceous-Tertiary ophiolitic Nicoya Complex of the Osa Peninsula, southern Costa Rica. *Tectonophysics*, 147, 193–220.
- Bray, R. & Kurtz, L. (1945). Determination of total, organic, and available forms of phosphorus in soils. *Soil Sci.*, 59, 39–46.
- Cleveland, C.C., Townsend, A.R., Taylor, P., Alvarez-Clare, S., Bustamante, M.M.C., Chuyong, G. *et al.* (2011). Relationships among net primary productivity, nutrients and climate in tropical rainforest: a pan-tropical analysis. *Ecol. Lett.*, 14, 939–947.
- Cleveland, C.C., Houlton, B.Z., Smith, B.K., Marklein, A.R., Reed, S.C., Parton, W. *et al.* (2013). Patterns of new versus recycled primary production in the terrestrial biosphere. *Proc. Natl Acad. Sci. USA*, 110, 12733–12737.
- D'Angelo, E., Crutchfield, J. & Vandiviere, M. (2001). Rapid, sensitive, microscale determination of phosphate in water and soil. *J. Environ. Qual.*, 30, 2206–2209.
- Davidson, E.A., de Carvalho, C.J.R., Vieira, I.C.G., Figueiredo, R.D.O., Moutinho, P., Ishida, F.Y. *et al.* (2004). Nitrogen and phosphorus limitation of biomass growth in a tropical secondary forest. *Ecol. App.*, 14, 150–163.
- Davidson, E.A., de Carvalho, C.J.R., Figueira, A.M., Ishida, F.Y., Ometto, J.P.H.B., Nardoto, G.B. *et al.* (2007). Recuperation of nitrogen cycling in Amazonian forests following agricultural abandonment. *Nature*, 447, 995–998.
- Doane, T.A. & Horwath, W.R. (2003). Spectrophotometric determination of nitrate with a single reagent. *Anal. Lett.*, 36, 2713–2722.
- Fisher, J.B., Sitch, S., Malhi, Y., Fisher, R.A., Huntingford, C. & Tan, S.Y. (2010). Carbon cost of plant nitrogen acquisition: A mechanistic, globally applicable model of plant nitrogen uptake, retranslocation, and fixation. *Global Biogeochem. Cy.*, 24, GB1014. DOI: 10.1029/2009GB003621.
- Frossard, E., Bünemann, E.K., Oberson, A., Jansa, J. & Kertesz, M.A. (2011). Phosphorus and sulfur in soil. In: *Handbook of soil sciences* (eds Huang, P.M., Sumner, M.E. & Yuncong, L.). 2nd edn. CRC, Boca Raton, pp. 1–15.
- Gerber, S., Hedin, L.O., Oppenheimer, M., Pacala, S.W. & Shevliakova, E. (2010). Nitrogen cycling and feedbacks in a global dynamic land model. *Global Biogeochem. Cycles*, 24, GB1001, doi:10.1029/2008GB003336.
- Hardy, R.W.F., Holsten, R.D., Jackson, E.K. & Burns, R.C. (1968). The acetylene-ethylene assay for N₂ fixation: laboratory and field evaluation. *Plant Phys.*, 43, 1185–1207.
- Harris, D., Pacovsky, R.S. & Paul, E.A. (1985). Carbon economy of soybean-*Rhizobium-Glomus* associations. *New Phytol.*, 101, 427–440.
- Hedin, L.O., Brookshire, E.N.J., Menge, D.N.L. & Barron, A.R. (2009). The nitrogen paradox in tropical forest ecosystems. *Annu. Rev. Ecol. Syst.*, 40, 613–635.
- Herder, G.D. & Parniske, M. (2009). The unbearable naivety of legumes in symbiosis. *Curr. Opin. Plant Biol.*, 12, 491–499.
- Houlton, B.Z., Wang, Y.P., Vitousek, P.M. & Field, C.B. (2008). A unifying framework for dinitrogen fixation in the terrestrial biosphere. *Nature*, 454, 327–330.
- Javaid, A. (2010). Role of arbuscular mycorrhizal fungi in nitrogen fixation in legumes. In: *Microbes for Legume Improvement* (eds Khan, M.S. *et al.*). Springer Vienna, Vienna, pp. 409–426.
- Jia, Y., Gray, V.M. & Straker, C.J. (2004). The influence of *Rhizobium* and arbuscular mycorrhizal fungi on nitrogen and phosphorus accumulation by *Vicia faba*. *Ann. Bot.*, 94, 251–258.

- Kaschuk, G., Kuyper, T.W., Leffelaar, P.A., Hungria, M. & Giller, K.E. (2009). Are rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses?. *Soil Biol. Biochem.*, 41, 1233–1244.
- Keller, A.B., Reed, S.C., Townsend, A.R. & Cleveland, C.C. (2013). Effects of canopy tree species on belowground biogeochemistry in a lowland wet tropical forest. *Soil Biol. Biochem.*, 58, 61–69.
- Khaliq, A. & Sanders, F.E. (2000). Effects of vesicular-arbuscular mycorrhizal inoculation on the yield and phosphorus uptake of field-grown barley. *Soil Biol. Biochem.*, 32, 1691–1696.
- Koske, R.E. & Gemma, J.N. (1989). A modified procedure for staining roots to detect VA mycorrhizas. *Mycol. Res.*, 92, 486–505.
- Lambers, H., Raven, J., Shaver, G. & Smith, S. (2008). Plant nutrient-acquisition strategies change with soil age. *Trends Ecol. Evol.*, 23, 95–103.
- Marklein, A.R. & Houlton, B.Z. (2011). Nitrogen inputs accelerate phosphorus cycling rates across a wide variety of terrestrial ecosystems. *New Phytol.*, 193, 696–704.
- McGill, W.B. & Cole, C.V. (1981). Comparative aspects of cycling of organic C, N, S and P through soil organic matter. *Geoderma*, 26, 267–286.
- McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L. & Swan, J.A. (1990). A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytol.*, 115, 495–501.
- Menge, D.N.L., Levin, S.A. & Hedin, L.O. (2009). Facultative versus obligate nitrogen fixation strategies and their ecosystem consequences. *Am. Nat.*, 174, 465–477.
- Olander, L.P. & Vitousek, P.M. (2000). Regulation of soil phosphatase and chitinase activity by N and P availability. *Biogeochemistry*, 49, 175–190.
- Ortas, I., Harris, P.J. & Rowell, D.L. (1996). Enhanced uptake of phosphorus by mycorrhizal sorghum plants as influenced by forms of nitrogen. *Plant Soil*, 184, 255–262.
- Saiya-Cork, K.R., Sinsabaugh, R.L. & Zak, D.R. (2002). The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biol. Biochem.*, 34, 1309–1315.
- Sanders, F.E. & Tinker, P.B. (1971). Mechanism of absorption of phosphate from soil by *Endogone* mycorrhizas. *Nature*, 233, 278–279.
- Sinsabaugh, R.L., Saiya-Cork, K., Long, T., Osgood, M.P., Neher, D.A., Zak, D.R. *et al.* (2003). Soil microbial activity in a Liquidambar plantation unresponsive to CO₂-driven increases in primary production. *Appl. Soil Ecol.*, 24, 263–271.
- Smith, S.E. & Read, D.J. (2008). *Mycorrhizal Symbiosis*, 3rd edn. Academic Press, San Diego.
- Solorzano, L. (1969). Determination of ammonia in natural waters by phenolhypo-chlorite method. *Limnol. Oceanogr.*, 14, 799–801.
- Sprent, J.I. & Raven, J.A. (1985). Evolution of nitrogen fixing symbioses. *P. Roy. Soc. Edinb. B.*, 85, 215–237.
- ter Steege, H., Pitman, N.C.A., Phillips, O.L., Chave, J., Sabatier, D., Duque, A. *et al.* (2006). Continental-scale patterns of canopy tree composition and function across Amazonia. *Nature*, 443, 444–447.
- Sullivan, B.W., Smith, W.K., Townsend, A.R., Nasto, M.K., Reed, S.C., Chazdon, R.L. *et al.* (2014). Spatially robust estimates of biological nitrogen (N) fixation imply substantial human alteration of the tropical N cycle. *Proc. Natl Acad. Sci. USA*, 111, 8101–8106.
- Townsend, A.R., Cleveland, C.C., Asner, G.A. & Bustamante, M.M.C. (2007). Controls over foliar N: P ratios in tropical rain forests. *Ecology*, 88, 107–118.
- Townsend, A., Asner, G. & Cleveland, C. (2008). The biogeochemical heterogeneity of tropical forests. *Trends Ecol. Evol.*, 23, 424–431.
- Treseder, K.K. & Vitousek, P.M. (2001). Effects of soil nutrient availability on investment in acquisition of N and P in Hawaiian rain forests. *Ecology*, 82, 1–9.
- Vitousek, P.M. & Farrington, H. (1997). Nutrient limitation and soil development: experimental test of a biogeochemical theory. *Biogeochemistry*, 37, 63–75.
- Vitousek, P.M. & Howarth, R.W. (1991). Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry*, 13, 87–115.
- Vitousek, P.M., Cassman, K., Cleveland, C.C., Crews, T.E., Field, C.B., Grimm, N.B. *et al.* (2002). Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry*, 57, 1–45.
- Wright, S.J., Yavitt, J.B., Wurzbarger, N., Turner, B.L., Tanner, E.V.J., Sayer, E.J. *et al.* (2011). Potassium, phosphorus, or nitrogen limit root allocation, tree growth, or litter production in a lowland tropical forest. *Ecology*, 92, 1616–1625.

SUPPORTING INFORMATION

Additional Supporting Information may be downloaded via the online version of this article at Wiley Online Library (www.ecologyletters.com).

Editor, Nancy Johnson
Manuscript received 25 June 2014
Manuscript accepted 6 July 2014