Biol Fertil Soils (2016) 52:665–674
DOI 10.1007/s00374-016-1109-5

ORIGINAL PAPER

# Biological nitrogen fixation and its response to nitrogen input in two mature tropical plantations with and without legume trees

**Mianhai Zheng**[1,3] · **Hao Chen**[2] · **Dejun Li**[2] · **Xiaomin Zhu**[1,3] · **Wei Zhang**[1] · **Shenglei Fu**[1] · **Jiangming Mo**[1]

Received: 7 September 2015 / Revised: 20 March 2016 / Accepted: 24 March 2016 / Published online: 6 April 2016


**Abstract** Rates of biological nitrogen fixation (BNF) were measured in ecosystem. In this study, we measured rates of BNF in ecosystem compartments (bulk soil, forest floor, rhizosphere soil, and nodule) in two mature tropical plantations in southern China with legume trees (*Acacia auriculiformis*, AA) and with non-legume trees (*Eucalyptus urophylla*, EU) after 4 years of nitrogen (N) fertilization (0, 50, and 100 kg N $ha^{-1}$ $year^{-1}$). BNF rates of bulk soil were comparable between plantations, while rates of rhizosphere soil were significantly higher in the EU plantation and rates of forest floor were significantly higher in the AA plantation. Thus, total BNF rates were comparable between plantations (AA = 6.04 kg N $ha^{-1}$ $year^{-1}$; EU = 6.42 kg N $ha^{-1}$ $year^{-1}$). In the AA plantation, N addition significantly decreased BNF rates in all measured compartments and thus the total rates. In the EU plantation, N addition did not change BNF rates of forest floor, but significantly decreased rates of bulk soil and increased rates of rhizosphere soil; thus, total rates did not change. Our findings provide evidence that forest type is an important factor regulating the effects of external N input on BNF, and suggest that elevated atmospheric N deposition in recent decades will suppress total N fixation in mature forests with legume trees but not in those with non-legume trees.

**Keywords** Biological nitrogen fixation · Nitrogen addition · Mature plantation · Legume tree · Non-legume tree · Nitrogenase activity

**Electronic supplementary material** The online version of this article (doi:10.1007/s00374-016-1109-5) contains supplementary material, which is available to authorized users.

✉ Jiangming Mo
mojm@scib.ac.cn

[1] Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China

[2] Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China

[3] University of Chinese Academy of Sciences, Beijing 100039, China

## Introduction

Biological nitrogen (N) fixation (BNF) represents a primary source of new N input in terrestrial ecosystems (Vitousek et al. 2013; Sullivan et al. 2014) and it strengthens when ambient N is limited but weakens when ambient N is adequate (Vitousek and Howarth 1991). As N frequently limits terrestrial net primary production and other ecological processes (Vitousek and Howarth 1991), the N fixed via BNF benefits terrestrial ecosystems, and thus, terrestrial BNF often occurs at high rates. For example, Galloway et al. (1995) estimated pre-industrial BNF in terrestrial ecosystems to be 90–130 Tg N $year^{-1}$, about an order of magnitude higher than that of lightning. Cleveland et al. (1999), using the century terrestrial ecosystem model, estimated the rates to be 100–290 Tg N $year^{-1}$. Recent reviews using a revised global BNF model (Sullivan et al. 2014) and N flux data (Vitousek et al. 2013) reported conservative estimates of ~44 and ~58 Tg N $year^{-1}$. In spite of these varying estimates caused by methodological differences, BNF does contribute large amounts of N to terrestrial ecosystems annually, particularly to forests that account for nearly half of all fixed N (Cleveland et al. 1999).

Diazotrophs are widely distributed in terrestrial ecosystems as symbionts of legume plants (i.e., in nodules), in

association with epiphytes such as mosses and lichens, and free-living in soil, forest floor, and foliage (Reed et al. 2011). Diazotrophs are thought to be abundant in forest ecosystems and BNF is widely observed in forests both with and without legume trees (Reed et al. 2011). For example, in unmanaged forests with legume trees, symbiotic nodules achieve high fixing rates, ranging from 1 to 160 kg N $ha^{-1}$ $year^{-1}$ (Reed et al. 2011). However, as legume trees are not always present, many forests without legume trees often fix N via free-living diazotrophs in above- and belowground ecosystem compartments. For example, diazotrophs in aboveground compartments, including moss, lichen, and leaves, were found to fix N at 0.2–7.7 kg N $ha^{-1}$ $year^{-1}$ in many boreal (Zackrisson et al. 2004; DeLuca et al. 2007; Lagerström et al. 2007) and tropical forests (Matzek and Vitousek 2003; Benner and Vitousek 2007; Cusack et al. 2009). Diazotrophs in belowground compartments, such as forest floor, and bulk and rhizosphere soils, may play more important roles. Although BNF in rhizosphere soil, which is home to abundant diazotrophs (Villadas et al. 2007), has not yet been well estimated, BNF rates of bulk soil and forest floor have been estimated to range from 0.1 to 21 kg N $ha^{-1}$ $year^{-1}$ in many volcanic sites (Vitousek 1999; Crews et al. 2000, 2001), and tropical (Reed et al. 2007, 2008; Wurzburger et al. 2012) and temperate forests (Pérez et al. 2010) where N-fixing legume trees are rare. This range even reached 0.1–60 kg N $ha^{-1}$ $year^{-1}$ in some tropical evergreen forests (Reed et al. 2011). Despite lower rates of free-living BNF than nodule BNF, recent evidence suggests that the N fixed by free-living diazotrophs can also be available for plant and microbial uptake (Pérez et al. 2010), and can, in part, replenish N losses from leaching and de-nitrification (Maheswaran and Gunatilleke 1990; Kreibich and Kern 2003). Thus, BNF in forests without legume trees may be as important as that in forests with legume trees.

Controls on BNF differ in different ecosystem compartments (Reed et al. 2011), but at a whole-ecosystem scale, BNF appears to be sensitive to N input. It is estimated that rates of BNF at both regional (Sullivan et al. 2014) and global (Vitousek et al. 2013) scales have decreased during recent decades, likely because of elevated atmospheric N deposition caused by human activities. The negative effect of N on BNF is further supported by several studies in forests dominated by legume trees. For example, Batterman et al. (2013b) found that both BNF and nodule biomass of legume trees declined after N addition. Using forest succession gradients, Pearson and Vitousek (2001) and Batterman et al. (2013a) found that BNF rates of nodule decreased across successions mainly because of improved soil N level. The mechanisms underlying these negative effects may be the loss of N fixer competitive advantage after external N input (DeLuca et al. 1996), and/or a reduction in the energy cost of BNF by N fixers when ambient N is adequate (Markham and Zekveld 2007).

However, N addition does not always decrease BNF, as can be seen in many forests dominated by non-legume trees. For example, Vitousek and Hobbie (2000) found a very weak BNF response in litter to long-term N fertilization in some wet forests dominated by *Metrosideros polymorpha*. Reed et al. (2007) found that BNF of both bulk soil and litter showed no response to N fertilization at any season in two mature rainforests dominated by non-legume trees. Cusack et al. (2009) also found no N fertilization effect on BNF in moss, lichen, and epiphyll in two tropical forests without legume trees. It is unclear why N addition fails to downregulate BNF in these forests and whether a negative response or no response of BNF to N addition may be related to the presence or absence of legume trees.

In the absence of human disturbance, similar-stage forests with legume trees often have richer soil N in than those without legume trees (Nygren and Leblanc 2009; Bouillet et al. 2013). Crews (1999) suggested that BNF decreases when soil N has exceeded a "threshold" at which N fixers change their N-acquiring strategy from fixing N (high cost) to obtaining soil N (low cost). This suggests that N input may more easily decrease BNF rates in forests with legume trees than in those without legume trees. However, as natural forests often mix legume and non-legume species, it is unclear whether the effects of N addition on BNF may be connected with different soil N levels driven by different forest types, and comparative studies on responses of BNF to increased N deposition between forests with and without legume trees are still absent.

Forest plantations occupy about 264 million hectare worldwide (FAO 2010), but studies of the effects of N addition on plantation BNF are rare (Binkley et al. 2003b). China has approximately 24 % of world plantation areas (Zhang et al. 2014). Among the planted trees, *Acacia* spp. (legumes) and *Eucalyptus* spp. (non-legumes) represent two of the most abundant native tree species in southern China (Chen et al. 2011). *Acacia* and *Eucalyptus* plantations have different soil N levels when in their mature stages (Zhang et al. 2012b), because *Acacia* can increase soil N via nodule BNF whereas *Eucalyptus* cannot. This offers us an opportunity to test whether forest type (with/without legume trees) regulates the effects of N addition on BNF.

In this study, we compared rates of BNF and responses to N addition in different forest compartments in two mature plantations in southern China, one with legume trees (*Acacia auriculiformis*, AA) and one with non-legume trees (*Eucalyptus urophylla*, EU). We measured rates of BNF after N addition (0, 50, and 100 kg N $ha^{-1}$ $year^{-1}$) to below, i.e., bulk soil, forest floor, rhizosphere soil, and nodule; aboveground compartments such as moss, lichen, and leaf were absent or fixed little N in our plantations and thus were excluded from this study. Since initial soil N levels were

significantly higher in the AA plantation (total N (TN) = 2.0 ± 0.1 g $kg^{-1}$; dissolved inorganic N (DIN) = 24.3 ± 1.7 mg $kg^{-1}$) than in the EU plantation (TN = 1.5 ± 0.2 g $kg^{-1}$; DIN = 17.9 ± 0.3 mg $kg^{-1}$), we hypothesized that: (1) rates of BNF would be lower in the AA plantation than in the EU plantation and (2) N addition would decrease rates of BNF in the AA plantation but not in the EU plantation.

## Material and methods

### Study site

This study was conducted in two tropical plantations at Heshan National Field Research Station of Forest Ecosystems (112° 50′ E, 22° 34′ N) which is located in Heshan county of Guangdong Province, southern China. This region has a typical monsoon climate and the average annual temperature and precipitation from were 21.7 °C and 1295 mm, respectively (Chen et al. 2011). Background N deposition in precipitation was about 43.1 kg N $ha^{-1}$ $year^{-1}$ from July 2010 to June 2012 (Huang et al. 2014). The soils are categorized as Acrisols (Chen et al. 2011).

We selected two distinct plantations, one dominated by *A. auriculiformis* and the other by *E. urophylla*. The understory layer was dominated by ferns, and no N-fixing plants occurred in that layer. Since both plantations were aged over 30 years (Zhang et al. 2012b) with crown closure or slight self-thinning, they could be classified as mature (Zhang et al. 2012a). Detail information of the tree structure is given in Table S1. A fertilization experiment was initiated in July 2010 with three blocks and three treatments: control (no fertilization), medium N addition (MN, 50 kg N $ha^{-1}$ $year^{-1}$) and high N addition (HN, 100 kg N $ha^{-1}$ $year^{-1}$). Each block included three 10 m × 10 m plots. In total, there were nine plots in each plantation. The distance between plots was 10 m. N was applied as ammonium nitrate ($NH_4NO_3$) dissolved in 10 L of water and sprayed below the canopy every other month with a backpack sprayer starting from August 2010 to July 2014, with a total of 24 times of N application. The control plot only received the same volume of water.

### Sample collection and standing stock estimation

Sample collection was conducted in July 2014 (within the growing season) when the trees could grow well with enough fresh leaves, and thus allowed us to collect fresh leaves and measure crown areas (Fig. 3). In addition, because some other soil processes and functions were also measured in July by our previous studies (Zhang et al. 2012b, 2014), which allowed us to use previous findings to further support the mechanisms in this study if necessary. Three samples of forest floor were randomly collected from each plot using a metal frame (0.2 × 0.2 m) and then mixed together. After forest floor sampling, the bulk soil underneath was sampled to a depth of 10 cm using a 2.5-cm soil corer and the samples were mixed by plot. All the fresh forest floor and bulk soil samples were weighed and portions of them were oven-dried at 65 °C (forest floor) and 105 °C (bulk soil) for 48 h to determine moisture content. We then calculated the area density of forest floor (kg $m^{-2}$ forest floor) and bulk soil (kg $m^{-2}$ bulk soil) in each plot. The rest of the samples were used for measuring BNF rates and chemical properties.

In each plot, two well-grown trees were chosen. Nodules were found and collected in the AA plantation, while rhizosphere samples were collected in both plantations. Preliminary experiments showed that nodules in the AA plantation mainly occurred in the topsoil, especially the top 5 cm. Therefore, we randomly selected three points within a 2-m radius around the base of each individual tree, and used a small shovel to dig a rectangular pit (10 × 20 cm) to a depth of 10 cm. Nodule samples were removed from the soil, cleaned, and checked for activity by coloration. At the same time, we collected rhizosphere samples following the method of Fujii et al. (2012). Rhizosphere soil was sampled by slightly shaking the fine root systems until the loose surrounding soil was removed and then the soil closely adhering to the roots was collected. Samples of nodule and rhizosphere soil were separately mixed by plot. After collection, portions of each nodule and rhizosphere sample were oven-dried for 24 h at 65 and 105 °C, respectively, and then weighed. Nodule density was expressed as grams per nodule per square meter. Since the rhizosphere soil was only the parts adhering to the roots, its density was calculated as an area-based density (kg rhizosphere soil $m^{-2}$) in this study.

### BNF measurement

To estimate rates of BNF, we measured nitrogenase activity using an acetylene reduction assay (ARA) (Hardy et al. 1968), which uses the ability of nitrogenase to reduce $N_2$ to $NH_3$ and also to reduce acetylene ($C_2H_2$) to ethylene ($C_2H_4$). It is worth noting that the method of ARA had some potential problems for quantitative estimation of N fixation, including the following three main aspects: first, some microbes may also produce or consume $C_2H_4$ which is also the end product of $C_2H_2$ reduction; second, the ARA method may induce N limitation which in turn may induce nitrogenase synthesis and thus overestimation of BNF; third, the theoretical ratio of 3:1 to convert nitrogenase activity to $N_2$ fixation may have a slight fluctuation depending on the fields (Welsh 2000). For the first aspect, our preliminary experiment found no (or very weak) natural production and consumption of $C_2H_4$ by the samples (forest floor, bulk and rhizosphere soils, and nodule), and thus the natural production and consumption of $C_2H_4$ could be disregarded in our study. For the second aspect, we admitted that the ARA method may overestimate BNF, but this method

is still widely used in many forest studies (Zackrisson et al. 2004; Barron et al. 2008; Reed et al. 2008; Menge and Hedin 2009; Sullivan et al. 2014), and our study also adopted this method in order to compare our results with theirs, particularly for the results of the control plots ("BNF between plantations" section). Additionally, because our study mainly focused on the effects of treatments and forest types rather than the pure estimation of BNF, any possible overestimation of BNF caused by this method is of minor importance. In this study, each sample (~6 g of forest floor, ~12 g of bulk soil, ~12 g of rhizosphere soil, and ~0.1 g of nodule attached to a short root segment) was sealed into a 120-mL gas-tight glass jar with a lid fitted with butyl rubber septa. Ten percent of the headspace (12 mL) in the jar was removed and replaced with the same volume of $C_2H_2$ gas. All samples were incubated in situ on the forest floor to simulate ambient light and temperature. Incubation times were 30–60 min for nodule and 7 h for other compartments, because our preliminary experiment showed that nitrogenase activity in the non-nodule compartments declined after more than 7-h incubation. At the end of incubation, the headspace gas from each jar was mixed, sampled, and stored in a gas-tight vacuum Labco Exetainer, and returned to the laboratory for analysis within 48 h.

After measurement, all samples were dried for 48 h (65 °C for forest floor and nodule, 105 °C for bulk and rhizosphere soils) to calculate the dry mass. Because we would like to understand the effects of treatments and forest types on BNF rates, we used the theoretical ratio 3:1 (mol $C_2H_2$ reduced/mol $N_2$ fixed) to convert nitrogenase activity to BNF rates (Hardy et al. 1968). This ratio has been used in many tropical forests (Pearson and Vitousek 2001; Reed et al. 2008; Cusack et al. 2009), but it should be considered as a potential estimation rather than a quantitative estimation. Rates of BNF were expressed in units of kilograms N per hectare per year for forest floor and bulk soil. At a mean density of 4 AA trees or 12 EU trees per 100 $m^2$ in our study sites (unpublished data), rates of BNF for nodule and rhizosphere soil were also converted into units of kilograms N per hectare per year.

### Dissolved inorganic N analyses

Concentrations of dissolved inorganic N (DIN, including $NH_4^+$ and $NO_3^-$) of bulk and rhizosphere soil were measured. Each 10-g fresh sample was extracted with 50-mL 2 M KCl solution, and $NH_4^+$ and $NO_3^-$ concentrations were measured spectrophotometrically following the method of Bremner and Mulvaney (1982).

### Statistical analyses

Effects of N addition on nitrogenase activity, rates of BNF, nodule density, crown area, diameter at breast height (DBH), DIN concentration, fresh leaf N content, and standing stock were determined in a one-way analysis of variance (ANOVA). A paired *t* test was used to compare nitrogenase activity, rates of BNF, standing stock, crown area, DBH, moisture content, DIN concentration, and fresh leaf N content in control plots between the two plantations. All statistical analyses were performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistically significant differences were recognized at the level of $P < 0.05$ unless otherwise stated.

## Results

### BNF in control plots

Because the ARA method was used for all the ecosystem compartments, this allowed us to compare the nitrogenase activity and BNF rates between the treatments or the plantations, similar with the method used by Cusack et al. (2009). Nitrogenase activity of bulk soil (0.43–0.54 nmol $C_2H_4\,g^{-1}\,h^{-1}$) was similar to that of rhizosphere soil (0.33–0.66 nmol $C_2H_4\,g^{-1}\,h^{-1}$), but significantly lower than that of forest floor (0.94–1.05 nmol $C_2H_4\,g^{-1}\,h^{-1}$) in both plantations (Fig. 1). Nitrogenase activity of both bulk and rhizosphere soils were significantly higher in the EU plantation than in the AA plantation, but nitrogenase activity of forest floor was similar between the two plantations. In the AA plantation, however, nitrogenase activity of nodule (1820.06 nmol $C_2H_4\,g^{-1}\,h^{-1}$) was three orders of magnitude higher than of other compartments. Total nitrogenase activity was significantly higher in the AA plantation (1821.86 nmol $C_2H_4\,g^{-1}\,h^{-1}$) than in the EU plantation (2.14 nmol $C_2H_4\,g^{-1}\,h^{-1}$). Accordingly, nitrogenase activity in control plots varied among ecosystem compartments and plantation types.

Similarly, BNF rates in control plots varied depending on ecosystem compartments and plantation types (Fig. 2). Rates of BNF were comparable among forest floor (0.40–1.05 kg N $ha^{-1}$ $year^{-1}$), rhizosphere soil (0.51–1.82 kg N $ha^{-1}$ $year^{-1}$), and nodule (0.67 kg N $ha^{-1}$ $year^{-1}$), but significantly higher in bulk soil (3.90–4.17 kg N $ha^{-1}$ $year^{-1}$) in both plantations. Compared to the EU plantation, the AA plantation had significantly higher rates of forest floor but significantly lower rates of rhizosphere soil. However, BNF rate of bulk soil was similar between the AA and EU plantation. Accordingly, total rates of BNF were comparable between plantations (EU = 6.42 kg N $ha^{-1}$ $year^{-1}$; AA = 6.04 kg N $ha^{-1}$ $year^{-1}$).

### Effects of N addition on BNF

In the AA plantation, N addition marginally or significantly suppressed nitrogenase activity of all compartments except for nodule (Fig. 1). In the EU plantation, N addition significantly suppressed nitrogenase activity of bulk soil

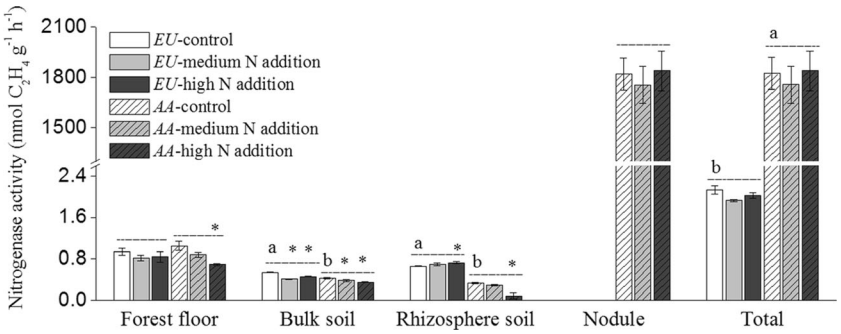


**Fig. 1** Effects of N addition on nitrogenase activity in each ecosystem compartment (forest floor, bulk and rhizosphere soils, and nodule) and on total nitrogenase activity in each plantation (*EU Eucalyptus urophylla*, *AA Acacia auriculiformis*). For each compartment, *different letters* indicate significant differences ($P < 0.05$) between plantations in control plots, while *asterisk* indicates significant differences ($P < 0.05$) between the corresponding treatment and control. *Error bars* represent standard errors ($n = 3$)

but not of forest floor. Nitrogenase activity, however, was significantly stimulated in rhizosphere soil in the EU plantation after HN addition. Total nitrogenase activity did not change with N addition in either plantation. Thus, the effects of N addition on nitrogenase activity may be regulated by ecosystem compartments and plantation types.

Responses of BNF rates to N addition were similar to those of nitrogenase activity for all compartments except for nodule (Fig. 2). In the AA plantation, although nitrogenase activity of nodule BNF had no response to either MN or HN addition, BNF rates of nodule were significantly decreased after HN addition. As a result, N addition significantly decreased total BNF rates in the AA plantation but not in the EU plantation. In the AA plantation, the negative effects of N addition on total BNF rates contrasted to the non-significant effects of N addition on total nitrogenase activity, indicating a potential role of nodule in regulating total BNF, because N addition significantly suppressed BNF rates but not nitrogenase activity of nodule (Figs. 1 and 2). The significant decreases in BNF rates of nodule after N addition were likely caused by the significant decreases in nodule biomass (Table 2).

## Effects of N addition and plantation types on plant parameters and environmental factors

Total DIN concentrations ($NH_4^+$ and $NO_3^-$) of bulk soil in control plots were significantly higher in the AA plantation than in the EU plantation, but both $NH_4^+$ and total DIN concentrations of rhizosphere soil were significantly higher in the EU plantation than in the AA plantation (Table 1). DIN concentrations ($NO_3^-$, $NH_4^+$, and total) of bulk soil showed significant or slight increases in both plantations after N addition. By contrast, DIN concentrations of rhizosphere soil significantly increased in the AA plantation but not in the EU plantation after N addition.

Standing stock of bulk soil, forest floor, and rhizosphere soil in control plots were significantly higher in the AA plantation than in the EU plantation (Table 2). Medium N addition significantly decreased standing stock of bulk soil in the AA plantation but not in the EU plantation. However, HN addition had no significant effect on standing stock of bulk soil in both plantations. Standing stock of forest floor had no significant response to MN or HN addition in either plantation. Both MN

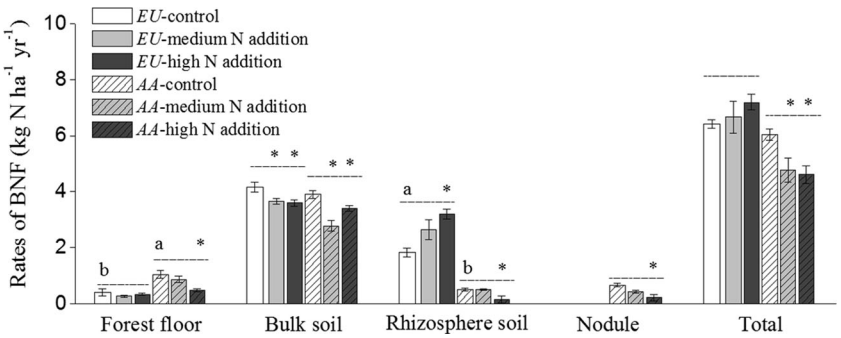


**Fig. 2** Effects of N addition on rates of BNF in each ecosystem compartment (forest floor, bulk and rhizosphere soils, and nodule) and on total rates of BNF in each plantation (*EU Eucalyptus urophylla*, *AA Acacia auriculiformis*). For each compartment, *different letters* indicate significant differences ($P < 0.05$) between plantations in control plots, while *asterisk* indicates significant differences ($P < 0.05$) between the corresponding treatment and control. *Error bars* represent standard errors ($n = 3$)

**Table 1** Effects of N addition on DIN concentrations in bulk and rhizosphere soils in each plantation

| Plantation type | | EU | | | AA | | |
|---|---|---|---|---|---|---|---|
| Treatment | | C | MN | HN | C | MN | HN |
| Bulk soil | $NO_3^-$ (mg $kg^{-1}$) | 11.82 (2.12) b | 20.18 (0.72) a | 20.72 (1.27) a | 18.19 (2.14) | 17.74 (2.67) | 26.82 (3.88) |
| | $NH_4^+$ (mg $kg^{-1}$) | 6.11 (1.84) b | 9.18 (0.87) ab | 11.79 (1.52) a | 6.11 (1.02) b | 5.58 (0.60) b | 12.09 (2.69) a |
| | Total DIN (mg $kg^{-1}$) | 17.93 (0.31) Bb | 29.36 (0.45) a | 32.51 (2.63) a | 24.30 (1.66) Ab | 23.31 (2.81) b | 38.92 (5.67) a |
| Rhizosphere soil | $NO_3^-$ (mg $kg^{-1}$) | 16.97 (1.18) | 15.69 (1.02) | 14.82 (0.96) | 18.15 (0.98) c | 21.40 (0.58) b | 24.87 (0.88) a |
| | $NH_4^+$ (mg $kg^{-1}$) | 14.94 (1.25) A | 12.08 (1.28) | 12.66 (1.27) | 8.72 (0.63) Bb | 10.80 (0.51) ab | 12.39 (0.63) a |
| | Total DIN (mg $kg^{-1}$) | 31.91 (1.51) A | 27.77 (2.18) | 27.48 (2.21) | 26.87 (0.57) Bc | 32.20 (0.22) b | 37.26 (1.51) a |

Values are means with standard errors in brackets ($n = 3$). Different capital and small letters indicate significant difference ($P < 0.05$) between forests and among treatments, respectively

*C* control, *MN* medium N addition, *HN* high N addition, *DIN* dissolved inorganic N, *EU Eucalyptus urophylla*, *AA Acacia auriculiformis*

and HN addition significantly increased standing stock of rhizosphere soil in the EU plantation but not in the AA plantation. High N addition significantly decreased standing stock of nodule in the AA plantation.

Fresh leaf N content in control plots was significantly higher in the AA plantation than in the EU plantation (Fig. 3a). N addition significantly increased fresh leaf N content in the EU plantation but not in the AA plantation. Crown areas in control plots were significantly higher in the AA plantation than in the EU plantation (Fig. 3b). High N addition significantly increased crown areas in the EU plantation but not in the AA plantation. However, DBH in both plantations remained constant following N addition (Fig. 3c).

Moisture content of bulk soil was significantly higher in the EU plantation than in the AA plantation, but moisture content of forest floor showed an opposite trend (Fig. S1). Moisture content of rhizosphere soil was comparable between plantations. These results suggest that moisture content in control plots were different depending on the compartments and plantation types.

## Discussion

### BNF between plantations

Our results showed that total rates of BNF were comparable between the AA (6.04 kg N $ha^{-1}$ $year^{-1}$) and the EU (6.42 kg N $ha^{-1}$ $year^{-1}$) plantations, which did not support our hypothesis that the N-rich AA plantation should be lower in BNF than the N-poor EU plantation. This finding is also inconsistent with the results (measured by ARA method) of previous studies in which total BNF rates seem to be lower in mature tropical forests with legume trees (Monk et al. 1981; Pearson and Vitousek 2001) than in those without legume trees (Reed et al. 2007, 2008; Cusack et al. 2009). However, the above-mentioned studies in legume tree forests only measured rates of nodule and not of other compartments, making it difficult to draw a comprehensive conclusion. Our study in the mature AA plantation showed that forest floor (1.05 kg N $ha^{-1}$ $year^{-1}$) and bulk (3.90 kg N $ha^{-1}$ $year^{-1}$) and rhizosphere (0.51 kg N $ha^{-1}$ $year^{-1}$) soils still fixed N in spite of

**Table 2** Effects of N addition on standing stock of bulk soil, forest floor, rhizosphere soil, and nodule in each plantation

| Plantation type | EU | | | AA | | |
|---|---|---|---|---|---|---|
| Treatment | C | MN | HN | C | MN | HN |
| Bulk soil (kg $m^{-2}$) | 94.09 (2.57) B | 109.27 (3.91) | 96.91 (6.28) | 112.35 (5.18) Aa | 89.68 (9.09) b | 117.77 (3.48) a |
| Forest floor (kg $m^{-2}$) | 0.50 (0.12) B | 0.41 (0.05) | 0.52 (0.13) | 1.24 (0.21) A | 1.20 (0.11) | 0.86 (0.09) |
| Rhizosphere soil (kg $m^{-2}$) | 2.32 (0.03) Bc | 2.71 (0.03) b | 2.91 (0.05) a | 2.58 (0.04) A | 2.54 (0.05) | 2.58 (0.07) |
| Nodule (g $m^{-2}$) | | | | 0.60 (0.06) a | 0.37 (0.06) ab | 0.20 (0.10) b |

Standing stock of bulk and rhizosphere soils, and nodule referred to the depth of 0–10 cm, and standing stock of forest floor referred to the full thickness from freshly fallen leaves to bulk soil surface. Values are means with standard errors in brackets ($n = 3$). Different capital letters and small letters indicate significant difference ($P < 0.05$) between forests and among treatments, respectively

*C* control, *MN* medium N addition, *HN* high N addition, *EU Eucalyptus urophylla*, *AA Acacia auriculiformis*

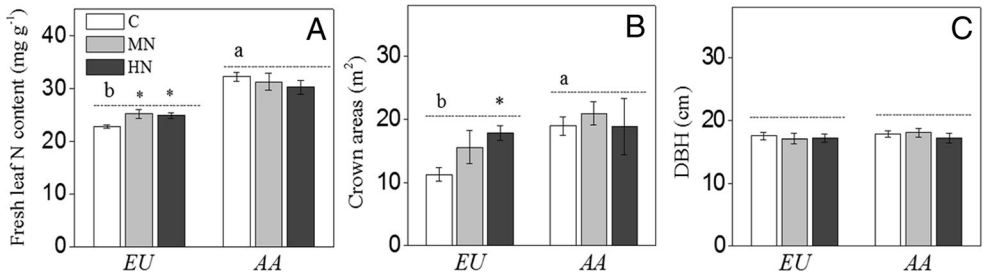


**Fig. 3** Effects of N addition on fresh leaf N content (**a**), crown areas (**b**), and diameter at breast height (DBH) (**c**) in the two plantations. *C* control, *MN* medium N addition, *HN* high N addition, *EU Eucalyptus urophylla*, *AA Acacia auriculiformis*. *Different letters* indicate significant differences ($P < 0.05$) between plantations. *Asterisk* indicates significant differences ($P < 0.05$) between each treatment and control. *Error bars* represent standard errors ($n = 3$)

low rates of nodule (0.67 kg N ha$^{-1}$ year$^{-1}$). This finding suggests that mature plantations with legume trees may still have total rates of BNF equal to plantations without legume trees.

BNF rates of bulk soil were comparable between the two plantations (Fig. 2), which contradicted our expectation that lower rates would be found in the AA plantation due to the richer initial N levels in bulk soil (Table 1). This finding, however, is consistent with those from studies in the tropics where BNF rates (measured by ARA method) of bulk soil were similar between forests with legume trees (Gei 2014) and without legume trees (Maheswaran and Gunatilleke 1990; Reed et al. 2008; Barron et al. 2008; Cusack et al. 2009). Actually, nitrogenase activity per gram of bulk soil was significantly lower in the AA plantation than in the EU plantation (Fig. 1), indicating that bulk soil with richer N in the AA plantation did show lower diazotroph activity. However, we found this plantation had significantly higher standing stock of bulk soil than the EU plantation (Table 2), suggesting that the AA plantation had a higher biomass of diazotrophs per unit area. Although direct data about soil diazotroph biomass is not available in this study, previous studies revealed that soils with legume trees had a higher abundance and diversity of diazotrophs than soils with non-legume trees (Diallo et al. 2004; Villadas et al. 2007). This, in combination with our findings, suggests that plantations with legume trees may have the higher biomass but lower activity of diazotrophs in bulk soil than plantations without legume trees, and thus, the rates of BNF per hectare of bulk soil may not significantly differ between such plantations.

As expected, BNF rates of rhizosphere soil were significantly higher in the EU plantation than in the AA plantation (Fig. 2), suggesting a more important role for rhizospheric BNF in plantations without legume trees. We infer that this result is related to tree species (non-legume versus legume) which may have different impacts on rhizospheric diazotrophs. Theoretically, non-legume trees should provide a favorable niche for rhizospheric diazotrophs that may indirectly provide N for host trees via BNF (Santi et al. 2013). This view is supported by our study, because we found that both nitrogenase activity and DIN concentrations of rhizosphere soil were significantly higher in the EU plantation than in the AA plantation (Fig. 1 and Table 1). By contrast, legume and other N-fixing trees rely mainly on nodule for N, regardless of soil N status (Markham and Zekveld 2007), indicating greater competitiveness in nodule BNF than in other pathways, such as rhizospheric BNF. In our mature AA plantation, nodule still had extremely high activity (Fig. 1) and fixed more N than rhizospheric diazotrophs (Fig. 2), despite nodule biomass (Table 2) being magnitudes lower than it was in younger stages (Ding et al. 1994). This demonstrates that nodule rather than rhizosphere soil is still the main source of fixed N for mature AA trees. To our knowledge, there has been so far no report available to estimate the role of rhizospheric BNF in forests. Our findings provide new insights into rhizospheric BNF in forest plantations and suggest that the rates of rhizospheric BNF may depend on forest type (with/without legume trees).

Contrary to our expectation, BNF rates of forest floor were significantly higher in the AA plantation than in the EU plantation (Fig. 2). This unexpected result can also be accounted for by tree species (legume versus non-legume) as follows. First, N-fixing legume trees are less limited by N and often grow relatively faster (Nichols et al. 2001; Pearson and Vitousek 2001) than non-legume trees (Binkley et al. 2003a). In this study, we found a significantly larger crown area in the AA plantation than in the EU plantation (Fig. 3b) and a crown closure in the AA plantation but not in the EU plantation. On the one hand, since forest canopy has net retention of atmospheric N in precipitation (Clark et al. 1998), the larger and more closed crown of the AA plantation may retain more atmospheric N that directly suppresses on BNF in forest floor (Cusack et al. 2009). On the other hand, the more closed crown of the AA plantation may result in lower evaporation rates and thus higher moisture content in forest floor (Fig. S1), which in turn provides favorable anoxic microsites for diazotrophs (Reed et al. 2007; Cusack et al. 2009). Therefore, the AA plantation showed higher BNF rates of forest floor than the EU plantation. Second, legume trees often produce more litterfall onto forest floor than non-legume trees

(Binkley et al. 1992; Zhang et al. 2012a, b) due to their fast growth. In our study, standing stock of forest floor was more than twofold higher in the AA plantation than in the EU plantation (Table 2). Higher standing stock has been found to contribute higher rates of BNF of forest floor per unit area (Matzek and Vitousek 2003; Menge and Hedin 2009). Accordingly, our result suggests that forest floor may have higher BNF rates in mature plantations with legume trees than in those without legume trees.

### Effects of N addition on BNF

As expected, N addition significantly decreased total rates of BNF in the AA plantation because of the decreased fixing rates in all compartments (Fig. 2). First, N addition significantly decreased BNF rates of nodule, as previous studies have also reported (Markham and Zekveld 2007; Batterman et al. 2013b). This phenomenon is not surprising because N fixers prefer to take up inorganic N since BNF costs more energy (Crews 1999). In this study, we found that the decreased fixing rates of nodule were caused by a decrease in nodule biomass (Table 2) but not in nitrogenase activity (Fig. 1). This suggests that tropical legume trees may be "facultative" strategy fixers (Batterman et al. 2013b; Menge et al. 2014) that reduce the energy cost of nodule production when soil N improved after N addition (Table 1). However, legume trees may still rely on nodule for N in spite of rich N in soil (Markham and Zekveld 2007) because high N addition never completely stops either nodule BNF (Fig. 2) or nodule production (Table 2).

Second, N addition also significantly decreased BNF rates of bulk soil, forest floor, and rhizosphere soil in the AA plantation (Fig. 2). This finding suggests that external N addition would not only decrease BNF rates of symbiotic nodules but also of free-living compartments (bulk and rhizosphere soils and forest floor) in legume tree forests, resulting in more serious suppression of total BNF than previously thought. We infer that the negative responses of BNF in free-living compartments were likely related to excess N in the AA plantation, based on two lines of evidence: (1) although N addition marginally and/or significantly increased N concentrations in bulk and rhizosphere soils (Table 1), and forest floor (Zhang et al. 2014) in the AA plantation, it did not increase microbial biomass N (Zhang et al. 2012b) or fresh leaf N content (Fig. 3a); and (2) the excess N in the AA plantation following N addition was lost via increased $N_2O$ gas emission (Zhang et al. 2014). Thus, external N added to this N-rich plantation would not be utilized any more, and it would instead work against BNF and nitrogenase activity in all free-living compartments (Figs. 1 and 2).

Consistent with our hypothesis, N addition did not change total rates of BNF in the EU plantation (Fig. 2). We found that although N addition significantly decreased BNF rates of bulk soil, it significantly increased rates of the rhizosphere soil and did not affect rates of forest floor (Fig. 2). Our result from the forest floor was consistent with those from other forests without legume trees (Vitousek and Hobbie 2000; Reed et al. 2007; Cusack et al. 2009), while our result from bulk soil was only consistent with that from Panama forests (Barron et al. 2008) but not from other forests (Reed et al. 2007; Cusack et al. 2009) where BNF rates of bulk soil had no response to N addition. In our EU plantation, the opposite responses of bulk and rhizosphere soils to N addition were interesting, because we expected no BNF response in this N-poor plantation. However, the underlying mechanism is currently unclear, because little is understood about how diazotroph communities vary from bulk (Diallo et al. 2004) to rhizosphere (Villadas et al. 2007) soil. In the EU plantation, we found that rhizospheric DIN concentrations showed a slight though not significant decrease after N addition, but DIN concentrations in bulk soil were significantly increased (Table 1). This led to lower DIN concentrations in rhizosphere (27.48–27.77 mg $kg^{-1}$) than in bulk soil (29.36–32.51 mg $kg^{-1}$) in N-treated plots. In light of this, we assume that diazotrophs may move from bulk to rhizosphere soil for the favorable lower N microsite in rhizosphere soil after N addition, thus leading to decreased fixing rates of bulk soil and increased fixing rates of rhizosphere soil. Two explanations can support this assumption. First, *Eucalyptus* can grow fast by absorbing large amounts of N from rhizosphere after N addition. In the EU plantation, N addition never increases rhizospheric DIN concentrations (Table 1), but it does significantly increase crown areas (Fig. 3b), fresh leaf N content (Fig. 3a), and leaf litter N content (Zhang et al. 2014). This evidence suggests that plenty of rhizospheric N may be absorbed by the EU trees for growth after N addition, leaving a low level of N in rhizosphere. Second, rhizosphere with low N may provide a favorable microsite for the free-living diazotrophs in bulk soil to move into. Bashan (1999) suggested that several groups of diazotrophs would move "actively" from bulk into rhizosphere soil if rhizosphere had favorable colonization conditions. This phenomenon likely occurs in our EU plantation, as reflected by the favorable lower N level in rhizosphere soil than in bulk soil after N addition (Table 1). In addition, our study implies that diazotrophs may also "passively" move into rhizosphere soil via root growth, as evidenced by a significant increase in standing stock of rhizosphere soil after N addition (Table 2). Movement of diazotrophs from bulk to rhizosphere soil after N addition was further supported by a significant decrease in nitrogenase activity of bulk soil and a significant increase in nitrogenase activity of rhizosphere soil (Fig. 1). Accordingly, our findings suggest that in plantations with non-legume trees, diazotrophs in bulk soil may move to rhizosphere soil after N addition for the lower rhizospheric N level, and that the decreased BNF rates of bulk soil were actually offset by the increased

rates of rhizosphere soil. Thus, N addition will not change total rates of BNF in mature plantations with non-legume trees.

## Conclusions

To our knowledge, this is the first comparative study of BNF in two mature tropical forest plantations with and without legume trees, and of the BNF response to N addition. Two important findings were discovered in this study. First, although the two plantations had similar rates of BNF overall, rates of rhizosphere soil were significantly higher in the EU plantation whereas rates of forest floor were significantly higher in the AA plantation. Second, external N addition significantly decreased BNF rates of all measured compartments, and thus, total rates in the AA plantation; by contrast, N addition did not change total BNF rates in the EU plantation, which upregulated BNF rates of rhizosphere soil to offset the suppressed BNF rates of bulk soil. This finding provides a new line of evidence that forest type (with/without legume trees) regulates the effects of N input on BNF, and suggests that elevated atmospheric N deposition in recent decades will suppress total N fixation in mature forests with legume trees but not in those with non-legume trees.

**Acknowledgments** This work was supported by National Natural Science Foundation of China (Nos. 41273143 and 41473112) and Provincial Natural Science Foundation of Guangdong (No. 431021001). We wish to acknowledge Shengxing Fu for his skillful assistance in field work and Dr. Xing Lu for his instruction in laboratory analysis. We thank the editors and two reviewers for their constructive comments and suggestions on the manuscript.

## References

Barron AR, Wurzburger N, Bellenger JP, Wright SJ, Kraepiel AM, Hedin LO (2008) Molybdenum limitation of asymbiotic nitrogen fixation in tropical forest soils. Nat Geosci 2:42–45

Bashan Y (1999) Interactions of *Azospirillum* spp. in soils: a review. Biol Fertil Soils 29:246–256

Batterman SA, Hedin LO, Breugel MV, Ransijn J, Craven DJ, Hall JS (2013a) Key role of symbiotic dinitrogen fixation in tropical forest secondary succession. Nature 502:224–227

Batterman SA, Wurzburger N, Hedin LO (2013b) Nitrogen and phosphorus interact to control tropical symbiotic $N_2$ fixation: a test in *Inga punctata*. J Ecol 101:1400–1408

Benner JW, Vitousek PM (2007) Development of a diverse epiphyte community in response to phosphorus fertilization. Ecol Lett 10: 628–636

Binkley D, Dunkin KA, Debell D, Ryan MG (1992) Production and nutrient cycling in mixed plantations of *Eucalyptus* and *Albizia* in Hawaii. Forest Sci 38:393–408

Binkley D, Senock R, Bird S, Cole TG (2003a) Twenty years of stand development in pure and mixed stands of *Eucalyptus saligna* and nitrogen-fixing *Facaltaria moluccana*. Forest Ecol Manag 182:93–102

Binkley D, Senock R, Cromack K (2003b) Phosphorus limitation on nitrogen fixation by *Facaltaria* seedlings. Forest Ecol Manag 186: 171–176

Bouillet JP, Laclau JP, Goncalves JLD, Voigtlaender M, Gava JL, Leite FP, Hakamada R, Mareschal L, Mabiala A, Tardy F, Levillain J, Deleporte P, Epron D, Nouvellon Y (2013) *Eucalyptus* and *Acacia* tree growth over entire rotation in single- and mixed-species plantations across five sites in Brazil and Congo. Forest Ecol Manag 301: 89–101

Bremner J, Mulvaney C (1982) Nitrogen-total. In: Page AL (ed) Part 2. Chemical and microbiological properties, method of soil analysis. American Society of Agronomy, Madison, pp 595–624

Chen DM, Zhang CL, Wu JP, Zhou LX, Lin YB, Fu SL (2011) Subtropical plantations are large carbon sinks: evidence from two monoculture plantations in South China. Agr Forest Meteorol 151: 1214–1225

Clark KL, Nadkarni NM, Schaefer D, Gholz HL (1998) Atmospheric deposition and net retention of ions by the canopy in a tropical montane forest, Monteverde, Costa Rica. J Trop Ecol 14:27–45

Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth RW, Hedin LO, Perakis SS, Latty EF, Von Fischer JC, Elseroad A (1999) Global patterns of terrestrial biological nitrogen ($N_2$) fixation in natural ecosystems. Global Biogeochem Cy 13:623–645

Crews TE (1999) The presence of nitrogen fixing legumes in terrestrial communities: evolutionary vs ecological considerations. Biogeochemistry 46:233–246

Crews TE, Farrington H, Vitousek PM (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 TE, Kurina LM, Vitousek PM (2001) Organic matter and nitrogen accumulation and nitrogen fixation during early ecosystem development in Hawaii. Biogeochemistry 52:259–279

Cusack DF, Silver W, McDowell WH (2009) Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. Ecosystems 12:1299–1315

DeLuca TH, Drinkwater LE, Wiefling BA, DeNicola DM (1996) Free-living nitrogen-fixing bacteria in temperate cropping systems: influence of nitrogen source. Biol Fert Soils 23:140–144

DeLuca TH, Zackrisson O, Gentili F, Sellstedt A, Nilsson MC (2007) Ecosystem controls on nitrogen fixation in boreal feather moss communities. Oecologia 152:121–130

Diallo MD, Willems A, Vloemans N, Cousin S, Vandekerckhove TT, de Lajudie P, Neyra M, Vyverman W, Gillis M, Van der Gucht K (2004) Polymerase chain reaction denaturing gradient gel electrophoresis analysis of the $N_2$-fixing bacterial diversity in soil under *Acacia tortilis* ssp. *raddiana* and *Balanites aegyptiaca* in the dryland part of Senegal. Environ Microbiol 6:400–415

Ding MM, Yi WM, Liao LY, Fu SL, Yu ZY (1994) Effect of ecological conditions on nodulation nitrogen fixation of *Acacia Mangium*. J Trop Subtrop Botany 2:15–21 (in Chinese with English Abstract)

FAO (2010) Global forest resources assessment 2010. United Nations Food and Agriculture Organization (UN FAO), Rome

Fujii K, Aoki M, Kitayama K (2012) Biodegradation of low molecular weight organic acids in rhizosphere soils from a tropical montane rain forest. Soil Biol Biochem 47:142–148

Galloway JN, Schlesinger WH, Levy H, Michaels A, Schnoor JL (1995) Nitrogen fixation: anthropogenic enhancement-environmental response. Global Biogeochem Cy 9:235–252

Gei MG (2014) Biological nitrogen fixation in tropical dry forests of Costa Rica: patterns and controls. Dissertation, University of Minnesota Digital Conservancy

Hardy RWF, Holsten R, Jackson E, Burns R (1968) The acetylene-ethylene assay for $N_2$ fixation: laboratory and field evaluation. Plant Physiol 43:1185–1207

Huang J, Zhang W, Zhu XM, Gilliam FS, Chen H, Lu XK, Mo JM (2014) Urbanization in China changes the composition and main sources of wet inorganic nitrogen deposition. Environ Sci Pollut R 22:1–9

Kreibich H, Kern J (2003) Nitrogen fixation and denitrification in a floodplain forest near Manaus, Brazil. Hydrol Process 17:1431–1441

Lagerström A, Nilsson MC, Zackrisson O, Wardle D (2007) Ecosystem input of nitrogen through biological fixation in feather mosses during ecosystem retrogression. Funct Ecol 21:1027–1033

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

Markham JH, Zekveld C (2007) Nitrogen fixation makes biomass allocation to roots independent of soil nitrogen supply. Botany 85:787–793

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

Menge DNL, Hedin LO (2009) Nitrogen fixation in different biogeochemical niches along a 120,000-year chronosequence in New Zealand. Ecology 90:2190–2201

Menge DNL, Lichstein JW, Angeles-Perez G (2014) Nitrogen fixation strategies can explain the latitudinal shift in nitrogen-fixing tree abundance. Ecology 95:2236–2245

Monk D, Pate J, Loneragan W (1981) Biology of *Acacia pulchella* R. Br. with special reference to symbiotic nitrogen fixation. Aust J Bot 29:579–592

Nichols JD, Rosemeyer ME, Carpenter FL, Kettler J (2001) Intercropping legume trees with native timber trees rapidly restores cover to eroded tropical pasture without fertilization. Forest Ecol Manag 152:195–209

Nygren P, Leblanc HA (2009) Natural abundance of $^{15}N$ in two cacao plantations with legume and non-legume shade trees. Agroforest Syst 76:303–315

Pearson HL, Vitousek PM (2001) Stand dynamics, nitrogen accumulation, and symbiotic nitrogen fixation in regenerating stands of Acacia koa. Ecol Appl 11:1381–1394

Pérez CA, Carmona MR, Armesto JJ (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

Reed SC, Cleveland CC, Townsend AR (2007) Controls over leaf litter and soil nitrogen fixation in two lowland tropical rain forests. Biotropica 39:585–592

Reed SC, Cleveland CC, Townsend AR (2008) Tree species control rates of free-living nitrogen fixation in a tropical rain forest. Ecology 89:2924–2934

Reed SC, Cleveland CC, Townsend AR (2011) Functional ecology of free-living nitrogen fixation: a contemporary perspective. Annu Rev Ecol Evol S 42:489–512

Santi C, Bogusz D, Franche C (2013) Biological nitrogen fixation in non-legume plants. Ann Bot-London 111:743–767

Sullivan BW, Smith WK, Townsend AR, Nasto MK, Reed SC, Chazdon RL, Cleveland CC (2014) Spatially robust estimates of biological nitrogen (N) fixation imply substantial human alteration of the tropical N cycle. Proc Natl Acad Sci U S A 111:8101–8106

Villadas PJ, Fernandez-Lopez M, Ramirez-Saad H, Toro N (2007) Rhizosphere-bacterial community in *Eperua falcata* (Caesalpiniaceae) a putative nitrogen-fixing from French Guiana rainforest. Microb Ecol 53:317–327

Vitousek PM (1999) Nutrient limitation to nitrogen fixation in young volcanic sites. Ecosystems 2:505–510

Vitousek PM, Hobbie S (2000) Heterotrophic nitrogen fixation in decomposing litter: patterns and regulation. Ecology 81:2366–2376

Vitousek PM, Howarth RW (1991) Nitrogen limitation on land and in the sea: how can it occur? Biogeochemistry 13:87–115

Vitousek PM, Menge DN, Reed SC, Cleveland CC (2013) Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Phil Trans R Soc B 368:20130119. doi:10.1098/rstb.2013.0119

Welsh DT (2000) Nitrogen fixation in seagrass meadows: regulation, plant-bacteria interactions and significance to primary productivity. Ecol Lett 3:58–71

Wurzburger N, Bellenger JP, Kraepiel AM, Hedin LO (2012) Molybdenum and phosphorus interact to constrain asymbiotic nitrogen fixation in tropical forests. PLoS ONE 7, e33710

Zackrisson O, DeLuca TH, Nilsson MC, Sellstedt A, Berglund L (2004) Nitrogen fixation increases with successional age in boreal forests. Ecology 85:3327–3334

Zhang H, Guan DS, Song MW (2012a) Biomass and carbon storage of *Eucalyptus* and *Acacia p*lantations in the Pearl River Delta, South China. Forest Ecol Manag 277:90–97

Zhang W, Zhu XM, Liu L, Fu SL, Chen H, Huang J, Lu XK, Liu ZF, Mo JM (2012b) Large difference of inhibitive effect of nitrogen deposition on soil methane oxidation between plantations with N-fixing tree species and non-N-fixing tree species. J Geophys Res 117:G00N16

Zhang W, Zhu XM, Luo YQ, Rafique R, Chen H, Huang J, Mo JM (2014) Responses of nitrous oxide emissions to nitrogen and phosphorus additions in two tropical plantations with N-fixing vs. non-N-fixing tree species. Biogeosciences 11:4941–4951