



Different responses of asymbiotic nitrogen fixation to nitrogen addition between disturbed and rehabilitated subtropical forests



Mianhai Zheng^{a,b,c}, Wei Zhang^a, Yiqi Luo^b, Taiki Mori^a, Qinggong Mao^a, Senhao Wang^{a,c}, Juan Huang^a, Xiankai Lu^a, Jiangming Mo^{a,*}

^a Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China

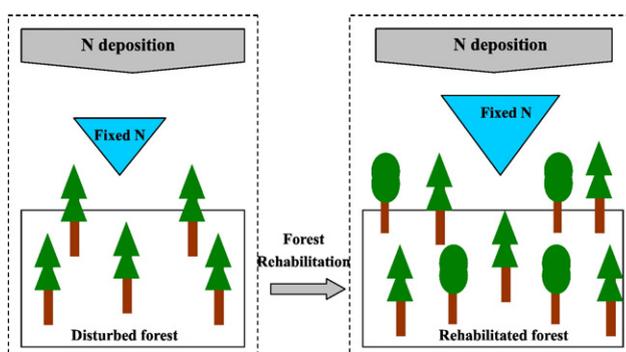
^b Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA

^c University of Chinese Academy of Sciences, Beijing 100039, China

HIGHLIGHTS

- N fixation was measured in a disturbed and a rehabilitated forest after N addition.
- N fixation was comparable between the disturbed and rehabilitated forests.
- N addition suppressed N fixation in all compartments in the disturbed forest.
- N addition did not affect N fixation in any compartment in the rehabilitated forest.
- Forest rehabilitation may change the response of N fixation to N deposition.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 12 April 2017

Received in revised form 3 June 2017

Accepted 5 June 2017

Available online 9 June 2017

Editor: Elena Paoletti

Keywords:

Asymbiotic nitrogen fixation

Nitrogen addition

Rehabilitated forest

Disturbed forest

Acetylene reduction

ABSTRACT

Asymbiotic nitrogen (N) fixation is an important source of new N in ecosystems, and is sensitive to atmospheric N deposition. However, there is limited understanding of asymbiotic N fixation and its response to N deposition in the context of forest rehabilitation. In this study, we measured N fixation rates (acetylene reduction) in different ecosystem compartments (i.e. soil, forest floor, moss *Syrrhopodon armatus*, and canopy leaves) in a disturbed and a rehabilitated subtropical forest in southern China, under 12 years of N treatments: control, low N addition (50 kg N ha⁻¹ yr⁻¹), and medium N addition (100 kg N ha⁻¹ yr⁻¹). The rehabilitated forest had higher nutrient (e.g. N) availability than the disturbed forest. In control plots, N fixation rates in forest floor were higher in the rehabilitated forest than in the disturbed forest, but N fixation rates in other compartments (soil, *S. armatus*, and canopy leaves) were comparable between the forests. Nitrogen addition significantly suppressed N fixation in soil, forest floor, *S. armatus*, and canopy leaves in the disturbed forest, but had no significant effect on those compartments in the rehabilitated forest. The main reasons for the negative effects of N addition on N fixation in the disturbed forest were NH₄⁺ inhibition (soil), the P and C limitation (forest floor), and the reduced N dependence on canopy N-fixers (*S. armatus* and canopy leaves). We conclude that asymbiotic N fixation does not decline with increasing N availability after rehabilitation in the study forests. The inhibitory effects of N addition on asymbiotic N fixation occurred in the disturbed forest but not in the rehabilitated forest, indicating that forest rehabilitation may change the response of ecosystem function (i.e. N fixation) to N deposition, which merits further study in other tropical and subtropical regions.

© 2017 Elsevier B.V. All rights reserved.

* Corresponding author at: South China Botanical Garden, Chinese Academy of Sciences, Dinghu, Zhaoqing 526070, Guangdong, China.

E-mail address: mojm@scib.ac.cn (J. Mo).

1. Introduction

Nitrogen (N) is essential for living organisms and is often the limiting factor for terrestrial net primary production (NPP) (Vitousek and Howarth, 1991; Galloway and Cowling, 2002). Asymbiotic N fixation performed by free-living microbes constitutes an important N source in terrestrial ecosystems, contributing 1–20 kg N ha⁻¹ yr⁻¹ to the ecosystem N pool (Reed et al., 2011). The N supply via N fixation influences the N balances and carbon (C) sequestration at both regional and global scales (Cleveland et al., 1999; Reed et al., 2011; Vitousek et al., 2002; Vitousek et al., 2013).

Asymbiotic N fixation occurs in various ecosystem compartments (Reed et al., 2011), including soil (Barron et al., 2008; Wurzburger et al., 2012), forest floor (Crews et al., 2000; Matson et al., 2015), mosses and/or lichens in the canopy (Benner and Vitousek, 2007) and on the forest floor (DeLuca et al., 2007; Gundale et al., 2011), leaf surface (Freiberg, 1998; Cusack et al., 2009), and inside of the leaves (Moyes et al., 2016). Nitrogen-fixing microbes are sensitive to environmental factors, such as temperature and moisture (Reed et al., 2011). Most commonly, warm and wet conditions favor asymbiotic N fixation (Reed et al., 2007; Cusack et al., 2009; Rousk and Michelsen, 2016). Nutrient availability regulates N fixation. For example, nitrogenase activity is commonly down-regulated by mineral N (e.g. NH₄⁺) (Crews et al., 2001; Zackrisson et al., 2004; DeLuca et al., 2007; Cusack et al., 2009; Matson et al., 2015). In many tropical forests, long-term N addition (e.g. N deposition) has been shown to increase soil acidity and reduce soil phosphorus (P) availability (Matson et al., 1999). Therefore, P addition partially relieved the inhibitory effects of N addition on N fixation in several tropical forests (Matson et al., 2015; Zheng et al., 2016b). Contrary to the effects of N addition, P addition often up-regulates N fixation (Crews et al., 2000; Reed et al., 2007; Matson et al., 2015). This is because P participates in the biosynthesis of ATP, which is needed due to the high-energy requirement of N fixation (Alberty, 2005). Carbohydrate is an important energy source for N fixation of heterotrophic and autotrophic diazotrophs. The carbohydrate needed for heterotrophic diazotrophs is derived from readily available C provided via organic matter mineralization (Crews et al., 2001; Reed et al., 2011). In contrast, autotrophic diazotrophs acquire carbohydrate via photosynthesis (Diáková et al., 2016). Therefore, nutrient (C, N, and P) availability is an important control on N fixation.

Biological N fixation is quite common in both natural and managed ecosystems (Vitousek et al., 2002; Reed et al., 2011), among which forest ecosystems merit great attention (Cleveland et al., 1999). It is estimated that nearly half of the N fixed in natural ecosystems is in forests (Cleveland et al., 1999), which indicates an important role of N fixation in these ecosystems. However, because of natural and anthropogenic disturbances (e.g. fire, deforestation, land management, and harvest), natural forest area worldwide has declined from 3961 M ha in 1990 to 3721 M ha in 2015 (Keenan et al., 2015). External disturbances have a profound impact on forest structure and function, often leading to forest degradation, thereby altering soil microbial community (Liu et al., 2014) and soil N dynamics (Mo et al., 2003; Zhang et al., 2008; Fang et al., 2008). Current efforts of forest conservation, expansion, and afforestation have led to a rapid increase in forest area at a rate of 6.8–7.3 million ha yr⁻¹ (FAO, 2010). Forest rehabilitation may change nutrient availability and plant biomass, and further influence asymbiotic N fixation. For example, previous studies in temperate and boreal regions reported an increase in N fixation rates across forest rehabilitation (Pérez et al., 2004; Zackrisson et al., 2004; Stuver et al., 2015), possibly because rehabilitation increased the biomass of N-fixers (Pérez et al., 2004; Stuver et al., 2015) or decreased N availability (Zackrisson et al., 2004). However, no such study has been conducted in tropical/subtropical forests where climate conditions (e.g. temperature and moisture) are distinct from those in high-latitude regions. Owing to afforestation, planted forests in tropical regions increased from ~30 K ha to ~57 K ha between 1990 and 2015 (Keenan et al., 2015).

Hence, it is essential to investigate the responses of asymbiotic N fixation to forest rehabilitation in tropical/subtropical regions.

Atmospheric N deposition has shown a dramatic increase in recent decades, with the greatest increases observed in tropical/subtropical regions (Galloway and Cowling, 2002; Galloway et al., 2008). Elevated N deposition has a number of negative effects on tropical forests, such as soil acidification, nutrient imbalance, and a decrease in plant biodiversity and productivity (Matson et al., 1999; Lu et al., 2010, 2014). Nitrogen deposition also changes the richness and abundance of N-fixing microbial community (Piceno and Lovell, 2000; Berthrong et al., 2014; Freedman et al., 2013) and N fixation rates in forest soils (Cusack et al., 2009; Zheng et al., 2016a). Many previous studies found that N addition had no or negative effects on asymbiotic N fixation in tropical mature forests (Crews et al., 2001; Reed et al., 2007; Barron et al., 2008; Cusack et al., 2009; Matson et al., 2015). A few studies reported that N addition suppressed moss N fixation in the boreal rehabilitated forests that had experienced past disturbance events (Zackrisson et al., 2004; DeLuca et al., 2007). However, to our knowledge, no study has reported how N deposition regulates asymbiotic N fixation after forest rehabilitation in tropical/subtropical regions. Forest rehabilitation often increases tree biomass, tree species (e.g. through natural invasion), and litterfall mass (Ren and Peng, 1999; Tang et al., 2006; Stuver et al., 2015), which promotes the accumulation of soil organic matter (Crews et al., 2001; Mo et al., 2003) and thereby provides abundant C resource for microbial growth (Banning et al., 2011; Liu et al., 2014). In addition to the increased organic matter, forest rehabilitation also increases canopy density, woody residues, soil N level (e.g. through biological N fixation and N deposition), and moisture content of forest substrates (Mo et al., 2003; Pérez et al., 2004; Tang et al., 2006; Stuver et al., 2015). These improved habitat conditions, particularly the elevated N level, may be disadvantageous to N-fixers, and further influence their capacity for N fixation under atmospheric N deposition.

In this study, we aimed to elucidate asymbiotic N fixation and its response to N deposition after rehabilitation in subtropical forests. We measured N fixation rates (acetylene reduction) in different ecosystem compartments (i.e. soil, forest floor, moss *S. armatus*, and canopy leaves) in a disturbed and a rehabilitated subtropical forest in southern China, under the following treatments: control, low N addition (50 kg N ha⁻¹ yr⁻¹), and medium N addition (100 kg N ha⁻¹ yr⁻¹). To determine whether nutrient availability affects N fixation, we measured NH₄⁺, NO₃⁻, and available P (AP) concentrations in the soil, and total organic carbon (TOC), total N (TN), and total P (TP) concentrations in all ecosystem compartments. The disturbed forest has experienced human disturbances (harvesting of understory vegetation and litter) in recent decades. Despite human disturbances in the past, the rehabilitated forest has been well protected and therefore invaded by native broadleaf species (Mo et al., 2003). Compared with the disturbed forest, the rehabilitated forest had higher plant biomass, microbial biomass (Tang et al., 2006), and N concentrations in forest compartments (Tables 2–4). Thus, we hypothesized that (1) the rehabilitated forest would have lower N fixation rates than the disturbed forest, given the improvement in nutrient conditions (e.g. elevated N level) after rehabilitation; and (2) N addition would suppress N fixation more in the disturbed forest than in the rehabilitated forest, given that the disturbed forest with low N level might readily reach the N threshold.

2. Materials and methods

2.1. Site description

This study was conducted in Dinghushan Biosphere Reserve (DHSBR), which is located in the center of Guangdong Province, southern China (112°10' E, 23°10' N). There are two types of forests in the reserve: a mixed pine and broadleaf forest (rehabilitated) and a pine forest (disturbed). The rehabilitated and disturbed forests occupy about 50% and 20% of the reserve, respectively (Mo et al., 2003). Both

forests originated from eroded sites and were subsequently planted with *Pinus* (*P.*) *massoniana* after the 1930s. The disturbed forest experienced continuous human disturbances (harvesting of understory vegetation and litter) until the late 1990s (Mo et al., 2003). Although the rehabilitated forest also experienced human disturbances, it was well protected after the 1950s. Owing to natural invasion and colonization by native broadleaf species, the rehabilitated forest had developed into a mixed pine and broadleaf forest (Mo et al., 2003). *Pinus massoniana* and *Schima superba* are the dominant tree species in the rehabilitated forest, occupying 94.6% of the total basal area, whereas *P. massoniana* is the dominant tree species in the disturbed forest, representing 95.1% of the total basal area (Fang et al., 2005). Due to well protection and rehabilitation, the rehabilitated forest had higher plant biomass, microbial biomass, and litter input than the disturbed forest (Tang et al., 2006). The rehabilitated forest also had a higher nutrient (C, N, and P) availability than the disturbed forest (Tables 2–4).

The reserve has a typical humid monsoon climate. Mean annual precipitation is 1927 mm, 75% of which occurs from March to August and 6% from December to February (Huang and Fan, 1982). Mean annual temperature is 21 °C, with the mean coldest (January) and warmest (July) monthly temperature being 12.6 and 28.0 °C, respectively. In 2004–2005, inorganic N deposition was 24 and 26 kg N ha⁻¹ yr⁻¹ for the rehabilitated and disturbed forests, respectively, with an additional input of 15–20 kg N ha⁻¹ yr⁻¹ as dissolved organic N (Fang et al., 2008). Both forest soils are lateritic red earth developed from sandstone. The soil depth is 30–60 and <30 cm in the rehabilitated and disturbed forests, respectively (Mo et al., 2003).

2.2. Experimental treatments

This experiment was initiated in July 2003, with three treatments (each with three replicate plots) in each forest: control, low N addition (LN, 50 kg N ha⁻¹ yr⁻¹), and medium N addition (MN, 100 kg N ha⁻¹ yr⁻¹). We chose these fertilization rates, because background N deposition rate was high (40–50 kg N ha⁻¹ yr⁻¹) in the study forests. This fertilization design is reasonable, because previous studies have reported that some ecosystem processes (e.g. greenhouse gas emission, soil N fluxes, and litter decomposition) had significant responses to medium and high N addition (150 kg N ha⁻¹ yr⁻¹; another experiment in a nearby N-saturated forest) but not to low N addition in our forests (Fang et al., 2005, 2007; Zhang et al., 2008). Each plot of 10 m × 20 m was surrounded by a 10-m wide buffer strip to prevent any inter-plot disturbance. All plots were laid out randomly. In N-addition plots, NH₄NO₃ was weighed and mixed with 20 L of water, and sprayed on the forest floor (4.17 and 8.33 kg N ha⁻¹ for LN- and MN-addition plots, respectively) using a backpack sprayer monthly from July 2003 to July 2015. The N solutions were sprayed on the forest floor from a height of ~1 m. Canopy leaves and epiphytes did not receive the added N directly. Each control plot only received 20 L of water. We used the method of understory N addition (N was added on the forest floor) for two reasons. First, this allows us to compare our results with those of many previous studies that used the understory N addition method. Second, we would like to understand whether long-term understory N addition has a significant effect on canopy N fixation, because some previous studies have reported a slight but not significant decrease in canopy N fixation after short-term (3–4 years) understory N addition (Cusack et al., 2009; Matson et al., 2015). We note that the effects of understory N addition on canopy N fixation (indirect N effects) may not be equal to the effects of canopy N addition; this requires further study.

2.3. Sampling

Samples were collected in July 2015. In each plot, five samples of forest floor were randomly collected using a metal frame (20 cm × 20 cm). 'Forest floor' refers to the full thickness from freshly fallen leaves to the

mineral soil surface, including recognizable leaves and fine woody (<2 cm diameter) tissues (i.e. the Oi horizon). Underneath the forest floor, mineral soil was sampled to a depth of 10 cm using a 2.5-cm soil corer, with a total of five soil samples collected per plot. Canopy leaves were sampled using a 20-m pole pruner from the dominant tree species (*P. massoniana* and *S. superba* in the rehabilitated forest, and *P. massoniana* in the disturbed forest). In each plot, canopy leaves were collected from three individuals of each tree species in the upper, middle, and lower layers. Leaves were removed from branches and mixed by individual tree, with a total of six and three samples in each plot in the rehabilitated and disturbed forests, respectively. In both forests, mosses occurred widely at the base (~2 m above the ground) of tree trunk. Among the mosses, only one species (*Syrrhopodon armatus* Mitt.) was found to fix N and thus was sampled. Moss *S. armatus* was collected by slightly scraping three 5 cm × 5 cm pieces from the tree trunk. The moss samples were mixed by trunk, with a total of six and three samples per plot in the rehabilitated and disturbed forests, respectively. The moss samples were stored under cold and dark conditions, and analyzed within 24 h.

All samples were weighed, and portions were oven-dried at 105 °C (soil) or 65 °C (forest floor, canopy leaves, and moss) for 48 h to determine moisture content. Standing stocks of soil (g soil m⁻²) and forest floor (g forest floor m⁻²) were estimated by their dry weight and sampling areas. The density of *S. armatus* was estimated by the mean percent cover on each tree. Percent cover was estimated by randomly placing eight 10 cm × 10 cm quadrats on each tree trunk, and visually estimating the percent cover of *S. armatus* in each quadrat. Tree surface area was calculated by assuming trees are cylinders, multiplying height by circumference (calculated by diameter at breast height). Therefore, standing stocks of *S. armatus* per unit of ground area (g moss m⁻²) were estimated by *S. armatus* density, tree surface area, and mean tree density (1933 and 767 tree ha⁻¹ for the rehabilitated and disturbed forests, respectively; Fang et al., 2005). Standing stocks of canopy leaves per unit of ground area (g canopy leaves m⁻²) were estimated by specific leaf area (measured by LI-3000A; Li-Cor) and the leaf area index of 11.28 and 6.61 for the rehabilitated and disturbed forests, respectively (Ren and Peng, 1999).

2.4. Measurement of asymbiotic N fixation

Asymbiotic N fixation was measured using the acetylene reduction assay (Hardy et al., 1968), which takes advantage of the ability of nitrogenase to reduce acetylene (C₂H₂) to ethylene (C₂H₄). Fresh samples of each compartment (~5 g of forest floor, ~13 g of soil, ~7 g of canopy leaves, and ~3 g of moss *S. armatus*) were sealed into a 120-mL gas-tight glass jar, with 10% of the headspace (12 mL) replaced with pure C₂H₂ (99.99%). Previous studies have shown that diazotrophs occurred on the leaf surface (epiphylls; e.g. Freiberg, 1998; Cusack et al., 2009) and in leaves (endophytes; e.g. Moyes et al., 2016). Our study measured N fixation of a whole leaf (i.e. N fixation by both epiphylls and endophytes), similar to that of Reed et al. (2008). All samples were incubated for 24 h in situ to approximate ambient light and temperature. After incubation, headspace gas from each jar was mixed, sampled, and stored in a 12-mL evacuated Exetainer™ (Labco, High Wycombe, U.K.), and analyzed within 24 h. In the laboratory, C₂H₄ concentrations were determined using a Shimadzu GC14 gas chromatography equipped with a flame ionization detector and a Poropak N column (injector/detector/column temperature: 70 °C/150 °C/250 °C). Background C₂H₄ concentrations in C₂H₂ gas were measured during the field incubation and subtracted. Our preliminary experiment showed that the C₂H₄ concentrations naturally produced by samples were below the detection limit. It is noted that this study only measured N fixation during the wet season. It remains inconclusive whether N fixation is higher in the wet season (Reed et al., 2007; Winbourne et al., 2017) or in the dry season (Matson et al., 2015) in tropical forests. Regardless of the seasonal

effects, this study focused on N fixation between forest types and among N addition treatments.

2.5. Measurement of nutrient availability

Soil NH_4^+ and NO_3^- concentrations were measured by extraction in 50 mL of 2 M KCl solution, and analyzed spectrophotometrically (Bremner and Mulvaney, 1982). Inorganic N (IN) concentrations were calculated as the sum of NH_4^+ and NO_3^- concentrations. Soil AP concentrations were measured spectrophotometrically after extraction with acid-ammonium fluoride solution (Anderson and Ingram, 1989). Concentrations of TN and TP were measured by micro-Kjeldahl digestion, followed by the indophenol blue and the Mo-Sb colorimetric methods, respectively, with a UV-8000 spectrophotometer (Liu, 1996). Concentrations of TOC were measured by potassium dichromate oxidation titration with Fe^{2+} solution (Liu, 1996).

2.6. Statistical analyses

Nutrient availability and acetylene reduction rates in canopy leaves were presented as the average of two tree species in the rehabilitated forest. Values of each variable were averaged by plot before analyses. A one-way analysis of variance (ANOVA) was used to determine the differences for each variable between the control and treatment plots in each forest. An independent-samples *t*-test was used to compare each variable in control plots of the forests. Data were tested to fulfill the assumptions of normality and homogeneity of variances. All statistical analyses were conducted in SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistically significant differences were recognized at $P < 0.05$, unless otherwise stated.

3. Results

3.1. Asymbiotic N fixation

In control plots, *S. armatus* had the highest acetylene reduction rates ($9.29\text{--}10.23 \text{ nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ h}^{-1}$), followed by forest floor ($1.80\text{--}2.60 \text{ nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ h}^{-1}$), canopy leaves ($0.16\text{--}0.18 \text{ nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ h}^{-1}$), and soil ($0.05\text{--}0.06 \text{ nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ h}^{-1}$) (Fig. 1). Acetylene reduction rates in forest floor were significantly higher in the rehabilitated forest than in the disturbed forest, whereas acetylene reduction rates in soil, *S. armatus*, and canopy leaves did not differ significantly between the two forests. In the disturbed forest, acetylene reduction rates in soil, forest floor, and canopy leaves were significantly lower in N-addition plots than in control plots by 36.7–44.9%, 36.1–38.3%, and 19.7–38.2%, respectively. In contrast, there was no significant difference in acetylene reduction rates in all compartments between the control and N-addition plots in the rehabilitated forest.

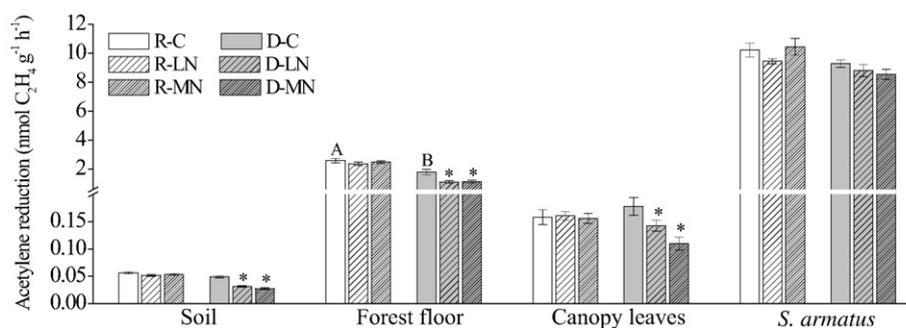


Fig. 1. Effects of N addition on N fixation rates (acetylene reduction) in different compartments in the rehabilitated (R) and disturbed (D) forests. C: control; LN: low nitrogen addition; MN: medium nitrogen addition; R: rehabilitated forest; D: disturbed forest. Different letters within each compartment represent significant differences between the forests, and "*" represents a significant difference between the control and treatment plots ($P < 0.05$). Error bars represent standard errors of means ($n = 3$).

3.2. Standing stocks

In control plots, soil had the highest standing stocks ($107.6\text{--}118.5 \text{ kg m}^{-2}$), followed by forest floor ($1.36\text{--}1.79 \text{ kg m}^{-2}$), canopy leaves ($1.33\text{--}1.47 \text{ kg m}^{-2}$), and *S. armatus* ($0.01\text{--}0.02 \text{ kg m}^{-2}$) (Fig. 2). Compared with the disturbed forest, the rehabilitated forest had significantly higher standing stocks of canopy leaves, and significantly lower standing stocks of soil and forest floor. Standing stocks of *S. armatus* did not differ significantly between the forests. Standing stocks of forest floor and *S. armatus* were significantly lower in N-addition plots than in control plots by 8.4–9.5% and 17.0–21.6%, respectively, in the disturbed forest. There was no significant difference in standing stocks of all ecosystem compartments between the control and N-addition plots in the rehabilitated forest.

3.3. Nutrient availability

Soil nutrient availability in control plots did not differ significantly between the forests (Table 1). However, compared with the disturbed forest, the rehabilitated forest had significantly higher TN, TP, and TOC concentrations in forest floor (Table 2), and significantly higher TN and TOC concentrations in *S. armatus* (Table 3) and canopy leaves (Table 4). In the rehabilitated forest, there was no significant difference in nutrient availability between the control and N-addition plots (Tables 1–4), except for the forest floor TOC/TP ratios, which were significantly higher in MN-addition plots than in control plots by 25.5% (Table 2). In the disturbed forest, soil NH_4^+ concentrations and IN/AP ratios were significantly higher in MN-addition plots than in control plots by 97.0% and 106.9%, respectively. Soil TOC/TN ratios were significantly lower in MN-addition plots than in control plots by 39.9% in the disturbed forest. Forest floor TN/TP ratios and TN concentrations were significantly higher in LN-addition plots than in control plots by 35.0% and 34.8%, respectively, in the disturbed forest. Forest floor TOC/TN ratios were significantly lower in LN- and MN-addition plots than in control plots by 23.4% and 17.1%, respectively, in the disturbed forest. In the disturbed forest, TP, TOC, and TOC/TN ratios of *S. armatus* were significantly lower in MN-addition plots than in control plots by 14.3%, 11.0%, and 21.3%, respectively; however, TN/TP ratios of *S. armatus* were significantly higher in MN-addition plots than in control plots by 30.0%. Total N and TOC concentrations in canopy leaves were significantly higher in MN-addition plots than in control plots by 39.9% and 8.0%, respectively, whereas TOC/TN ratios of canopy leaves were significantly lower in MN-addition plots than in control plots by 23.3% in the disturbed forest.

4. Discussion

4.1. Asymbiotic N fixation in control plots

To our knowledge, many N fixation studies focused on understory compartments (e.g. soil and leaf litter) (Maheswaran and Gunatilleke,

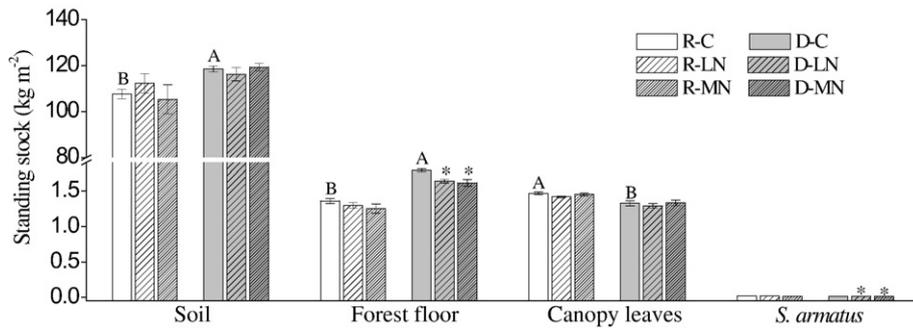


Fig. 2. Effects of N addition on standing stocks of different compartments in the rehabilitated (R) and disturbed (D) forests. C: control; LN: low nitrogen addition; MN: medium nitrogen addition; R: rehabilitated forest; D: disturbed forest. Different letters within each compartment represent significant differences between the forests, and “*” represents a significant difference between the control and treatment plots ($P < 0.05$). Error bars represent standard errors of means ($n = 3$).

1990; Vitousek, 1994; Crews et al., 2000; Pérez et al., 2004; Reed et al., 2007; Barron et al., 2008; Wurzbürger et al., 2012), and only a few studies reported asymbiotic N fixation in both canopy and understory compartments in mature forests (Reed et al., 2008; Cusack et al., 2009). For example, Reed et al. (2008) found that asymbiotic N fixation occurred in soil, leaf litter, and canopy leaves (0.08–0.27, 3.84–22.37, and 0.00–0.14 nmol C₂H₄ g⁻¹ h⁻¹, respectively), which brought large quantities of N into a mature tropical forest in Costa Rica. Cusack et al. (2009) found that asymbiotic N fixation occurred in various ecosystem compartments, including soil, forest floor, mosses, lichens, and canopy leaves (0.06–0.11, 1.20–2.00, 5.60–11.00, 0.03–0.05, and 0.008–0.01 nmol C₂H₄ g⁻¹ h⁻¹, respectively) in two mature tropical forests in Puerto Rico. Our study, conducted in a rehabilitated and a disturbed subtropical forest, also found high N fixation rates in soil, forest floor, moss *S. armatus*, and canopy leaves (0.05–0.06, 1.80–2.60, 9.29–10.23, and 0.16–0.18 nmol C₂H₄ g⁻¹ h⁻¹, respectively). Compared with mature forests, nutrient availability is relatively low in many disturbed and rehabilitated forests (Mo et al., 2003; Pérez et al., 2004; Zhang et al., 2008; Stuver et al., 2015). Therefore, asymbiotic N fixation may be an important N source for the disturbed and rehabilitated forests in our study region.

Rehabilitation process improved nutrient conditions in forests, because we found that the rehabilitated forest had significantly higher C, N, and P concentrations in forest floor, *S. armatus*, and canopy leaves compared with the disturbed forest (Tables 2–4). Despite the presence of better nutrient conditions (e.g. elevated N level) after rehabilitation, the rehabilitated forest did not show lower N fixation rates than the disturbed forest (Fig. 1), which rejects our initial hypothesis. Our findings do not support the traditional notion that biological N fixation often declines across ecosystem succession (Crews et al., 2000; Barron et al., 2011; Batterman et al., 2013). However, our findings are consistent with those reported in some temperate and boreal forests (Pérez et al., 2004; Zackrisson et al., 2004; Stuver et al., 2015), despite only one

ecosystem compartment (e.g. mosses or leaf litter) being investigated in these forests. For example, Zackrisson et al. (2004) found that moss (*P. schreberi*) N fixation increased linearly with time since fire in a series of successional forests in northern Sweden. Stuver et al. (2015) found that moss (*P. schreberi*) N fixation increased after clear-cutting in some boreal forests in northern Sweden. Similarly, our study found that moss (*S. armatus*) N fixation was slightly, though not significantly, higher in the rehabilitated forest than in the disturbed forest (Fig. 1). In the post-fire temperate forests in southern Chile, Pérez et al. (2004) found that litter N fixation increased from early to mid successional stages, and slightly decreased in late successional stages but remaining higher than in early stages. Our study also found significantly higher N fixation in forest floor (including leaf litter) in the rehabilitated forest compared with the disturbed forest (Fig. 1). Reasons for the increases in asymbiotic N fixation with succession may be the increases in biomass of N-fixers, such as mosses (Stuver et al., 2015) and litter (Pérez et al., 2004), and the N limitation in late successional stages (Zackrisson et al., 2004). These phenomena, however, were not observed in our forests, because standing stocks of *S. armatus* and forest floor in the rehabilitated forest were comparable to or even lower than those in the disturbed forest (Fig. 2). In addition, the rehabilitated forest had higher N availability in ecosystem compartments than the disturbed forest (Tables 2–4).

A potential explanation for our results is that asymbiotic N-fixers may be less sensitive to increasing N availability after forest rehabilitation, given that background N deposition was high in the past two decades. In the study region, N deposition rates were 35.6 kg N ha⁻¹ yr⁻¹ in 1990 (Huang et al., 1994), 50–52 kg N ha⁻¹ yr⁻¹ in 2004–2005 (Fang et al., 2008), 34.4 kg N ha⁻¹ yr⁻¹ in 2009–2010 (Lu et al., 2013), and 21.3 kg N ha⁻¹ yr⁻¹ in 2013 (Zhu et al., 2015). Due to long-term high N deposition, some ecosystem processes may be less sensitive to increasing N availability. For example, our previous studies showed that litter decomposition rates (Fang et al., 2007) and soil

Table 1
Effects of N addition on soil nutrient availability in the rehabilitated and disturbed forests.

Forest type	Rehabilitated forest			Disturbed forest		
	C	LN	MN	C	LN	MN
NO ₃ ⁻ (mg/kg)	3.22 ± 0.44	3.57 ± 0.36	3.29 ± 0.26	3.46 ± 0.44	3.65 ± 0.35	3.42 ± 0.68
NH ₄ ⁺ (mg/kg)	1.69 ± 0.36	2.00 ± 0.46	3.26 ± 0.61	3.04 ± 0.78b	3.89 ± 0.77ab	5.99 ± 0.22a
AP (mg/kg)	0.97 ± 0.25	0.69 ± 0.15	0.97 ± 0.38	1.47 ± 0.29	1.78 ± 0.36	1.07 ± 0.35
TN (g/kg)	1.97 ± 0.25	2.17 ± 0.13	2.24 ± 0.18	1.37 ± 0.22	1.58 ± 0.25	1.89 ± 0.16
TP (g/kg)	0.25 ± 0.03	0.29 ± 0.03	0.29 ± 0.01	0.30 ± 0.01	0.31 ± 0.02	0.32 ± 0.02
TOC (g/kg)	32.14 ± 4.03	36.42 ± 3.41	35.03 ± 3.51	23.30 ± 1.62	20.72 ± 1.27	19.92 ± 2.59
IN/AP ratio	5.58 ± 0.94	9.11 ± 2.46	8.07 ± 3.02	4.81 ± 1.10b	4.43 ± 0.57b	9.95 ± 1.86a
TOC/TN ratio	16.95 ± 3.28	16.88 ± 2.13	15.66 ± 1.20	17.63 ± 2.07a	13.49 ± 1.32ab	10.59 ± 1.20b
TN/TP ratio	8.23 ± 1.93	7.76 ± 1.24	7.92 ± 0.85	4.54 ± 0.54	5.10 ± 0.83	5.90 ± 0.62
TOC/TP ratio	131.43 ± 25.12	128.68 ± 18.48	122.98 ± 12.64	77.89 ± 1.53	66.82 ± 7.09	61.38 ± 4.83

Note: values are means ± standard errors ($n = 3$). C: control; LN: low nitrogen addition; MN: medium nitrogen addition; AP: available phosphorus; IN: inorganic nitrogen; TN: total nitrogen; TP: total phosphorus; TOC: total organic carbon. Different lower case letters represent significant differences among the treatments ($P < 0.05$).

Table 2
Effects of N addition on nutrient availability in forest floor in the rehabilitated and disturbed forests.

Forest type	Rehabilitated forest			Disturbed forest			
	Treatment	C	LN	MN	C	LN	MN
TN (g/kg)		22.33 ± 1.17A	20.69 ± 1.22	20.28 ± 2.05	17.65 ± 1.00Bb	23.80 ± 1.16a	21.35 ± 1.46ab
TP (g/kg)		1.17 ± 0.03A	1.03 ± 0.08	0.98 ± 0.05	0.99 ± 0.05B	0.99 ± 0.08	1.06 ± 0.11
TOC (g/kg)		521.59 ± 12.86A	542.65 ± 6.96	550.58 ± 13.36	455.39 ± 9.65B	470.86 ± 7.04	455.24 ± 7.41
TOC/TN ratio		23.52 ± 1.59	26.41 ± 1.53	27.77 ± 3.10	25.91 ± 0.99a	19.86 ± 0.79b	21.47 ± 1.13b
TN/TP ratio		19.18 ± 1.27	20.04 ± 0.66	20.65 ± 1.91	17.94 ± 1.73b	24.21 ± 1.89a	20.17 ± 0.77ab
TOC/TP ratio		448.44 ± 23.20b	529.91 ± 38.99ab	562.81 ± 25.31a	461.53 ± 27.91	480.82 ± 41.25	434.05 ± 33.11

Note: values are means ± standard errors ($n = 3$). C: control; LN: low nitrogen addition; MN: medium nitrogen addition; TN: total nitrogen; TP: total phosphorus; TOC: total organic carbon. Different lower case letters represent significant differences among the treatments ($P < 0.05$).

nitrous oxide emission rates (Zhang et al., 2008) in the rehabilitated forest did not differ significantly from those in the disturbed forest. Even with low and medium N addition, soil nitrous oxide emission (Zhang et al., 2008) and microbial community structures (Liu et al., 2014) did not change in our rehabilitated forest. Although N availability increased after rehabilitation (Tables 1–4), asymbiotic N fixation did not decline (Fig. 1). This result provides an insight into a long-lasting N paradox – many N-rich tropical forests sustain N richness and up-regulate N cycles (Houlton et al., 2006; Hedin et al., 2009; Brookshire et al., 2012). However, more field studies are needed to investigate the responses of asymbiotic N fixation to increasing N availability in tropical and subtropical regions, particularly in those with high N deposition.

4.2. Effects of N addition on asymbiotic N fixation

Consistent with our hypothesis, N addition suppressed asymbiotic N fixation in the disturbed forest, but not in the rehabilitated forest (Fig. 1). The results observed in the rehabilitated forest differ from previous findings in the boreal forests. For example, Zackrisson et al. (2004) and DeLuca et al. (2007) found that N addition (0.56 and 3.18 kg N ha⁻¹ week⁻¹, for a total of 8 weeks) reduced N fixation rates of feather mosses (*Pleurozium schreberi*) in late-successional boreal forests after fire disturbance. We suggest that soil N status may explain the results in our rehabilitated forest. Compared with the boreal forests (Zackrisson et al., 2004; DeLuca et al., 2007), our rehabilitated forest had a higher soil N level, and thus asymbiotic N fixation might have been less sensitive to further N addition during the experiment. To date, no study has reported how N addition affects asymbiotic N fixation in disturbed forests.

Soil N fixation is often down-regulated by inorganic N (e.g. NH₄⁺) (Cusack et al., 2009; Reed et al., 2011). In a laboratory study, Drozd et al. (1972) observed that nitrogenase activity declined with the supply of NH₄⁺. Cusack et al. (2009) reported that soil N fixation decreased after inorganic N addition in two tropical forests in Puerto Rico. In our study, N addition increased soil NH₄⁺ concentrations in the disturbed forest, which could account for the decreased soil N fixation (Fig. 1). However, N addition did not increase soil NH₄⁺ concentrations in the rehabilitated forest (Table 1), possibly because the added N was taken up by the plants. Compared with the disturbed forest, the rehabilitated forest had more tree species and a higher stem density (Fang et al., 2005),

indicating that more N was required for plant growth. Our previous study found that fine root biomass tended to increase after N addition in the rehabilitated forest (Zhu et al., 2013). Therefore, the added N was likely utilized for biomass increment and was therefore, not available to inhibit soil N fixation in the rehabilitated forest (Fig. 1). Based on our results, we conclude that soil NH₄⁺ may be an important determinant of soil N fixation in the disturbed forest.

Nitrogen addition suppressed N fixation in forest floor in the disturbed forest, which could be attributed to the changes in stoichiometric ratios. In our disturbed forest, N addition significantly increased N concentrations in forest floor, which in turn increased N/P ratios but decreased C/N ratios (Table 2). Since P and C are important nutrient and energy substrates for N-fixing microbes, respectively (Alberly, 2005), the elevated N/P ratios and reduced C/N ratios are closely related to the decline in N fixation (Maheswaran and Gunatilleke, 1990; Matzek and Vitousek, 2003; Pérez et al., 2010; Reed et al., 2011). In contrast, C/N and N/P ratios of forest floor did not change after N addition in the rehabilitated forest. Total N concentrations in forest floor showed a slight but not significant decrease in the rehabilitated forest, indicating that a portion of N in forest floor tissues might lose after N addition. Therefore, N addition did not suppress N fixation in forest floor in the rehabilitated forest. Some lines of evidence showed that N addition had a negative effect on N fixation in leaf litter (Silvester, 1989; Crews et al., 2001) and forest floor (Cusack et al., 2009). Our findings showed that N addition had a negative effect on N fixation in the disturbed forest floor, possibly due to the changes in N/P and C/N ratios caused by increased N concentrations.

Understory N addition suppressed N fixation in canopy leaves in the disturbed forest, which has not been reported before. Nitrogen addition did not significantly affect canopy N fixation in our rehabilitated forest, which was similar to the results reported in some other tropical forests (Cusack et al., 2009; Matson et al., 2015). Our previous study found that the disturbed forest was N limited (Fang et al., 2008), and in this study, we found that N concentrations in canopy leaves were lower in the disturbed forest compared with the rehabilitated forest (Table 4). In this case, understory N addition will stimulate plant uptake of N, and thereby increase N concentrations in canopy leaves in the disturbed forest (Table 4). Therefore, N addition reduced the dependence of the disturbed forest on leaf N fixation, which in turn decreased N fixation in canopy leaves.

Table 3
Effects of N addition on nutrient availability in *Syrrhopodon armatus* in the rehabilitated and disturbed forests.

Forest type	Rehabilitated forest			Disturbed forest			
	Treatment	C	LN	MN	C	LN	MN
TN (g/kg)		15.88 ± 0.81A	16.71 ± 1.66	15.46 ± 0.91	12.60 ± 0.83B	13.34 ± 1.25	14.20 ± 0.87
TP (g/kg)		0.73 ± 0.04	0.69 ± 0.02	0.70 ± 0.03	0.70 ± 0.02a	0.66 ± 0.03ab	0.60 ± 0.02b
TOC (g/kg)		501.39 ± 13.40A	491.46 ± 16.63	512.18 ± 16.57	454.42 ± 9.60Ba	453.66 ± 15.05a	404.34 ± 10.46b
TOC/TN ratio		31.70 ± 1.42	29.83 ± 2.14	33.48 ± 3.00	36.35 ± 2.35a	34.45 ± 2.30ab	28.60 ± 1.12b
TN/TP ratio		21.75 ± 1.64	24.17 ± 2.21	22.23 ± 2.18	18.19 ± 1.45b	20.11 ± 1.73ab	23.65 ± 0.61a
TOC/TP ratio		684.87 ± 24.51	711.46 ± 17.79	731.97 ± 31.15	655.64 ± 32.56	685.74 ± 25.56	675.35 ± 12.10

Note: values are means ± standard errors ($n = 3$). C: control; LN: low nitrogen addition; MN: medium nitrogen addition; TN: total nitrogen; TP: total phosphorus; TOC: total organic carbon. Different capital letters represent significant differences between the forests, while different lower case letters represent significant differences among the treatments ($P < 0.05$).

Table 4
Effects of N addition on nutrient availability in canopy leaves in the rehabilitated and disturbed forests.

Forest type	Rehabilitated forest			Disturbed forest		
	Treatment	C	LN	MN	C	LN
TN (g/kg)	21.08 ± 0.62A	21.19 ± 0.80	23.23 ± 1.43	14.33 ± 1.24Bb	17.79 ± 1.10ab	20.04 ± 1.22a
TP (g/kg)	0.77 ± 0.03	0.77 ± 0.04	0.80 ± 0.05	0.85 ± 0.04	0.88 ± 0.04	0.89 ± 0.03
TOC (g/kg)	550.49 ± 10.82A	575.49 ± 11.29	562.28 ± 12.15	507.86 ± 9.19Bb	526.96 ± 8.68ab	548.27 ± 9.27a
TOC/TN ratio	26.15 ± 0.76B	27.24 ± 1.16	24.47 ± 2.16	35.90 ± 2.68Aa	29.82 ± 1.69ab	27.53 ± 1.52b
TN/TP ratio	27.42 ± 1.42A	27.67 ± 1.43	29.37 ± 2.52	17.08 ± 2.34B	20.31 ± 1.53	22.58 ± 1.67
TOC/TP ratio	717.19 ± 45.91	751.00 ± 22.59	712.14 ± 50.02	601.95 ± 44.50	600.75 ± 16.39	617.96 ± 32.62

Note: values are means ± standard errors ($n = 3$). C: control; LN: low nitrogen addition; MN: medium nitrogen addition; TN: total nitrogen; TP: total phosphorus; TOC: total organic carbon. Different capital letters represent significant differences between the forests, while different lower case letters represent significant differences among the treatments ($P < 0.05$).

Although N addition had no significant effect on *S. armatus* N fixation per unit mass (Fig. 1), N addition significantly reduced *S. armatus* biomass in the disturbed forest (Fig. 2). We found that *S. armatus* N fixation per unit area significantly decreased following N addition (157.48 ± 11.89 , 123.83 ± 7.69 , 113.51 ± 6.72 $\text{nmol m}^{-2} \text{h}^{-1}$ for the control, LN-addition, and MN-addition plots, respectively; $P = 0.03$) in the disturbed forest. Nitrogen addition increased *S. armatus* TN concentrations in the disturbed forest but not in the rehabilitated forest (Table 3), which might account for the different responses of *S. armatus* N fixation to N addition between the forests. The lack of significant response to N addition in our rehabilitated forest is similar to that reported in two tropical rainforests in Puerto Rico with N-rich soils (Cusack et al., 2009). Our results in the disturbed forest are consistent with those in many boreal forests (Gundale et al., 2011; Ackermann et al., 2012; Gundale et al., 2013; Leppänen et al., 2013), where productivity was limited by N. This suggests that the difference in N status between the disturbed and rehabilitated forests (Tables 1–4) may affect the response of moss N fixation to N input.

We infer that the disturbed forest may reduce the dependence on moss N fixation after N addition. Three lines of evidence could support this mechanism. First, N addition may reduce P leaching from leaves of host trees to the mosses, as evidenced by the reduced P concentrations in *S. armatus* after N addition (Table 3). The decrease in P concentrations would suppress moss N fixation, because P participates in the biosynthesis of ATP and nitrogenase proteins (Alberty, 2005). Second, the reduced P concentrations might further affect moss photosynthesis (Fritz et al., 2012), and thus reduced *S. armatus* C concentrations (Table 3). Carbon is an important energy substrate needed for N fixation (Alberty, 2005). Third, the reduced P and C concentrations would constrain *S. armatus* growth and decrease *S. armatus* biomass (Fig. 2).

5. Conclusions

This study elucidated asymbiotic N fixation and its response to N addition in a disturbed and a rehabilitated subtropical forest. In contrast to the previous notion that biological N fixation often declines across ecosystem succession, our study found that asymbiotic N fixation remained constant (i.e. soil, moss *S. armatus*, and canopy leaves) or even increased (i.e. forest floor) after rehabilitation in the study subtropical forests. Asymbiotic N fixation in soil, forest floor, *S. armatus*, and canopy leaves decreased following N addition in the disturbed forest, which contrasts to the lack of significant response in any of the compartments in the rehabilitated forest. Although asymbiotic N fixation is often regulated by multiple factors, our study aimed to identify a main mechanism accounting for the effects of N addition on N fixation in each ecosystem compartment. In the disturbed forest, the inhibition of N addition on N fixation may be due to NH_4^+ inhibition (soil), the P and C limitation (forest floor), and the reduced N dependence on canopy N-fixers (*S. armatus* and canopy leaves). Based on our results, we conclude that forest rehabilitation may increase N availability, which does not necessarily down-regulate asymbiotic N fixation. Nitrogen addition suppressed asymbiotic N fixation in the disturbed forest but not in the rehabilitated forest, indicating that forest rehabilitation changes the response of

asymbiotic N fixation to N addition. This deserves further study in other tropical and subtropical regions, particularly in those with elevated N deposition.

Acknowledgement

This study was financially supported by the National Natural Science Foundation of China (41473112 and 31370011), the Natural Science of Guangdong Province (2014A030311023). We thank Shaowei Chen for the skillful assistance in laboratory work. We appreciate two anonymous reviewers for their constructive comments and suggestions on the manuscript.

References

- Ackermann, K., Zackrisson, O., Rousk, J., Jones, D.L., DeLuca, T.H., 2012. N_2 fixation in feather mosses is a sensitive indicator of N deposition in boreal forests. *Ecosystems* 15, 986–998.
- Alberty, R.A., 2005. Thermodynamics of the mechanism of the nitrogenase reaction. *Biophys. Chem.* 114, 115–120.
- Anderson, J.M., Ingram, J., 1989. *Tropical Soil Biology and Fertility*. CAB International, Wallingford.
- Banning, N.C., Gleeson, D.B., Grigg, A.H., Grant, C.D., Andersen, G.L., Brodie, E.L., Murphy, D., 2011. Soil microbial community successional patterns during forest ecosystem restoration. *Appl. Environ. Microbiol.* 77, 6158–6164.
- Barron, A.R., Wurzbarger, N., Bellenger, J.P., Wright, S.J., Kraepiel, A.M., Hedin, L.O., 2008. Molybdenum limitation of asymbiotic nitrogen fixation in tropical forest soils. *Nat. Geosci.* 2, 42–45.
- Barron, A.R., Purves, D.W., Hedin, L.O., 2011. Facultative nitrogen fixation by canopy legumes in a lowland tropical forest. *Oecologia* 165, 511–520.
- Batterman, S.A., Hedin, L.O., van Breugel, M., Ransijn, J., Craven, D.J., Hall, J.S., 2013. Key role of symbiotic dinitrogen fixation in tropical forest secondary succession. *Nature* 502, 224–227.
- Benner, J.W., Vitousek, P.M., 2007. Development of a diverse epiphyte community in response to phosphorus fertilization. *Ecol. Lett.* 10, 628–636.
- Berthrong, S.T., Yeager, C.M., Gallegos-Graves, L., Steven, B., Eichorst, S.A., Jackson, R.B., Kuske, C.R., 2014. Nitrogen fertilization has a stronger effect on soil nitrogen-fixing bacterial communities than elevated atmospheric CO_2 . *Appl. Environ. Microbiol.* 80, 3103–3112.
- Bremner, J., Mulvaney, C., 1982. Nitrogen-total. In: A.L., P. (Ed.) Second ed. *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties*. American Society of Agronomy, pp. 595–624.
- Brookshire, E.J., Hedin, L.O., Newbold, J.D., Sigman, D.M., Jackson, J.K., 2012. Sustained losses of bioavailable nitrogen from montane tropical forests. *Nat. Geosci.* 5, 123–126.
- Cleveland, C.C., Townsend, A.R., Schimel, D.S., Fisher, H., Howarth, R.W., Hedin, L.O., Perakis, S.S., Latty, E.F., Von Fischer, J.C., Elseroad, A., 1999. Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. *Glob. Biogeochem. Cycles* 13, 623–645.
- Crews, T.E., Farrington, H., Vitousek, P.M., 2000. Changes in asymbiotic, heterotrophic nitrogen fixation on leaf litter of *Metrosideros polymorpha* with long-term ecosystem development in Hawaii. *Ecosystems* 3, 386–395.
- Crews, T.E., Kurina, L.M., Vitousek, P.M., 2001. Organic matter and nitrogen accumulation and nitrogen fixation during early ecosystem development in Hawaii. *Biogeochemistry* 52, 259–279.
- Cusack, D.F., Silver, W., McDowell, W.H., 2009. Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. *Ecosystems* 12, 1299–1315.
- DeLuca, T.H., Zackrisson, O., Gentili, F., Sellstedt, A., Nilsson, M.C., 2007. Ecosystem controls on nitrogen fixation in boreal feather moss communities. *Oecologia* 152, 121–130.
- Diáková, K., Biasi, C., Capek, P., Martikainen, P.J., Marushchak, M.E., Patova, E.N., Santrucková, H., 2016. Variation in N_2 fixation in subarctic tundra in relation to landscape position and nitrogen pools and fluxes. *Arct. Antarct. Alp. Res.* 48, 111–125.
- Drozd, J.W., Tubbs, R.S., Postgate, J.R., 1972. A chemostat study of the effect of fixed nitrogen sources on nitrogen fixation, membranes and free amino acids in *Azotobacter chroococcum*. *J. Gen. Microbiol.* 73, 221–232.

- Fang, Y.T., Zhu, W.X., Mo, J.M., Zhou, G.Y., Gundersen, P., 2005. Dynamics of soil inorganic nitrogen and their responses to nitrogen additions in three subtropical forests, south China. *J. Environ. Sci.* 18, 752–759.
- Fang, H., Mo, J.M., Peng, S.L., Li, Z.A., Wang, H., 2007. Cumulative effects of nitrogen additions on litter decomposition in three tropical forests in southern China. *Plant Soil* 297, 233–242.
- Fang, Y.T., Gundersen, P., Mo, J.M., Zhu, W.X., 2008. Input and output of dissolved organic and inorganic nitrogen in subtropical forests of South China under high air pollution. *Biogeosciences* 5, 339–352.
- FAO, 2010. Food and Agriculture Organization of the United Nations. Global Forest Resources 25 Assessment 2010: Main Report FAO Forestry Paper 163.
- Freedman, Z., Eisenlord, S.D., Zak, D.R., Xue, K., He, Z., Zhou, J., 2013. Towards a molecular understanding of N cycling in northern hardwood forests under future rates of N deposition. *Soil Biol. Biochem.* 66, 130–138.
- Freiberg, E., 1998. Microclimatic parameters influencing nitrogen fixation in the phyllosphere in a Costa Rican premontane rain forest. *Oecologia* 117, 9–18.
- Fritz, C., Dijk, G.V., Smolders, A.J.P., Pancotto, V.A., Elzenga, T.J.T.M., Roelofs, J.G.M., Grootjans, A.P., 2012. Nutrient additions in pristine Patagonian Sphagnum bog vegetation: can phosphorus addition alleviate (the effects of) increased nitrogen loads. *Plant Biol.* 14, 491–499.
- Galloway, J.N., Cowling, E.B., 2002. Reactive nitrogen and the world: 200 years of change. *Ambio* 31, 64–71.
- Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science* 320, 889–892.
- Gundale, M.J., Deluca, T.H., Nordin, A., 2011. Bryophytes attenuate anthropogenic nitrogen inputs in boreal forests. *Glob. Chang. Biol.* 17, 2743–2753.
- Gundale, M.J., Bach, L.H., Nordin, A., 2013. The impact of simulated chronic nitrogen deposition on the biomass and N₂ fixation activity of two boreal feather moss-cyanobacteria associations. *Biol. Lett.* 9, 20130797.
- Hardy, R.W., Holsten, R., Jackson, E., Burns, R., 1968. The acetylene-ethylene assay for N₂ fixation: laboratory and field evaluation. *Plant Physiol.* 43, 1185–1207.
- Hedin, L.O., Brookshire, E.J., Menge, D.N., Barron, A.R., 2009. The nitrogen paradox in tropical forest ecosystems. *Annu. Rev. Ecol. Syst.* 40, 613–635.
- Houlton, B.Z., Sigman, D.M., Hedin, L.O., 2006. Isotopic evidence for large gaseous nitrogen losses from tropical rainforests. *P. Natl. A. Sci. USA* 103, 8745–8750.
- Huang, Z.F., Fan, Z.G., 1982. The climate of Ding Hu Shan. *Trop. Subtrop. Forest Ecosyst.* 1, 11–23 (in Chinese).
- Huang, Z.L., Ding, M.M., Zhang, Z.P., Yi, W.M., 1994. The hydrological processes and nitrogen dynamics in a monsoon evergreen broad-leaved forest of Dinghu shan. *Acta Phytocologica Sinica* 18, 194–199 (in Chinese).
- Keenan, R.J., Reams, G.A., Achard, F., de Freitas, J.V., Grainger, A., Lindquist, E., 2015. Dynamics of global forest area: results from the FAO Global Forest Resources Assessment 2015. *For. Ecol. Manag.* 352, 9–20.
- Leppänen, S.M., Salemaa, M., Smolander, A., Mäkipää, R., Tiirola, M., 2013. Nitrogen fixation and methanotrophy in forest mosses along a N deposition gradient. *Environ. Exp. Bot.* 90, 62–69.
- Liu, G., 1996. Standard Methods for the Observation and Analysis of Chinese Ecosystem Research Network: Soil Analysis and Profile Description. Standards Press of China, Beijing (in Chinese).
- Liu, L., Gundersen, P., Zhang, W., Zhang, T., Chen, H., Mo, J., 2014. Effects of nitrogen and phosphorus additions on soil microbial biomass and community structure in two reforested tropical forests. *Sci. Rep.* UK 5, 14378.
- Lu, X.K., Mo, J.M., Gilliam, F.S., Zhou, G.Y., Fang, Y.T., 2010. Effects of experimental nitrogen additions on plant diversity in an old-growth tropical forest. *Glob. Chang. Biol.* 16, 2688–2700.
- Lu, X., Gilliam, F.S., Yu, G., Li, L., Mao, Q., Chen, H., Mo, J., 2013. Long-term nitrogen addition decreases carbon leaching in nitrogen-rich forest ecosystems. *Biogeosciences* 10, 3931–3941.
- Lu, X.K., Mao, Q.G., Gilliam, F.S., Luo, Y.Q., Mo, J.M., 2014. Nitrogen deposition contributes to soil acidification in tropical ecosystems. *Glob. Chang. Biol.* 20, 3790–3801.
- Maheswaran, J., Gunatilleke, I., 1990. Nitrogenase activity in soil and litter of a tropical lowland rain forest and an adjacent fernland in Sri Lanka. *J. Trop. Ecol.* 6, 281–289.
- Matson, P.A., McDowell, W.H., Townsend, A.R., Vitousek, P.M., 1999. The globalization of N deposition: ecosystem consequences in tropical environments. *Biogeochemistry* 46, 67–83.
- Matson, A.L., Corre, M.D., Burneo, J.I., Veldkamp, E., 2015. Free-living nitrogen fixation responds to elevated nutrient inputs in tropical montane forest floor and canopy soils of southern Ecuador. *Biogeochemistry* 122, 281–294.
- Matzek, V., Vitousek, P., 2003. Nitrogen fixation in bryophytes, lichens, and decaying wood along a soil-age gradient in Hawaiian montane rain forest. *Biotropica* 35, 12–19.
- Mo, J.M., Brown, S., Peng, S.L., Kong, G.H., 2003. Nitrogen availability in disturbed, rehabilitated and mature forests of tropical China. *For. Ecol. Manag.* 175, 573–583.
- Moyes, A.B., Kueppers, L.M., Pett-Ridge, J., Carper, D.L., Vandehey, N., O'Neil, J., Frank, A.C., 2016. Evidence for foliar endophytic nitrogen fixation in a widely distributed subalpine conifer. *New Phytol.* 210, 657–668.
- Pérez, C.A., Carmona, M.R., Aravena, J.C., Armesto, J.J., 2004. Successional changes in soil nitrogen availability, non-symbiotic nitrogen fixation and carbon/nitrogen ratios in southern Chilean forest ecosystems. *Oecologia* 140, 617–625.
- Pérez, C.A., Carmona, M.R., Armesto, J.J., 2010. Non-symbiotic nitrogen fixation during leaf litter decomposition in an old-growth temperate rain forest of Chiloé Island, southern Chile: effects of single versus mixed species litter. *Austral. Ecol.* 35, 148–156.
- Piceno, Y., Lovell, C., 2000. Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. *Microbial. Ecol.* 39, 32–40.
- Reed, S.C., Cleveland, C.C., Townsend, A.R., 2007. Controls over leaf litter and soil nitrogen fixation in two lowland tropical rain forests. *Biotropica* 39, 585–592.
- Reed, S.C., Cleveland, C.C., Townsend, A.R., 2008. Tree species control rates of free-living nitrogen fixation in a tropical rain forest. *Ecology* 89, 2924–2934.
- Reed, S.C., Cleveland, C.C., Townsend, A.R., 2011. Functional ecology of free-living nitrogen fixation: a contemporary perspective. *Annu. Rev. Ecol. Syst.* 42, 489–512.
- Ren, H., Peng, S., 1999. The characteristics of ecological energetics of the forest ecosystem in the successional process in Dinghushan, Guangdong, China. *Acta Ecol. Sin.* 19, 817–822.
- Rousk, K., Michelsen, A., 2016. Ecosystem nitrogen fixation throughout the snow-free period in subarctic tundra: effects of willow and birch litter addition and warming. *Glob. Chang. Biol.* 4, 1552–1563.
- Silvester, W., 1989. Molybdenum limitation of asymbiotic nitrogen fixation in forests of Pacific northwest America. *Soil Biol. Biochem.* 21, 283–289.
- Stuiver, B.M., Gundale, M.J., Wardle, D.A., Nilsson, M.C., 2015. Nitrogen fixation rates associated with the feather mosses *Pleurozium schreberi* and *Hylocomium splendens* during forest stand development following clear-cutting. *Forest Ecol Manag* 347, 130–139.
- Tang, X., Liu, S., Zhou, G., Zhang, D., Zhou, C., 2006. Soil-atmospheric exchange of CO₂, CH₄, and N₂O in three subtropical forest ecosystems in southern China. *Glob. Chang. Biol.* 12, 546–560.
- Vitousek, P.M., 1994. Potential nitrogen fixation during primary succession in Hawaii Volcanoes National Park. *Biotropica* 234–240.
- 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., Crews, T., Field, C.B., Grimm, N.B., Howarth, R.W., Marino, R., Martinelli, L., Rastetter, E.B., 2002. Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry* 57, 1–45.
- Vitousek, P.M., Menge, D.N., Reed, S.C., Cleveland, C.C., 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philos T R Soc B* 368, 20130119.
- Winbourne, J.B., Brewer, S.W., Houlton, B.Z., 2017. Iron controls over di-nitrogen fixation in karst tropical forest. *Ecology* 98, 773–781.
- Wurzburger, N., Bellenger, J.P., Kraepiel, A.M., Hedin, L.O., 2012. Molybdenum and phosphorus interact to constrain asymbiotic nitrogen fixation in tropical forests. *PLoS One* 7, e33710.
- Zackrisson, O., DeLuca, T.H., Nilsson, M.C., Sellstedt, A., Berglund, L., 2004. Nitrogen fixation increases with successional age in boreal forests. *Ecology* 85, 3327–3334.
- Zhang, W., Mo, J., Yu, G., Fang, Y., Li, D., Lu, X., Wang, H., 2008. Emissions of nitrous oxide from three tropical forests in southern China in response to simulated nitrogen deposition. *Plant Soil* 306, 221–236.
- Zheng, M.H., Chen, H., Li, D.J., Zhu, X.M., Zhang, W., Fu, S.L., Mo, J.M., 2016a. Biological nitrogen fixation and its response to nitrogen input in two mature tropical plantations with and without legume trees. *Biol. Fertil. Soils* 52, 1–10.
- Zheng, M.H., Li, D.J., Lu, X., Zhu, X.M., Zhang, W., Huang, J., Fu, S.L., Lu, X.K., Mo, J.M., 2016b. Effects of phosphorus addition with and without nitrogen addition on biological nitrogen fixation in tropical legume and non-legume tree plantations. *Biogeochemistry* 131, 65–76.
- Zhu, F.F., Yoh, M., Gilliam, F.S., Lu, X.K., Mo, J.M., 2013. Nutrient limitation in three lowland tropical forests in southern China receiving high nitrogen deposition: insights from fine root responses to nutrient additions. *PLoS One* 8, e82661.
- Zhu, J.X., He, N.P., Wang, Q.F., Yuan, G.F., Wen, D., Yu, G.R., Jia, Y.L., 2015. The composition, spatial patterns, and influencing factors of atmospheric wet nitrogen deposition in Chinese terrestrial ecosystems. *Sci. Total Environ.* 511, 777–785.