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Stoichiometry controls asymbiotic nitrogen fixation and its response to nitrogen inputs in a nitrogen-saturated forest

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Abstract. Lowland tropical forests with chronic nitrogen (N) deposition and/or abundant N-fixing organisms are commonly rich in N relative to other nutrients. The tropical N richness introduces a paradoxical relationship in which many tropical forests sustain high rates of asymbiotic N fixation despite the soil N richness and the higher energy cost of N fixation than of soil N uptake. However, the mechanism underlying this phenomenon remains unclear. Our study aims to test this phenomenon and examine potential mechanisms of nutrient concentrations vs. substrate stoichiometry in regulating N fixation using multiple linear regression models. We hypothesized that the rates of asymbiotic N fixation would be low in an N-rich forest under N deposition and substrate stoichiometry would explain the variation in N fixation better than nutrient concentrations. We conducted a chronic N-addition experiment in an N-saturated tropical forest in southern China and measured the N fixation rates, carbon (C), N, and phosphorus (P) concentrations, and stoichiometry in different substrates (soil, forest floor, mosses, and canopy leaves). Total N fixation rates were high (10.35–12.43 kg N·ha^{-1}·yr^{-1}) in this N-saturated forest because of the high substrate C:N and N:P stoichiometry (which explained 13–52% of the variation in N fixation, $P < 0.037$) rather than substrate nutrient concentrations ($P > 0.05$). Atmospheric N deposition (34–50 kg N·ha^{-1}·yr^{-1}) failed to down-regulate asymbiotic N fixation in this forest possibly because the N deposition rate was insufficient to inhibit N fixation or N deposition maintained high N fixation rates by increasing C sequestration in the substrates. Our N-addition experiment showed the insensitivity of N fixation in all the tested substrates to low N addition (50 kg N·ha^{-1}·yr^{-1}); however, medium and high N addition (100–150 kg N·ha^{-1}·yr^{-1}) stimulated the moss and foliar N fixation because of the increases in substrate C:N stoichiometry (which explained 30–34% of the variation in N fixation, $P < 0.001$). Overall, our results emphasize the importance of substrate (particularly mosses and foliage) stoichiometry as a driver of asymbiotic N fixation and sustained N richness in lowland tropical forests.

Key words: asymbiotic nitrogen fixation; leaky nitrostat model; nitrogen deposition; nitrogen-saturated forest; nutrient concentrations; substrate stoichiometry.

Introduction

Lowland tropical forests subjected to chronic nitrogen (N) deposition (Matson et al. 1999) and/or inhabited by abundant N-fixing organisms (i.e., legume species [Menge et al. 2014] and N-fixing microbes [Reed et al. 2008]) are commonly rich in N relative to other nutrients, as evidenced by their capacity to accumulate, recycle, and export large quantities of N (e.g., Fang et al. 2008, Hedin et al. 2009). The N richness of tropical forests introduces an N paradox in which asymbiotic N fixation (a process of N fixation performed by autotrophic or heterotrophic microbes; Reed et al. 2010, 2011) remains active though the soil is rich in N and N fixation is more energetically costly than soil N uptake (Gutschick 1981). For example, high rates of N fixation have been recorded in various substrates in N-rich tropical forests, such as the surface soil and litter (Reed et al. 2008, Cusack et al. 2009), decaying wood (Matzek and Vitousek 2003), canopy lichens (Forman 1975), and epiphylls (Goosem and Lamb 1986, Bentley 1987). The seemingly paradoxical observation of high N fixation rates in ecosystems that are not apparently limited by N underscores our incomplete understanding of the controls over N fixation.

The leaky nitrostat model proposed by Hedin et al. (2009) provides a mechanism by which asymbiotic N fixation remains active regardless of soil N richness; however, this model overlooks the potential effects of exogenous N inputs (e.g., atmospheric N deposition) on N fixation. Briefly, the model assumes that asymbiotic N fixation occurs in substrates (e.g., litter, epiphytes, and leaves) that are decoupled from soil N richness and are relatively poor in N (Hedin et al. 2009). Although this conceptual model explains why asymbiotic N fixers are active under the condition of soil N richness, it cannot explain why high N fixation rates sustain under chronic N deposition scenarios (e.g., Zheng et al.

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2016*a*, 2017). Previous modeling research indicates that N fixation has dramatically declined in tropical regions (Sullivan et al. 2014) and at global scales (Galloway et al. 2004, Vitousek et al. 2013) because of elevated anthropogenic N deposition. Furthermore, many manipulation experiments have revealed that N addition reduces N fixation rates in leaf litter (Crews et al. 2000, Winbourne et al. 2017), soil (Cusack et al. 2009, Zheng et al. 2016*a*), mosses (Gundale et al. 2011), and canopy leaves (Zheng et al. 2017), because N fixation is not energetically favorable when ambient N can be obtained. These findings indicate the importance of N deposition in regulating N fixation rates and the necessity of understanding its potential mechanisms.

Chronic N deposition could affect asymbiotic N fixation via controlling the concentrations of substrate N, P, and carbon (C) (Matson et al. 1999, Galloway et al. 2004). First, N deposition directly increases N concentrations in various substrates (e.g., mineral soil, forest floor, and epiphytes; Cusack et al. 2009, Zheng et al. 2016*a*), which inhibits nitrogenase synthesis and thus N fixation (Bentley 1987). Second, long-term N deposition causes soil acidification and leaching loss of P, thereby reducing concentrations of P (or increasing N:P ratios) in plant tissues and soils (Matson et al. 1999, Vitousek et al. 2010). Low P supply inhibits N fixation because P is needed for adenosine triphosphate (ATP) generation and the cell growth of N fixers (Alberty 2005). Third, N deposition increases the soil and litter C concentrations via the inhibition of soil respiration (e.g., Mo et al. 2008) and litter decomposition (e.g., Fang et al. 2007), which is beneficial to heterotrophic N fixers who acquire energy from organic matter (Gutschick 1981, Reed et al. 2011).

Nonetheless, in certain cases, single nutrient concentrations cannot explain the variation in N fixation rates well because N fixation is usually coregulated by multiple nutrients (e.g., N and P; Dynarski and Houlton 2018). Therefore, substrate stoichiometry, such as N:P and C:N ratios, may predict N fixation rates better than either N or P alone (Reed et al. 2011). For example, N-fixing microbes have a high capacity for N fixation when growing on C-rich but N-poor substrates (Vitousek and Hobbie 2000, Pérez et al. 2010) or when the substrates have low N but high P concentrations (Eisele et al. 1989). To date, our knowledge of the substrate stoichiometric control over N fixation has extended from freshwater and managed terrestrial ecosystems (e.g., Schindler 1977, Eisele et al. 1989, Smith 1992) to forests (Cusack et al. 2009, Pérez et al. 2010, Reed et al. 2010, 2013). Tropical forests have experienced the greatest increase in anthropogenic N deposition in recent decades (Galloway et al. 2004), and such deposited N altered the elemental stoichiometry in both plants and soils (Yue et al. 2016, Yu et al. 2017). However, to our knowledge, there is no published study addressing whether substrate stoichiometry regulates asymbiotic N fixation in N-rich tropical forests under chronic N deposition scenarios.

In this study, we investigated asymbiotic N fixation under atmospheric N deposition and in response to experimental N addition in an N-saturated tropical forest and tested the importance of nutrient concentrations vs. substrate stoichiometry in regulating N fixation. We hypothesized that (1) asymbiotic N fixation rates would be low in the N-saturated forest because of atmospheric N deposition and experimental N addition and (2) the variation in N fixation rates would be explained by substrate stoichiometry better than nutrient concentrations. We measured the N fixation rates, C, N, and P concentrations, and stoichiometry in different ecosystem compartments (soil, forest floor, mosses, and canopy leaves) in an N-saturated old-growth tropical forest (>400 yr) in southern China following 12 yr of N addition: control, low N, medium N, and high N (0, 50, 100, and 150 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$, respectively). Additionally, this forest has been subjected to high N deposition (34–50 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$) since 1990 due to the rapid development of industry in southern China. Our N-saturated forest can be representative of N-rich forests elsewhere because of the typical trait of high N losses from the soil (Fang et al. 2008) and the limitation of ecological processes (e.g., soil respiration) by P rather than N (Mo et al. 2008, Liu et al. 2012).

Methods

Site description

This study was conducted in Dinghushan Biosphere Reserve in the central area of Guangdong Province, southern China (112°10′ E, 23°10′ N). The study forest is an evergreen broadleaf forest and has been protected from human disturbance for more than 400 yr. The dominant tree species are *Castanopsis chinensis* Hance, *Schima superba* Chardn. & Champ., *Cryptocarya chinensis* (Hance) Hemsl., and *Machilus chinensis* (Champ. Ex Benth.) Hemsl. (Fang et al. 2005). Symbiotic N-fixing trees (e.g., legume species) are rare in the study area.

The reserve has a typical humid monsoon climate. Mean annual precipitation is 1927 mm, with 75% of rainfall occurring between March and August and 6% occurring between December and February (Huang and Fan 1982). Mean annual temperature is 21°C, with January being the coldest month (12.6°C) and July being the warmest month (28.0°C; Huang and Fan 1982). The forest soil is lateritic red earth formed from sandstone and exceeds 60 cm in depth. The forest has experienced high rates of atmospheric N deposition (34–50 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$) since 1990 (Huang et al. 1994, Fang et al. 2008, Lu et al. 2013).

Experimental design

The experiment was initiated in July 2003 with four levels of N addition (each in three replicates): control, low N (LN, 50 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$), medium N (MN, 100 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$), and high N (HN, 150 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$). Each 10 × 20 m plot was surrounded by a 10 m wide buffer strip, and all the plots were randomly laid out. Solutions of NH_4NO_3 were sprayed on the forest floor monthly from July 2003 to July 2015 using a backpack sprayer. Fertilizer was weighed and mixed with 20 L of water for each plot except the control plots, which received only an equivalent volume of deionized water.

Sample collection

In July 2015, five forest floor samples were randomly collected from each plot using a metal frame (20 × 20 cm),

and the mineral soil underneath the forest floor was sampled to a depth of 10 cm using a 2.5-cm soil corer. Canopy leaves were sampled from four dominant tree species (*Ca. chinensis*, *S. superba*, *M. chinensis*, and *Cr. chinensis*) using a pole pruner. Specifically, leaves in the upper, middle, and lower layers were collected from three individuals of each tree species in each plot for a total of 12 leaf samples per plot (note that the three layers of leaves from individuals were mixed). The leaves were removed from branches and sorted by tree species for a total of 12 samples per plot. Lichens were not found in the study forest, whereas mosses were growing on the bases of trees (~2 m above the ground). The dominant moss species were *Syrrhopodon armatus* Mitt. (*S. armatus*), *Octoblepharum albidum* Hedw. (*O. albidum*), and *Sematophyllum subhumile* (Mull. Hal.) Fleisch. (*S. subhumile*), among which only *S. armatus* was determined to fix N and was thus sampled. The mosses were collected by gently scraping three 5 × 5 cm pieces from each of the 12 trees (where canopy leaves were sampled) and then mixed based on the trunk for a total of 12 samples per plot. All the samples were stored under cold and dark conditions and analyzed within 24 h. All the samples were weighed, and portions were oven dried at 65°C (forest floor, canopy leaves, and mosses) or 105°C (soil) for 48 h to determine the moisture content.

Acetylene reduction assay

N fixation rates were measured using the acetylene reduction assay (ARA; Hardy et al. 1968), which measures the ability of nitrogenase to reduce acetylene (C_2H_2) to ethylene (C_2H_4). Fresh samples (~5 g forest floor, ~13 g soil, ~7 g canopy leaves, or ~3 g mosses) were sealed into 120-mL gas-tight glass jars, with 10% of the headspace (12 mL) replaced with pure C_2H_2 (99.99%; Kodi Gas Chemical Industry, Foshan, China). All the samples were incubated for 24 h in situ to approximate ambient light and temperature conditions. We selected the incubation period of 24 h (long compared to some previous studies in tropical forests; e.g., Barron et al. 2008, Reed et al. 2008) because C_2H_4 production was not detectable in some of our substrates within a shorter time period. After incubation, the headspace gas from each jar was sampled, stored in a 12-mL evacuated Exetainer (Labco, High Wycombe, UK), and returned to the laboratory for analysis within 24 h. In the laboratory, C_2H_4 concentrations were measured using a GC14 gas chromatograph (Shimadzu Co., Tokyo, Japan) equipped with a flame ionization detector and a Poropak N column (Shimadzu Co; the injector, detector, and column temperatures were 70, 150, and 250°C, respectively). The background C_2H_4 concentrations of C_2H_2 gases (no sample) were measured during the field incubation and subtracted. The C_2H_4 concentrations naturally produced by the samples were also measured but were below the detection limit (C_2H_4 <5.66 ppb).

Estimate of N fixation rates

Annual N fixation rates (kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$) were scaled up using the acetylene reduction rates (nmol $C_2H_4{\cdot}g^{-1}{\cdot}h^{-1}$) to facilitate comparisons with published estimates (Appendix S1: Table S1). Note that extrapolations from a single sampling event are based on the assumption that N fixation rates are constant across seasons. Thus, annual N fixation rates represent potential rates rather than definitive rates. We calibrated the conversion ratio of C_2H_2 reduced to N_2 fixed by incubating each substrate sample (divided into three subsamples exposed to 10% $^{15}N_2$ [99 atom%], pure C_2H_2, and ambient air) for 24 h. The substrate samples were obtained from all the plots, and each plot had three replicate samples. After incubation, all the subsamples were dried at 60°C, ground to fine powder, and analyzed for $^{15}N/^{14}N$ and N% on an isotope ratio mass spectrometer (IsoPrime 100; Elementar Co., Langenselbold, Germany).

The soil bulk density was determined by the dry mass and sampling volume and converted to the standing stock (kg soil/m^2, based on the depth of 0–10 cm; Appendix S1: Table S2). The standing stock of the forest floor (kg forest floor/m^2) was estimated by the dry mass and sampling area. The moss density was estimated by the mean percent cover on the tree surface. Percent cover was estimated by randomly placing eight 10 × 10 cm quadrats on the tree surface and visually estimating the percent cover of mosses in each quadrat (Gundale et al. 2011). The tree surface area was calculated by assuming that trees are cylinders and multiplying height by circumference. The standing stock of mosses (dry mass) per unit of ground area (kg moss/m^2) was estimated by the moss density, tree surface area, and tree density (1,729 trees/ha; Fang et al. 2005). Canopy leaves were collected, and the specific leaf area (leaf area/dry mass) was estimated for each species and each plot. The leaf area was measured using a leaf-area meter (LI-3000A; Li-Cor, Lincoln, Nebraska, USA), and the dry mass was measured after oven drying at 65°C. Because leaves of different species are mixed in the canopy, the standing stock of canopy leaves (kg canopy leaves/m^2) in each plot was estimated by the mean specific leaf area of four species and a published leaf area index of 12.08 for the whole canopy strata (Ren et al. 1996). Because of the small within-plot variation in nitrogenase activity (Appendix S1: Table S3), we estimated the N fixation rates by assuming that N fixation was homogeneous in each plot. The annual N fixation rates (kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$) were scaled up using the standing stock (kg/m^2), acetylene reduction rates (nmol $C_2H_4{\cdot}g^{-1}{\cdot}h^{-1}$), and conversion ratio of C_2H_2 reduced to N_2 fixed (Appendix S1: Table S4).

Analyses of chemical properties

Total C (TC) concentrations of each substrate were measured by potassium dichromate oxidation titration with Fe^{2+} solution (Liu 1996). Total N (TN) and total P (TP) concentrations of each substrate were measured by micro-Kjeldahl digestion followed by the indophenol blue and the Mo-Sb colorimetric methods, respectively, using a UV-8000 spectrophotometer (Liu 1996). Soil NH_4^+ and NO_3^- concentrations were measured by extraction in 50 mL of a 2 mol/L KCl solution and analyzed spectrophotometrically (Bremner and Mulvaney 1982). Soil inorganic N (IN) concentrations were the sum of the NH_4^+ and NO_3^- concentrations. Soil available P (AP) concentrations were

measured spectrophotometrically after extraction with an acid-ammonium fluoride solution (Anderson and Ingram 1989).

Statistical analyses

We only used the arithmetic means for analyses because the differences among the arithmetic, logarithmic, and square-root transformed mean N fixation values were small. Data were tested to fulfill normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test) and then analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's HSD test for the treatment effects. Yet, the N fixation rates in certain layers (i.e., mosses and the forest floor) did not exhibit normal distributions and were thus analyzed using the Kruskal-Wallis H test followed by the Nemenyi test for multiple comparisons (Hollander et al. 2013). Multiple linear regression models were used to explore the multivariate effects of substrate nutrient concentrations and substrate stoichiometry on nitrogenase activity. Single linear regression models were used to explore the stoichiometric effects on nitrogenase activity under each treatment (i.e., control, LN-, MN-, and HN-plots) and across the N-addition treatment (i.e., all the plots combined). All statistical analyses were conducted using SPSS 19.0 for Windows (SPSS, Chicago, Illinois, USA). Statistically significant differences were recognized at $P < 0.05$.

Results

Asymbiotic N fixation

Mosses had the highest nitrogenase activity (10.64 ± 0.42 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$), followed by the forest floor (3.07 ± 0.07 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$), canopy leaves (0.15 ± 0.01 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$), and soil (0.09 ± 0.00 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$; Fig. 1a). LN addition did not affect the forest floor or soil nitrogenase activity, whereas MN and HN additions reduced the nitrogenase activity in these substrates ($F_{3,8} = 65.7$, $P < 0.001$ for the soil and $F_{3,8} = 34.2$, $P < 0.001$ for the forest floor). Neither LN nor MN additions affected the moss nitrogenase activity, whereas HN addition increased the moss nitrogenase activity ($F_{3,8} = 7.9$, $P = 0.009$). Foliar nitrogenase activity tended to increase across N additions, with significant responses observed for *S. superba* ($F_{3,8} = 5.6$, $P = 0.023$), *C. chinensis* ($F_{3,8} = 6.9$, $P = 0.013$) and *M. chinensis* ($F_{3,8} = 4.7$, $P = 0.036$).

The $^{15}N_2$ incubation results showed that the conversion ratios of per mol C_2H_2 reduced to per mol N_2 fixed were relatively higher in the soil (2.24–4.04), forest floor (1.78–3.75), and mosses (2.12–4.31) than those in the leaves (0.21–1.14; Appendix S1: Table S4). The standing stock of the soil (95–102 kg/m^2, based on the depth of 0–10 cm) was two to three orders of magnitude higher than that of the forest floor (1.07–1.22 kg/m^2), mosses (0.11–0.14 kg/m^2), and leaves (0.79–0.87 kg/m^2; Appendix S1: Table S2). Therefore, the N

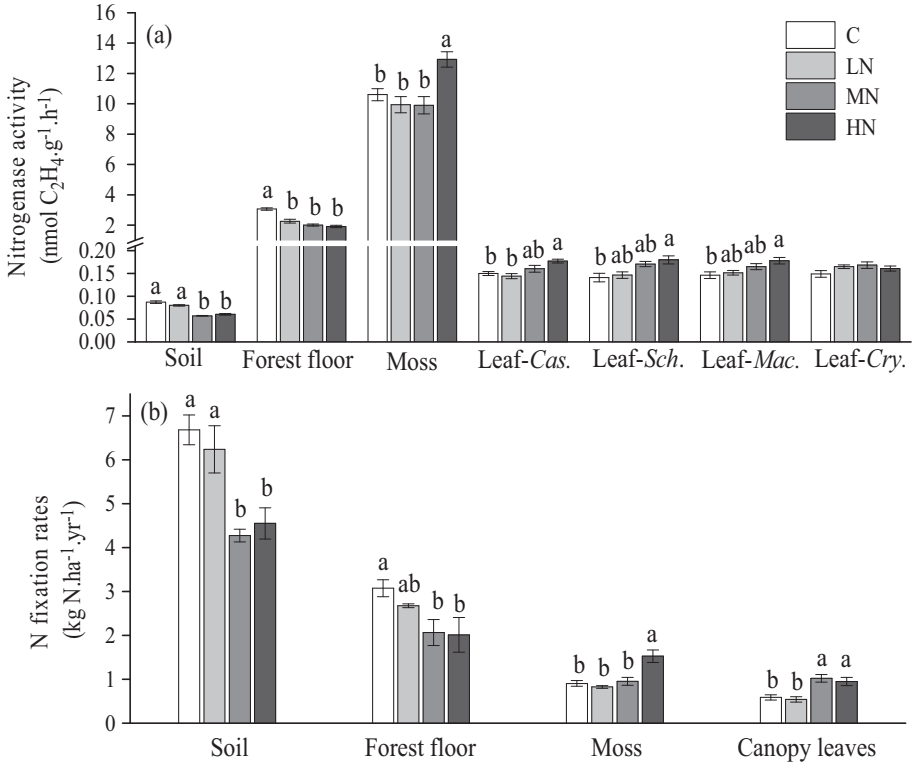

Fig. 1. Effects of (a) N addition on nitrogenase activity and (b) N fixation rates in different compartments. C, control; LN, low nitrogen addition; MN, medium nitrogen addition; HN, high nitrogen addition; Leaf-*Cas.*, *Castanopsis chinensis*; Leaf-*Sch.*, *Schima superba*; Leaf-*Mac.*, *Machilus chinensis*; Leaf-*Cry.*, *Cryptocarya chinensis*. Rates of N fixation were scaled up based on the nitrogenase activity, standing stock (Appendix S1: Table S2), and conversion ratios (Appendix S1: Table S3). Different lowercase letters represent significant differences among treatments ($P < 0.05$). Error bars represent standard errors of the means ($n = 3$; which are the plot replicates).

fixation rates per unit area, which were scaled up based on the nitrogenase activity, conversion ratios, and standing stock, were highest in the soil (6.65 ± 0.22 kg N·ha^{-1}·yr^{-1}), followed by the forest floor (3.08 ± 0.27 kg N·ha^{-1}·yr^{-1}), mosses (0.89 ± 0.03 kg N·ha^{-1}·yr^{-1}), and canopy leaves (0.58 ± 0.04 kg N·ha^{-1}·yr^{-1}) (Fig. 1b). Importantly, MN and/or HN additions reduced N fixation rates in the substrates (soil [$F_{3,8} = 10.4$, $P = 0.004$] and forest floor [$F_{3,8} = 4.7$, $P = 0.036$]) that fixed the most N, but the rates in the mosses ($F_{3,8} = 12.1$, $P = 0.002$) and leaves ($F_{3,8} = 10.4$, $P = 0.004$) increased and thereby sustained high total rates of ecosystem N fixation (7.89–11.26 kg N·ha^{-1}·yr^{-1}).

Nutrient concentrations and substrate stoichiometry in response to N addition

MN and HN additions increased the soil IN concentrations ($F_{3,8} = 4.7$, $P = 0.036$), and MN addition decreased the soil AP concentrations ($F_{3,8} = 4.5$, $P = 0.039$; Table 1). Soil IN:AP and N:P ratios increased following MN addition ($F_{3,8} = 4.4$, $P = 0.043$ and $F_{3,8} = 4.6$, $P = 0.037$, respectively). MN and HN additions increased the forest floor N concentrations ($F_{3,8} = 6.6$, $P = 0.015$) and N:P ratios ($F_{3,8} = 5.0$, $P = 0.031$) but decreased the forest floor C:N ratios ($F_{3,8} = 4.8$, $P = 0.033$; Table 1). HN addition increased the moss C concentrations ($F_{3,8} = 4.9$, $P = 0.032$) and C:N ratios ($F_{3,8} = 4.4$, $P = 0.041$; Table 1). MN and HN additions increased the foliar C concentrations ($F_{3,8} = 10.6$, $P = 0.004$), and MN addition increased the foliar C:N ratios ($F_{3,8} = 4.7$, $P = 0.036$; Table 1).

Stoichiometry controls over asymbiotic N fixation

Multiple regression models showed that the variation in nitrogenase activity was controlled by substrate stoichiometry rather than substrate nutrient concentrations (Table 2). Under each treatment, substrate C:N or N:P ratios explained the variation in nitrogenase activity in the soil (30–52%, $P < 0.037$) and forest floor (32–51%, $P < 0.031$), and substrate C:N ratios explained the variation in the mosses (13–43%, $P < 0.031$) and leaves (15–49%, $P < 0.019$). Across the N-addition treatments, the declines in the forest floor and soil nitrogenase activity could be

Table 1. Effects of N addition on nutrient concentrations and substrate stoichiometry in the study forest.

Compartment and variable	Treatment			
	C	LN	MN	HN
Soil				
IN (mg/kg)	8.09 (1.78)^{b}	8.79 (1.22)^{ab}	13.51 (1.85)^{a}	13.18 (0.74)^{a}
AP (mg/kg)	2.06 (0.40)^{a}	1.35 (0.29)^{ab}	0.80 (0.16)^{b}	1.31 (0.17)^{ab}
IN:AP	4.73 (2.15)^{b}	6.93 (1.11)^{b}	18.54 (4.80)^{a}	10.65 (2.24)^{ab}
C (mg/g)	39.98 (4.18)^{a}	40.72 (4.56)^{a}	46.94 (3.78)^{a}	41.79 (2.86)^{a}
N (mg/g)	2.93 (0.49)^{a}	2.71 (0.22)^{a}	3.48 (0.31)^{a}	3.28 (0.24)^{a}
P (mg/g)	0.34 (0.02)^{a}	0.31 (0.01)^{a}	0.30 (0.03)^{a}	0.30 (0.01)^{a}
C:N	14.50 (3.02)^{a}	15.50 (2.78)^{a}	13.54 (0.79)^{a}	12.97 (1.59)^{a}
N:P	8.57 (0.95)^{b}	8.69 (0.86)^{b}	11.69 (0.86)^{a}	10.79 (0.72)^{ab}
C:P	119.72 (16.74)^{a}	129.96 (12.39)^{a}	157.00 (3.75)^{a}	137.69 (8.99)^{a}
Forest floor				
C (mg/g)	523.82 (9.61)^{a}	550.53 (8.84)^{a}	543.56 (9.22)^{a}	543.39 (9.01)^{a}
N (mg/g)	17.33 (1.54)^{b}	22.24 (1.45)^{ab}	27.39 (2.12)^{a}	27.60 (2.32)^{a}
P (mg/g)	0.79 (0.07)^{a}	0.87 (0.03)^{a}	0.89 (0.08)^{a}	0.91 (0.05)^{a}
C:N	30.80 (3.21)^{a}	25.03 (2.10)^{ab}	20.10 (1.75)^{b}	20.00 (1.93)^{b}
N:P	21.90 (1.04)^{b}	25.56 (1.64)^{ab}	30.82 (1.56)^{a}	30.50 (2.92)^{a}
C:P	672.93 (71.64)^{a}	633.98 (26.63)^{a}	619.20 (59.97)^{a}	601.21 (40.35)^{a}
Mosses				
C (mg/g)	444.59 (15.21)^{b}	451.75 (14.71)^{ab}	477.94 (17.03)^{ab}	505.04 (18.85)^{a}
N (mg/g)	21.61 (0.56)^{a}	20.41 (0.57)^{a}	21.18 (0.33)^{a}	21.43 (0.59)^{a}
P (mg/g)	0.68 (0.02)^{a}	0.63 (0.06)^{a}	0.71 (0.04)^{a}	0.66 (0.03)^{a}
C:N	20.62 (1.02)^{b}	22.14 (0.43)^{ab}	22.56 (0.55)^{ab}	23.58 (0.85)^{a}
N:P	31.72 (0.92)^{a}	33.14 (3.91)^{a}	29.84 (1.32)^{a}	32.59 (1.93)^{a}
C:P	652.43 (19.00)^{a}	730.37 (71.08)^{a}	674.05 (42.01)^{a}	769.15 (60.24)^{a}
Canopy leaves				
C (mg/g)	507.48 (9.79)^{b}	536.33 (6.14)^{b}	597.84 (16.67)^{a}	601.04 (19.91)^{a}
N (mg/g)	23.40 (1.70)^{a}	21.16 (0.78)^{a}	22.81 (1.73)^{a}	24.03 (1.64)^{a}
P (mg/g)	0.82 (0.07)^{a}	0.75 (0.10)^{a}	0.88 (0.09)^{a}	0.80 (0.08)^{a}
C:N	21.87 (1.21)^{b}	25.41 (0.97)^{ab}	26.41 (1.40)^{a}	25.18 (1.40)^{ab}
N:P	28.82 (0.46)^{a}	29.21 (3.18)^{a}	26.37 (2.24)^{a}	30.78 (3.26)^{a}
C:P	631.24 (44.97)^{a}	747.92 (109.68)^{a}	703.23 (102.65)^{a}	777.87 (103.67)^{a}

Notes: Values are means with standard errors in brackets ($n = 3$). C, control; LN, low nitrogen addition; MN, medium nitrogen addition; HN, high nitrogen addition; IN, inorganic nitrogen; AP, available phosphorus. Different lowercase letters represent significant differences among treatments ($P < 0.05$).

TABLE 2. Multiple linear regression models of nitrogenase activity against nutrient concentrations and/or substrate stoichiometry in the study forest.

Dependent variable and Plots	Regression model	n	r^2	P
Soil nitrogenase activity				
Control	$Y = 0.001 \times$ C:N + 0.074	15	0.376	0.015
Low N	$Y = -0.002 \times$ N:P + 0.102	15	0.424	0.009
	$Y = -0.002 \times$ N:P + 0.001 × IN:AP + 0.093	15	0.643	0.002
Medium N	$Y = -0.001 \times$ N:P + 0.064	15	0.519	0.002
High N	$Y = 0.001 \times$ C:N + 0.054	15	0.295	0.036
All the plots	$Y = -0.002 \times$ N:P + 0.096	60	0.349	<0.001
	$Y = -0.002 \times$ N:P + 0.001 × IN:AP + 0.097	60	0.436	<0.001
Forest floor nitrogenase activity				
Control	$Y = -0.046 \times$ N:P + 4.085	15	0.322	0.027
Low N	$Y = 0.029 \times$ C:N + 1.495	15	0.512	0.003
Medium N	$Y = -0.021 \times$ N:P + 2.646	15	0.512	0.002
High N	$Y = 0.019 \times$ C:N + 1.517	15	0.315	0.030
All the plots	$Y = 0.05 \times$ C:N + 1.075	60	0.524	<0.001
	$Y = 0.057 \times$ C:N − 0.004 × C + 2.859	60	0.592	<0.001
Moss nitrogenase activity				
Control	$Y = 0.234 \times$ C:N + 5.724	36	0.131	0.030
Low N	$Y = 0.304 \times$ C:N + 2.982	36	0.349	<0.001
Medium N	$Y = 0.228 \times$ C:N + 4.611	36	0.263	0.001
High N	$Y = 0.416 \times$ C:N + 2.681	36	0.429	<0.001
All the plots	$Y = 0.323 \times$ C:N + 3.452	144	0.300	<0.001
	$Y = 0.222 \times$ C:N + 0.014 × C−0.675	144	0.332	<0.001
Foliar nitrogenase activity				
Control	$Y = 0.001 \times$ C:N + 0.118	36	0.154	0.018
Low N	$Y = 0.002 \times$ C:N + 0.088	36	0.485	<0.001
	$Y = 0.002 \times$ C:N × 0.001 × N:P + 0.127	36	0.626	<0.001
Medium N	$Y = 0.003 \times$ C:N + 0.088	36	0.469	<0.001
	$Y = 0.003 \times$ C:N + 0.001 × C + 0.024	36	0.535	<0.001
High N	$Y = 0.003 \times$ C:N + 0.096	36	0.330	<0.001
All the plots	$Y = 0.003 \times$ C:N + 0.093	144	0.345	<0.001
	$Y = 0.002 \times$ C:N + 0.001 × C + 0.019	144	0.473	<0.001

Notes: Independent variables used for the stepwise selection procedure included inorganic N (IN), available P (AP), total C, total N, total P, IN:AP, C:N, N:P, and C:P for the soil, and the same variables (except for IN, AP, and IN:AP) for the forest floor, mosses, and leaves. Non-significant terms ($P > 0.05$) were excluded in the models. All the plots included both the control and treatment plots. The values of n and r^2 represent the sample sizes and determination coefficients, respectively.

explained by the substrate C:N (52%, $P < 0.001$) and N:P ratios (34%, $P < 0.001$), respectively (Fig. 2a, b), and the increases in the moss and foliar nitrogenase activity could be explained by the substrate C:N ratios (30%, $P < 0.001$ and 34%, $P < 0.001$, respectively; Fig. 2c, d). Therefore, substrate stoichiometry plays an important role in regulating asymbiotic N fixation.

Discussion

Contrary to our hypothesis that N fixation rates should have been low in this already N-saturated forest, we found that asymbiotic N fixation remained active in all the tested substrates (Fig. 1) and the total N fixation rates were high (10.35–12.43 kg N·ha^{-1}·yr^{-1}; Appendix S1: Table S1). This finding is consistent with earlier findings in humid tropical forests where high rates of N fixation were observed in the epiphytes (e.g., lichens [Forman 1975], epiphylls [Goosem and Lamb 1986, Bentley 1987]) and supports recent findings that asymbiotic N fixation is active in numerous substrates (e.g., soil, litter, and foliage [Reed et al. 2008, Cusack et al. 2009]; bryophytes, lichens, and decaying wood [Matzek and Vitousek 2003]) despite the forest soils being rich in N. These findings together lend support to the leaky nitrostat hypothesis that N-fixing microbes growing on certain substrates are decoupled from soil N richness and are therefore not controlled by soil N status (Hedin et al. 2009, Menge and Hedin 2009).

We found no evidence that the concentrations of a single nutrient (e.g., N or P) could account for the variation in N fixation of different ecosystem substrates; instead, substrate stoichiometry (i.e., C:N and N:P ratios) explained 13–52% of the variation in nitrogenase activity across different types of substrates (Table 2). This result supports our hypothesis that N fixation is controlled by substrate stoichiometry rather than by nutrient concentrations. On the one hand, substrate C:N stoichiometry plays an important role in sustaining high N fixation rates because N fixation is energy intensive and N fixers have a competitive advantage under low-N conditions. This mechanism was supported by previous studies, in which high rates of N fixation were observed in plant tissues (e.g., leaf litter [Winbourne et al. 2017] and fresh leaves [Cusack et al. 2009]) with high C:N ratios. Additional evidence is derived from litter decomposition assays

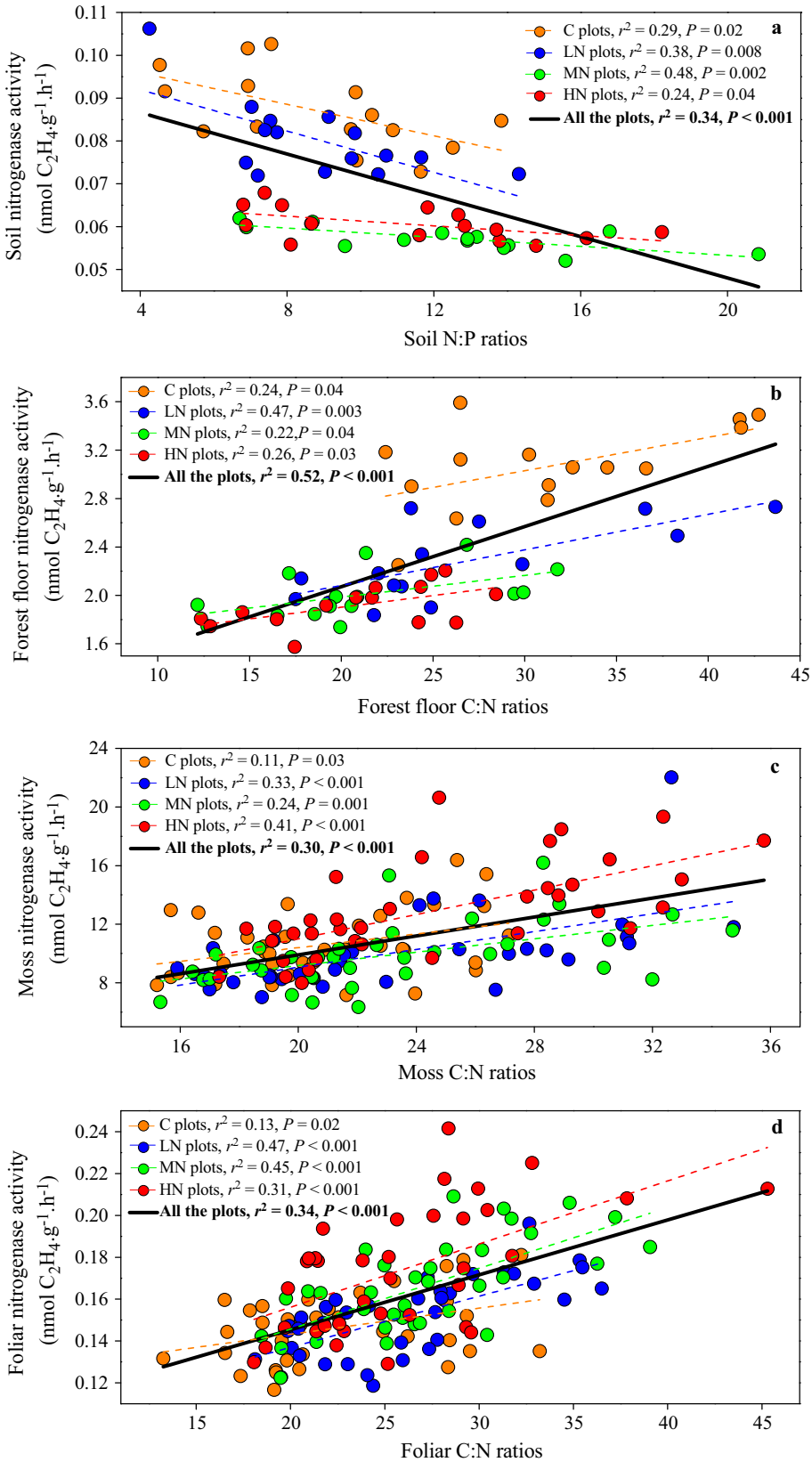

FIG. 2. Single linear regression models of nitrogenase activity against substrate stoichiometry under each treatment (control, low N [LN]), medium N [MN], and high N [HN] plots) and across the N-addition treatments (all the plots combined). The variables regulating nitrogenase activity (i.e., N:P ratios for the soil and C:N ratios for the other compartments) were selected from the multiple regression models (Table 2). Each of the treatments had three plot replicates, and each plot had five replicate samples for the soil (n = 15) and forest floor (n = 15) and 12 replicate samples for the mosses (n = 36) and leaves (n = 36).

showing that heterotrophic N fixation was up-regulated by the high availability of labile C (or low lignin content) but low availability of N (Vitousek and Hobbie 2000, Pérez et al. 2010). Therefore, high rates of asymbiotic N fixation in N-rich tropical forests may be driven by substrate C:N stoichiometry rather than by the heterogeneity of ecosystem N pools (i.e., local N limitation) as previously hypothesized (Hedin et al. 2009).

On the other hand, substrate N:P stoichiometry is also important in sustaining high asymbiotic N fixation given that P supply constrains N fixation rates (e.g., Reed et al. 2007, Zheng et al. 2016*b*). In our N-saturated forest, chronic N addition intensified soil P limitation, as evidenced by the decreases in soil P availability and the increases in soil IN:AP and N:P ratios (Table 1). Stepwise regression analysis showed that the substrate N:P stoichiometry explained 32–52% of the variation in the soil and forest floor N fixation (Table 2) and N addition inhibited soil N fixation partially via increases in soil N:P ratios (34%, $P < 0.001$; Fig. 2a). Under P-limiting conditions, N fixation rates remained high in this forest (Appendix S1: Table S1), but the potential mechanism is not clear. We propose that N-fixing microbes may hold an advantage in P acquisition when the ambient P supply is limiting. Although direct evidence is lacking, a previous model proposed by Houlton et al. (2008) has demonstrated that N fixation rates can be high in tropical sites assuming that N fixers can invest more N to acquire P in low-P soils. Recent findings support this model because some N-fixing tree species have higher production of extracellular phosphatase (a class of N-rich enzymes involved in P mineralization) than non-N-fixing species (e.g., Keller et al. 2013, Nasto et al. 2014). Therefore, sustaining high rates of N fixation may be a P-acquisition strategy performed by N-fixing microbes living in N-rich tropical forests, and additional experiments are needed to test this mechanism in the near future.

Inconsistent with our hypothesis, we did not observe down-regulation of asymbiotic N fixation by atmospheric N deposition (34–50 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$) in this forest (Appendix S1: Table S1). The following mechanisms could account for this phenomenon. First, the responses of the N-saturated forest to N addition depend on the N amounts, as evidenced by a lack of response of many ecological processes (e.g., litter decomposition and soil respiration) to low N addition (50 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$) and a negative response to medium and high N addition (100 and 150 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$, respectively; Fang et al. 2007, Mo et al. 2008). These findings suggest that the local N deposition rate (34–50 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$) may not be sufficiently high to inhibit N fixation in the study forest. Second, N-rich mature forests, including our forest, have been reported to sequester C in plant tissues, litter, and/or surface soils under scenarios of climate change and elevated N deposition (Zhou et al. 2006, Luyssaert et al. 2008), which may maintain high C:N ratios in these substrates and thus favor N fixation.

We hypothesized that experimental N addition would down-regulate N fixation rates, which was not supported by our results. Actually, we found no response of N fixation to low N addition in any of the compartments (Fig. 1). Although medium and high N additions inhibited the soil and forest floor N fixation, they stimulated the moss and foliar N fixation (Fig. 1), and the total N fixation rates remained high (8.79–11.26 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$). The divergent responses of the substrates are controlled by stoichiometry as demonstrated, across the N-addition treatments, by the substrate N:P and/or C:N ratios, which accounted for the declines in the soil and forest floor N fixation (34–52%) and the increases in the moss and foliar N fixation (30–34%; Fig. 2). This finding further supports our hypothesis that substrate stoichiometry controls asymbiotic N fixation. Our findings are consistent with previous findings in which N addition inhibited N fixation in the soil and forest floor (Barron et al. 2008, Cusack et al. 2009, Matson et al. 2015), and importantly, indicates that N addition can stimulate N fixation in some substrates (mosses and foliage).

Interestingly, we found that N addition stimulated the moss and foliar N fixation via increasing C sequestration and C:N ratios in these plant tissues (Table 1 and Fig. 2c, d). Two potential mechanisms may account for the increases in plant C concentrations. One is that N addition stimulates plant photosynthesis and thus C sequestration, assuming that primary production of plants is constrained by N supply. Yet, in N-saturated forests, plant growth is less limited by N and instead, excess N inputs often cause foliar nutrient imbalances, thereby inhibiting foliar photosynthesis (Aber et al. 1995). Our recent finding from this N-saturated forest showed that N addition had no or negative effects on the photosynthetic capacity of understory plants (Mao et al. 2018), indicating that the observed increases in plant C may not result from increased plant photosynthesis. We propose an alternative mechanism to be that N addition leads to reallocation of plant C from aboveground to belowground. Specifically, long-term N addition likely reduced plant C investments into the belowground tissues, as evidenced by the decreases in fine root biomass (Zhu et al. 2013), respiration (Mo et al. 2008) and dissolved organic C efflux from the primary rooting zones (Lu et al. 2013) in this forest. Therefore, the carbohydrates are stored in the foliage and leach to epiphytes, leading to increases in C concentrations and thus N fixation rates in the leaves and mosses.

In summary, our study revealed high rates of asymbiotic N fixation in an N-saturated tropical forest regardless of atmospheric N deposition and experimental N addition. This finding lends support to Hedin et al.'s (2009) leaky nitrostat model, in which asymbiotic N fixation is decoupled from and less controlled by soil N richness. Our findings also indicate that high asymbiotic N fixation is driven by the substrate stoichiometry (C:N ratio) rather than by N heterogeneity (within ecosystem pools) as hypothesized by the model. Moreover, our work extends the leaky nitrostat model to an N-saturated tropical forest that has experienced long-term N pollution and showed that asymbiotic N fixation was not down-regulated by atmospheric N deposition. This phenomenon can be explained by two mechanisms in our study: (1) the rate of ambient N deposition was insufficient to inhibit N fixation and/or (2) N deposition maintained high substrate C:N ratios by stimulating C sequestration in mosses and foliage, which favored N fixation in these two substrates. Importantly, experimental N additions inhibited the soil and forest floor N fixation but stimulated the moss and foliar N fixation via the controls over substrate stoichiometry, thereby sustaining high total

N fixation rates in this N-saturated forest. Although other factors (e.g., molybdenum, iron, and moisture) that may control asymbiotic N fixation were not considered in this study, our findings showed that substrate stoichiometry could explain 13–64% of the variation in asymbiotic N fixation in this N-saturated forest (Table 2). Overall, our work adds to the growing understanding of the mechanisms underlying high N fixation rates observed in N-rich tropical forests and emphasizes the role of substrate stoichiometry in driving tropical N fixation.

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Supporting Information

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