Seedling leaves allocate lower fractions of nitrogen to photosynthetic apparatus in nitrogen fixing trees than in non-nitrogen fixing trees in subtropical China

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Abstract

Photosynthetic-nitrogen use efficiency (PNUE) is a useful trait to characterize leaf physiology and survival strategy. PNUE can also be considered as part of ‘leaf economics spectrum’ interrelated with leaf nutrient concentrations, photosynthesis and respiration, leaf life-span and dry-mass investment. However, few studies have paid attention to PNUE of N-fixing tree seedlings in subtropical China. In this study, we investigated the differences in PNUE, leaf nitrogen (N) allocation, and mesophyll conductance ($g_m$) in Dalbergia odorifera and Erythrophleum fordii (N-fixing trees), and Betula alnoides and Castanopsis hystrix (non-N-fixing trees). PNUE of D. odorifera and E. fordii were significantly lower than those of B. alnoides and C. hystrix mainly because of their allocation of a lower fraction of leaf N to Rubisco ($P_R$) and bioenergetics ($P_B$). Mesophyll conductance had a significant positive correlation with PNUE in D. odorifera and E. fordii (N-fixing trees), and B. alnoides and C. hystrix (non-N-fixing trees). PNUE of D. odorifera and E. fordii were significantly lower than those of B. alnoides and C. hystrix mainly because of their allocation of a lower fraction of leaf N to Rubisco ($P_R$) and bioenergetics ($P_B$). Mesophyll conductance had a significant positive correlation with PNUE in D. odorifera, E. fordii, and B. alnoides, but the effect of $g_m$ on PNUE was different between species. The fraction of leaf N to cell wall ($P_{CW}$) had a significant negative correlation with $P_R$ in B. alnoides and C. hystrix seedling leaves, but no correlation in D. odorifera and E. fordii seedling leaves, which may indicate that B. alnoides and C. hystrix seedling leaves did not have enough N to satisfy the demand from both the cell wall and Rubisco. Our results indicate that B. alnoides and C. hystrix may have a higher competitive ability in natural ecosystems with fertile soil, and D. odorifera and E. fordii may grow well in N-poor soil. Mixing these non-N-fixing and N-fixing trees for afforestation is useful for improving soil N utilization efficiency in the tropical forests.
Nitrogen fixing tree allocate lower fraction of nitrogen to photosynthetic apparatus than non-nitrogen fixing

Introduction

Nitrogen (N) is very important for plants leaves, because main function of leaves–photosynthesis need a lot of N [1,2], and there was a positive correlation between photosynthetic capacity and N content in many species. However, there existed interspecific difference in the photosynthesis–N relationship [3]. Many researchers use photosynthetic-N use efficiency (PNUE, the ratio of light-saturated net CO₂ assimilation rate (A_{\text{max}}) to leaf N content per area (N_{\text{area}})) to show how efficiently N resources are used during photosynthesis, and studies have been conducted on a variety of species [3,5,6]. N-fixing species could convert N from the air through legume bacteria, and always have enough N in leaves [7–9]. Studies have shown that N-fixing trees had lower A_{\text{max}} and higher N_{\text{area}} which resulted in a lower PNUE [10, 11]. These contradicting results may imply that some N-fixing species use a different strategy to utilize N compared to non-N-fixing species.

Many factors could affect PNUE, and the most important factor is leaves photosynthetic N allocation [12]. Rubisco is the most abundant enzyme in C\textsubscript{3} plants [13], and it is the key factor in carbon assimilation [14]. Many researchers have found a positive correlation between leaf N fraction in Rubisco (P\textsubscript{R}) and PNUE in various plants [15–16]. Bioenergetics and the light-harvesting components could also influence PNUE in some plants [17]. Apart from photosynthetic, leaf cell walls, which could protect leave cell and influence leaf life-span also need a lot of N to synthesize [18]. Trade-offs may occur between N allocation to cell walls and Rubisco [18–20]. However, some studies have shown that these trade-offs only exist in individuals of the same species [16] or species lacking N in leaves [18, 21].

Carbon dioxide is an important raw material for photosynthesis [22], and CO₂ partial pressure is important for Rubisco activity; this is because O₂ is a competitive inhibitor of the C assimilatory reaction of Rubisco, promoting the Rubisco oxidation reaction [23]. A significant negative correlation between C\textsubscript{i} (intercellular CO₂ concentration)-C\textsubscript{o} (CO₂ concentration at carboxylation site) and PNUE was found in Populus cathayana [24]. Nitrogen is also involved in carbonic anhydrases and aquaporins [25]. These proteins play a role in mesophyll conductance (g\textsubscript{m}) by changing the nature of the diffusing molecule [26] and facilitating CO₂ diffusion through membranes [27]. Therefore, PNUE may be influenced by g\textsubscript{m} [25]. A significant positive correlation was found between mesophyll conductance (g\textsubscript{m}) and PNUE in six Populus genotypes [28].

What reason causes the low PNUE in N-fixing plants? One possible explanation is that the percentage of N in the photosynthetic apparatus is lower in the N-fixing trees [10, 11]. However, these studies neglect that g\textsubscript{m} and the fraction of leaf N to cell wall (P\textsubscript{CW}) could also influence PNUE [19, 20, 29]. We studied the factors that affect PNUE in both N-fixing and non-N-fixing large trees in a previous study and found P\textsubscript{R} and fraction of leaf N to bioenergetics (P\textsubscript{B}) to be the main factors; the effects of g\textsubscript{m} and P\textsubscript{CW} were relatively small [30], but the effects in N-fixing tree seedlings remained unclear.

Dalbergia odorifera, Erythrophleum fordii, Betula alnoides, and Castanopsis hystrix are suitable for forestation in southern subtropical China and have high economic values [31–34]. D. odorifera and E. fordii are both evergreen N-fixing trees, whereas B. alnoides and C. hystrix are both non-N-fixing, and deciduous and evergreen, respectively. The objectives of our study are as follows: 1) understand how PNUE varies among D. odorifera, E. fordii, B. alnoides, and C. hystrix seedlings; 2) quantify the relationship between PNUE related to leaf N allocation and diffusional conductances to CO₂ in seedlings.

Materials and methods

Study area and plant material

This study was carried out in Experimental Center of Tropical Forestry (22°7′19″–22°7′22″N, 106°44′40″–106°44′44″E) of the Chinese Academy of Forestry located in Guangxi Pingxiang.
China. The location has a subtropical monsoon climate with distinct dry and wet periods where the mean annual temperature is 21°C. The mean monthly minimum and maximum temperatures are 12.1°C and 26.3°C. The mean annual precipitation is 1400 mm, and it occurs mainly from April to September. Active accumulated temperature above 10°C is 6000–7600°C. The total annual sunshine duration is 1419 hours [35,36].

Seeds of *D. odorifera*, *E. fordii*, and *C. hystrix* were collected from a single tree for each species, and *B. alnoides* seedlings were somaclone. The seeds of *D. odorifera*, *E. fordii*, and *C. hystrix* were germinated in a seedbed in February 2014 and *B. alnoides* went through budding at the same time. When the seedlings were approximately 20 cm tall, 30 similarly sized seedlings per species were individually transplanted to pots (5.4 L, filled with washed river sand) and established in an open site at the Experimental Center of Tropical Forestry in March 2014. From April to June, each pot received the same nutrient solution (0.125 g N and 0.11 g P, Hyponex M. Scott & Sons, Marysville, OH, USA) once a week, and was watered every day to keep the soil moist. Natural light (100% of light in the field) was used for illumination.

**Determination of gas exchange measurements**

Gas exchange parameters were determined with a Li-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA) on sunny days from 8 am to 10 am in July and August 2014. Seven healthy and newly emerged leaves exposed to the sun in each tree species were chosen (one leaf per individual healthy tree). Photosynthetic response to photosynthetic photon flux density (PPFD) and intercellular CO₂ concentration (Cᵢ, μmol mol⁻¹) were determined for each leaf (seven repetitions in each species): Under 380 μmol mol⁻¹ of leaf chamber CO₂ concentration (the average air CO₂ concentration in the day time), the photosynthetic rates were measured under photon flux densities of 1500, 1200, 1000, 800, 600, 400, 200, 150, 100, 80, 50, 30, 20, 10 and 0 μmol m⁻² s⁻¹ [37]. Under a saturated PPFD, the photosynthetic rates were detected using the same leaf-under leaf chamber CO₂ concentrations of 380, 200, 150, 100, 80, 50, 380, 600, 800, 1000, 1200, 1500, 1800 and 2000 μmol mol⁻¹ [28]. Relative humidity of the air in the leaf chamber was maintained at 60–70%, and leaf temperature was set at 30°C. The net photosynthetic rate (Aᵦ, μmol m⁻² s⁻¹), stomatal conductance (gₛ, mol CO₂ m⁻² s⁻¹), and Cᵢ of each sampled leaf were recorded ten times after 200 s under each PPFD and CO₂ concentration. Then light-saturated net CO₂ assimilation rate (Aᵦ max, μmol m⁻² s⁻¹), light-saturated day respiration rate (Rᵥ, μmol m⁻² s⁻¹) and light- and CO₂-saturated net CO₂ assimilation rate (Aᵦ max, μmol m⁻² s⁻¹) were measured or calculated. For further details see Tang et al. [30].

**Determination of chlorophyll fluorescence and mesophyll conductance**

Fluorescence yield was measured with a Li–6400 leaf chamber fluorometer (6400–40, LI-COR, Lincoln, Nebraska, USA), using the same leaf with seven repetitions of each species. Chamber temperature was maintained at 28–32°C, and chamber air relative humidity was maintained at 60–70%. Chamber CO₂ concentration was set to 380 μmol mol⁻¹. PPFD was set to light saturation point. Constant values of fluorescence yield (ΔF/Φₘ) of each leaf sample were recorded 10 times after 200 s [38]. We used Loreto et al. [39] methods to calculate the photosynthetic electron transport rate (Jₑ, μmol m⁻² s⁻¹):

$$ Jₑ = PPFD \times \frac{ΔF}{Φₘ} \times Leafreflu \times PARDistPhotosys. $$ (1)

*Leafreflu* (leaf absorptance valued) and *PARDistPhotosys* (the fraction of quanta absorbed by photosystem II) were 0.85 [40] and 0.5 [39], respectively. We used the variable J method described by Harley et al. [41], which has been used in recent years [42–45] to calculate
mesophyll conductance ($g_m$, mol CO$_2$ m$^{-2}$ s$^{-1}$):

$$g_m = \frac{A_{\text{max}}'}{C_i - \left\{ \frac{1}{J_f} \ln \left[ \frac{A_{\text{max}}'}{J_f (R_d + R_i)} \right] \right\}^{\frac{1}{J_f}}}$$  

(2)

Where $R_d$, $C_i$, and $A_{\text{max}}'$ were determined from gas exchange measurements. The CO$_2$ photo compensation point ($I^*$, μmol mol$^{-1}$) value was 54.76 at 30˚C according to Bernacchi et al [46].

Because the Harley method should calibrate the ETR using Chl fluorescence and gas exchange under low O$_2$, we used the experience value instead ($\text{Leafreflu} = 0.85$) [30]. We also used Ethier and Livingston [47] and the exhaustive dual optimization (EDO) method [48] to calculate $g_m$. We used software based on the Ethier and Livingston method developed by Sharkey et al. [49] to get $g_m$, and uploaded our data through a website (http://www.leafweb.org) to get $g_m$ calculated by the EDO method.

**Determination of $V_{\text{cmax}}$ and $J_{\text{max}}$**

The mean value of $g_m$, calculated from three methods was used to calculate CO$_2$ concentration in chloroplasts ($C_c$, μmolmol$^{-1}$):

$$C_c = C_i - \frac{A_{\text{max}}'}{g_m}$$  

(3)

Then $C_c$ was used to fit an $A_n$-$C_c$ curve, followed by the maximum carboxylation rate ($V_{\text{cmax}}$, μmol m$^{-2}$ s$^{-1}$) calculated according to Farquhar et al. [14], and the maximum electron transport rate ($J_{\text{max}}$, μmol m$^{-2}$ s$^{-1}$) calculated according to Loustau et al. [50]. The fitting model used in vivo Rubisco kinetics parameters ($K_o$, $K_c$, and their activation energy) measured by Niinemets and Tenhunen [12].

**Analysis of quantitative limitations of photosynthetic capacity**

The relative controls on photosynthetic capacity imposed by stomatal conductance ($l_s$, %), mesophyll diffusion ($l_m$, %), and biochemical capacity ($l_b$, %) were calculated following the quantitative limitation analysis of Grassi and Magnani [51] as applied in Tomás et al [52], Peguero-Pina et al. [53, 54] and Nha et al. [55]. Different fractional limitations, $l_s$, $l_m$, and $l_b$ ($l_s + l_m + l_b = 1$) were calculated as:

$$l_s = \frac{g_{\text{tot}}/g_s \times \partial A_n}/g_{\text{tot}}/\partial C_c$$  

(4)

$$l_m = \frac{g_{\text{tot}}/g_m \times \partial A_n}/g_{\text{tot}}/\partial C_c$$  

(5)

$$l_b = \frac{g_{\text{tot}}}{g_{\text{tot}} + \partial A_n}/\partial C_c$$  

(6)

Where $g_s$ and $g_m$ were used in light-saturated and atmospheric CO$_2$ concentration was 380 μmol mol$^{-1}$, and $g_m$ was the mean value of three methods. The $g_{\text{tot}}$ is the total conductance to CO$_2$ from ambient air to chloroplasts (the sum of the inverse CO$_2$ serial conductances $g_s$ and $g_m$). The $\partial A_n/\partial C_c$ was calculated as the slope of $A_n$-$C_c$ response curves over a $C_c$ range of 50–100 μmol mol$^{-1}$ [53, 54].
**Determination of additional leaf traits**

Leaf samples used for gas exchange measurements and leaves which size was similar to leaves used for determine photosynthesis was taken. Leaf areas were measured with a scanner (Perfection v700 Photo, Epson, Nagano-ken, Japan). Leaf dry weights were measured using an analytic balance after being oven-dried at 80˚C for 48 h, then leaf mass per area (LMA, g m\(^{-2}\)) was calculated.

Dried leaf samples were ground into a dry flour. Organic carbon (C) concentration was determined by the potassium dichromate-sulfuric acid oxidation method (C\(_{\text{mass}}\), mg g\(^{-1}\)). Nitrogen concentration was determined by a VELP automatic Kjeldahl N determination apparatus (UDK-139, Milano, Italy), and leaf N per mass (N\(_{\text{mass}}\), mg g\(^{-1}\)) and per area (N\(_{\text{area}}\), g m\(^{-2}\)) values were calculated [30]. The PNUE (μmol mol\(^{-1}\) s\(^{-1}\)) was then calculated by the formula:

\[
\text{PNUE} = \frac{A_{\text{max}}}{N_{\text{area}}} \times 14
\]

Where 14 is the atomic mass of nitrogen.

Chlorophylls were extracted by direct immersion: 0.2 g of frozen leaves were cut into small pieces which were 5–10 mg. Leaf pieces were placed into a volumetric flask and 25 mL of 95% (v/v) alcohol was added. The flask was kept in the dark for 24 h. The absorbance of the extracts was measured at 665 nm and 649 nm with a Shimadzu visible-ultraviolet spectrophotometer (UV 2250, Fukuoka, Japan). Cell wall N content was calculated according to Onoda et al. [19]: 1 g of leaves were powdered in liquid N and suspended in sodium phosphate buffer (pH 7.5), the homogenate was centrifuged at 2500 g for 5 min, and the supernatant was discarded. The pellet was washed with 3% (w/v) SDS, amyloglucosidase (35 U ml\(^{-1}\), Rhizopus mold, Sigma, St Louis, MO, USA), and 0.2 M KOH, then heated and centrifuged. The pellet was then washed with distilled water and ethanol, and oven dried (75˚C) for 2 days. Nitrogen in the final pellet was determined using an automatic Kjeldahl apparatus (VELP Scientifica, Usmate, Italy). The fraction of leaf N allocated to cell walls (P\(_{\text{CW}}\)) represents the ratio of cell wall N content to the total N content.

**Calculation of N allocation in the photosynthetic apparatus**

The fraction of leaf N allocated to Rubisco (P\(_R\)), bioenergetics (P\(_B\)), and the light-harvesting components (P\(_L\)) (g g\(^{-1}\)) were calculated from \(V_{\text{cmax}}\), \(J_{\text{max}}\) and chlorophyll contents using the method of Niinemets and Tenhunen [12], which has been widely used in recent years [15, 56–58]:

\[
P_R = \frac{V_{\text{cmax}}}{6.25 \times V_{\text{cr}} \times \text{LMA} \times N_{\text{mass}}} \tag{8}
\]

\[
P_B = \frac{J_{\text{max}}}{8.06 \times J_{\text{mc}} \times \text{LMA} \times N_{\text{mass}}} \tag{9}
\]

\[
P_L = \frac{C_{\text{Chl}}}{C_B \times N_{\text{mass}}} \tag{10}
\]

Where \(C_{\text{Chl}}\) is the chlorophyll concentration (mmol g\(^{-1}\)), \(V_{\text{cr}}\) is the specific activity of Rubisco (μmol CO\(_2\) g\(^{-1}\) Rubisco s\(^{-1}\)), \(J_{\text{mc}}\) is the potential rate of photosynthetic electron transport (μmol electrons μmol\(^{-1}\)Cyt f s\(^{-1}\)), and \(C_B\) is the ratio of leaf chlorophyll to leaf N during light-harvesting (mmol Chl (g N\(^{-1}\)). Where \(V_{\text{cr}}, J_{\text{mc}},\) and \(C_B\) were calculated according to
Ninemets and Tenhunen [12]. The fraction of leaf N allocated to the photosynthetic apparatus (P\textsubscript{NP}) was calculated as the sum of P\textsubscript{R}, P\textsubscript{B}, and P\textsubscript{L}.

**Statistical analysis**

Differences between the seedling leaves were analyzed using one-way analysis of variance (ANOVA), and a post hoc test (Tukey's test) was conducted if the differences were significant. The significance of the correlation between each pair of variables was tested with a Pearson correlation (two-tailed). All analyses were carried out using Statistical Product and Service Solutions 17.0 (SPSS17.0, Chicago, IL, USA).

**Results**

**PNUE in four seedling leaves**

There were significant differences in PNUE between the leaves of the four seedlings (P < 0.001, Table 1). The PNUE in B. alnoides and C. hystrix seedling leaves were higher than those in D. odorifera and E. fordistii, which was mainly attributed to their lower N\textsubscript{area} and N\textsubscript{mass} values. The highest PNUE in B. alnoides (120.54 μmol mol\textsuperscript{-1} s\textsuperscript{-1}) was 2.6 times the lowest, found in E. fordistii (45.92 μmol mol\textsuperscript{-1} s\textsuperscript{-1}). However, N\textsubscript{area} and N\textsubscript{mass} in B. alnoides were 48.75% and 45.21% lower than in E. fordistii, respectively (Table 1). There were no significant differences between B. alnoides, C. hystrix, and D. odorifera seedling leaves in A\textsubscript{max}' and the value in E. fordistii (6.60 μmol m\textsuperscript{-2} s\textsuperscript{-1}) was the smallest (Table 1). The LMA of C. hystrix (100.13 g m\textsuperscript{-2}) was the highest (Table 1). E. fordistii and B. alnoides seedling leaves had higher C\textsubscript{max} than D. odorifera and C. hystrix, but C/N was higher in B. alnoides and C. hystrix seedling leaves than D. odorifera and E. fordistii (Table 1).

**Photosynthetic parameters in four seedling leaves**

Analysis of the quantitative limitations of photosynthesis revealed that photosynthetic capacity was mainly limited by diffusional processes (l\textsubscript{r} and l\textsubscript{m}), whereas biochemical limitations (l\textsubscript{b}) were only between 0.33% and 0.45% of the total for all studied species (Table 2).

Photosynthetic parameters were shown in Table 3 and Table 4. The V\textsubscript{cmax} and J\textsubscript{max} in E. fordistii were higher than the other three species (Table 3) but the statistically significant values

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**Table 1.** Light-saturated photosynthesis (A\textsubscript{max}), leaf N content per area (N\textsubscript{area}), leaf N content per mass (N\textsubscript{mass}), leaf C content per mass (C\textsubscript{mass}), C/N ratio, leaf mass per area (LMA), and photosynthetic-N use efficiency (PNUE) in seedling leaves of four species.

| Leaf traits   | D. alnoides | E. fordistii | B. alnoides | C. hystrix | F     |
|---------------|-------------|-------------|-------------|------------|-------|
| A\textsubscript{max} (µmolm\textsuperscript{-2}s\textsuperscript{-1}) | 8.04±0.46\textsuperscript{ab} | 6.60±0.50\textsuperscript{b} | 8.55±1.60\textsuperscript{a} | 8.16±0.18\textsuperscript{ab} | 3.441\textsuperscript{*} |
| N\textsubscript{area} (g m\textsuperscript{-2}) | 2.19±0.13\textsuperscript{a} | 2.01±0.12\textsuperscript{a} | 1.03±0.25\textsuperscript{b} | 1.02±0.06\textsuperscript{b} | 36.314\textsuperscript{***} |
| N\textsubscript{mass} (mg g\textsuperscript{-1}) | 31.70±0.76\textsuperscript{a} | 28.09±1.49\textsuperscript{b} | 15.36±1.04\textsuperscript{b} | 10.22±0.18\textsuperscript{c} | 106.219\textsuperscript{***} |
| C\textsubscript{mass} (mg g\textsuperscript{-1}) | 449.50±8.66\textsuperscript{b} | 516.65±13.98\textsuperscript{a} | 493.63±5.40\textsuperscript{a} | 479.65±4.66\textsuperscript{b} | 9.713\textsuperscript{**} |
| C/N (g g\textsuperscript{-1}) | 14.24±0.48\textsuperscript{a} | 18.70±1.05\textsuperscript{a} | 32.98±2.15\textsuperscript{b} | 47.02±1.09\textsuperscript{a} | 123.492\textsuperscript{***} |
| LMA (g m\textsuperscript{-2}) | 68.97±3.90\textsuperscript{b} | 71.35±0.89\textsuperscript{b} | 67.60±5.45\textsuperscript{b} | 100.13±2.60\textsuperscript{a} | 18.272\textsuperscript{**} |
| PNUE (µmolmol\textsuperscript{-1}s\textsuperscript{-1}) | 52.64±3.78\textsuperscript{b} | 45.92±2.24\textsuperscript{b} | 120.54±5.18\textsuperscript{a} | 112.01±4.62\textsuperscript{a} | 30.833\textsuperscript{***} |

Mean values (± SD) were shown (n = 7).
Different letters indicated significant differences between species (Tukey’s test, P < 0.05).
Statistically significant F-ratios were denoted by
*P < 0.05
**P < 0.01
***P < 0.001.

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Different letters indicated significant differences between species (Tukey's test, F-ratios) were lower than PNUE (Table 1). Stomatal conductance ($g_m$, 0.100 mol CO$_2$ m$^{-2}$ s$^{-1}$) and $C_i$ (292.88 µmol mol$^{-1}$) in B. alnoides seedling leaves were higher than the other three species (Table 4). Moreover, $g_{m-Harley}$ in B. alnoides (0.136 mol CO$_2$ m$^{-2}$ s$^{-1}$) was higher than the other three species but $g_{m-Either}$ (0.140 mol CO$_2$ m$^{-2}$ s$^{-1}$) and $g_{m-Gu}$ (0.160 mol CO$_2$ m$^{-2}$ s$^{-1}$) was highest in D. odorifera (Table 4). The $C_c$ in B. alnoides seedling leaves (all three methods) was higher than the other three species (Table 4).

**Leaf N allocation in four species seedling leaves**

There were significant differences in leaf N allocation between the four species ($P < 0.001$, Table 5). The $P_p$ was higher than $P_{CW}$ in four species seedling leaves (Table 5). The $P_p$ was 3.9 times of the $P_{CW}$ in D. odorifera, 5.4 times in E. fordii, 2.0 times in B. alnoides and 1.6 times in C. hystrix. Where $P_R > P_L > P_B$ in D. odorifera, E. fordii, and B. alnoides seedling leaves, and $P_R > P_L = P_B$ in C. hystrix seedling leaves.

The $P_p$ in B. alnoides and C. hystrix seedling leaves (both were 0.44 g g$^{-1}$) were higher than D. odorifera and E. fordii (both were 0.27 g g$^{-1}$). The $P_R$ and $P_B$ in B. alnoides and C. hystrix seedling leaves were also higher than in D. odorifera and E. fordii. The $P_L$ in B. alnoides was the highest (0.12 g g$^{-1}$), followed by D. odorifera (0.10 g g$^{-1}$), C. hystrix (0.07 g g$^{-1}$), and E. fordii (0.06 g g$^{-1}$).

**Relationship between PNUE and affecting factors**

There was a positive relationship between $g_m$ and PNUE ($P < 0.05$), in D. odorifera, E. fordii, and B. alnoides, but not in C. hystrix (Fig 1). Both $P_p$, $P_R$, and $P_B$ had a significant positive correlation with PNUE in these species ($P < 0.001$) (Fig 2).

**Table 2. Relative stomatal ($l_s$), mesophyll ($l_m$) and biochemical ($l_b$) photosynthesis limitations in four species seedling leaves.**

| Leaf traits | D. odorifera | E. fordii | B. alnoides | C. hystrix | F |
|------------|-------------|-----------|-------------|-----------|---|
| $l_s$ (%)  | 68.09±1.35a | 58.23±1.93b | 56.84±1.67b | 56.68±2.22b | 9.001*** |
| $l_m$ (%)  | 31.54±1.35b | 41.39±1.95a | 42.82±1.64a | 42.87±2.22a | 8.957*** |
| $l_b$ (%)  | 0.36±0.05  | 0.37±0.07  | 0.33±0.04  | 0.45±0.03  | 0.964 |

Mean values (± SD) were shown (n = 7).

Different letters indicated significant differences between species (Tukey's test, $P < 0.05$).

Statistically significant F-ratios were denoted by

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

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**Table 3. Maximum carboxylation rate ($V_{max}$) and maximum electron transport rate ($I_{max}$) in four species seedling leaves.**

| Leaf traits | D. odorifera | E. fordii | B. alnoides | C. hystrix | F |
|------------|-------------|-----------|-------------|-----------|---|
| $V_{max}$ (µmol m$^{-2}$ s$^{-1}$) | 78.13±4.59b | 99.83±9.37a | 72.98±1.51b | 82.78±1.47ab | 4.786** |
| $I_{max}$ (µmol m$^{-2}$s$^{-1}$) | 100.71±5.80bc | 128.76±11.20a | 98.38±5.37bc | 109.27±4.05ab | 3.822* |

Mean values (± SD) were shown (n = 7).

Different letters indicated significant differences between species (Tukey's test, $P < 0.05$).

Statistically significant F-ratios were denoted by

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

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Table 4. Stomatal conductance (g_s), mesophyll conductance (g_mm), intercellular CO₂ concentration (C_i), and CO₂ concentration at carboxylation site (C_c) in four species seedling leaves.

| Leaf traits | D. odorifera | E. fordii | B. alnoides | C. hystrix | F |
|-------------|--------------|-----------|-------------|------------|---|
| g_s (mol CO₂ m⁻² s⁻¹) | 0.067±0.004bc | 0.046±0.002c | 0.100±0.013a | 0.074±0.004b | 11.106*** |
| C_i (μmol mol⁻¹) | 25.54±6.44 | 235.61±6.19 | 292.88±5.94 | 256.78±5.24 | 7.348** |
| E_m-Harley (mol CO₂ m⁻² s⁻¹) | 0.11±0.013ab | 0.068±0.007b | 0.136±0.013a | 0.109±0.006b | 15.391*** |
| E_m-Ethier (mol CO₂ m⁻² s⁻¹) | 0.14±0.01a | 0.06±0.01c | 0.13±0.01ab | 0.099±0.01bc | 14.772*** |
| E_m-Gn (mol CO₂ m⁻² s⁻¹) | 0.16±0.01a | 0.06±0.01b | 0.13±0.02a | 0.09±0.01b | 19.390*** |
| C_c-Harley (μmol mol⁻¹) | 178.39±7.84a | 136.80±5.18a | 228.78±8.44a | 172.17±6.10b | 29.182*** |
| C_c-Ethier (μmol mol⁻¹) | 192.99±7.11b | 133.56±7.95c | 224.91±9.13a | 173.93±3.73b | 27.639*** |
| C_c-Gn (μmol mol⁻¹) | 200.92±6.72c | 127.42±9.56b | 225.58±12.27a | 157.86±9.56b | 20.268*** |

Data of CO₂ conductance was measured in light-saturated and atmospheric CO₂ concentration was 380 μmol mol⁻¹. Mean values (± SD) were shown (n = 7).

Different letters indicated significant differences between species (Tukey’s test, P < 0.05).

Statistically significant F-ratios were denoted by

* P < 0.05
** P < 0.01
*** P < 0.001.

The relationship between Pₖ and Pₐ in B. alnoides (P = 0.022) and C. hystrix (P = 0.011) seedling leaves were more significant than in D. odorifera (P = 0.409) and E. fordii (P = 0.637). Regression analysis of Pₖ with Pₐ in B. alnoides seedling leaves was within the shaded zone; C. hystrix was on the shaded zone; D. odorifera and E. fordii were under the shaded zone (Fig 3).

Discussion

The range of PNUE in these tree seedlings was 45.92–120.54 μmol mol⁻¹ s⁻¹ (Table 1) which was close to six Fagus sylvatica populations (68.74–122.22 μmol mol⁻¹ s⁻¹) [59] and four Quercus species (approximately 60–150 μmol mol⁻¹ s⁻¹) [20]; lower than P. cathayana (171.64–213.36 μmol mol⁻¹ s⁻¹) [17] and S. alterniflora (171.64–213.36 μmol mol⁻¹ s⁻¹) [15] under different N deposition. Wright et al. summed up PNUE in 710 species and the range was between 10 and 500 μmol mol⁻¹ s⁻¹ [60]; therefore, our results seem reasonable. Shrubs and trees

Table 5. Fraction of leaf N allocated to rubisco (P_R), bioenergetics (P_B), light-harvesting components (P_L), photosynthetic apparatus (P_P), cell wall (P_CW), and other parts (1-P_R-P_CW, P_Other) in four species seedling leaves.

| Leaf traits | D. odorifera | E. fordii | B. alnoides | C. hystrix | F |
|-------------|--------------|-----------|-------------|------------|---|
| P_R (g g⁻¹) | 0.13±0.01b | 0.16±0.01b | 0.26±0.03a | 0.30±0.01a | 22.130*** |
| P_B (g g⁻¹) | 0.03±0.002b | 0.04±0.003b | 0.07±0.007a | 0.07±0.003a | 18.111*** |
| P_L (g g⁻¹) | 0.10±0.01ab | 0.06±0.01c | 0.12±0.01a | 0.07±0.01b | 8.488*** |
| P_P (g g⁻¹) | 0.27±0.02b | 0.27±0.02b | 0.44±0.04b | 0.44±0.02a | 14.796*** |
| P_CW (g g⁻¹) | 0.07±0.004c | 0.05±0.002c | 0.22±0.010b | 0.27±0.011a | 182.914*** |
| P_Other (g g⁻¹) | 0.66±0.02a | 0.68±0.02a | 0.34±0.04b | 0.29±0.02b | 63.830*** |

Mean values (± SD) were shown (n = 7).

Different letters indicated significant differences between species (Tukey’s test, P < 0.05).

Statistically significant F-ratios were denoted by

* P < 0.05
** P < 0.01
*** P < 0.001.

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usually have a low PNUE and grasses usually have high value [60]. Fast growing herbaceous species may have a PNUE higher than 200 μmol mol⁻¹ s⁻¹, whereas values for evergreen woody species can be lower than 50 [1]. Our values are within the medium range.

The overall result highlights a substantial difference between N-fixing and non-N-fixing tree seedling leaves in PNUE (Table 1). The variation of PNUE may be attributable to plant evolution and natural selection [61]. Low PNUE species compensate for their low productivity with a long leaf life-span [20]; stress-tolerant species [62] and late successional species [63] usually have low PNUE values. Therefore, low PNUE in *D. odorifera* and *E. fordii* may lead to high stress-tolerance traits and increase competitiveness in poor soil [64]. Higher PNUE species such as *B. alnoides* and *C. hystrix* could grow faster [20] and have a stronger competitive ability in ecosystems with fertile soil [65]. The PNUE tended to be lower for species at the ‘slow-return’ end of the leaf economics spectrum [60], and according to the ‘leaf economics spectrum’, at the slow-return end are species with long leaf life-span, expensive high-LMA leaf construction, low nutrient concentrations, and low rates of photosynthesis and respiration [4], and therefore, it can be concluded that two N-fixing species were at the ‘slow-return’ end of the leaf economics [4, 60]. Because these species live in the same area, we believe that mix these non-N-fixing and N-fixing trees for afforestation is useful for improving soil N utilization efficiency in this place.
As PNUE is the ratio of $A_{\text{max}}$ and $N_{\text{area}}$, changes of $A_{\text{max}}$ and $N_{\text{area}}$ affect PNUE. We found significant $F$-ratios in $A_{\text{max}}$ between the four species’ seedling leaves was 3.441, lower than in $N_{\text{area}}$ which was 36.314. Therefore, a change of $N_{\text{area}}$ was the main reason affecting PNUE in these four species. We suspect that N-fixing species which could gain N from air by legume bacteria [7–9], may have both higher $N_{\text{area}}$ and $A_{\text{max}}$, but our results did not support this speculation. Which reason limited $A_{\text{max}}$ in two N-fixing species? firstly, relative stomatal ($l_s$), and mesophyll ($l_m$) were main reasons limited photosynthesis ability in these trees (Table 2), secondly, two N-fixing species didn’t show significant higher $g_s$, $g_m$, $C_c$, $V_{\text{cmax}}$, or $J_{\text{max}}$ than non-N-fixing species (Tables 3 and 4). Therefore, we believe that a large proportion of N in the leaves of N-fixing plants did not used for photosynthesis.

N-fixing trees $D$. odorifera and $E$. fordii had significantly higher $N_{\text{area}}$ than $B$. alnoides and $C$. hystrix (Table 1). Because $N_{\text{area}} = N_{\text{mass}} \times \text{LMA}$, $N_{\text{area}}$ may also be affected by LMA besides N content $N_{\text{mass}}$. The difference of LMA between species was far lower than the difference of $N_{\text{mass}}$ (Table 1). Therefore, the significantly higher $N_{\text{mass}}$ caused the significantly higher $N_{\text{area}}$ in $D$. odorifera and $E$. fordii. The low C/N ratio also showed high N in $D$. odorifera and $E$. fordii (Table 1). These results agreed with earlier studies [10, 11] and our study in five Fagaceae and five Leguminosae tree species [30]. However, one study reported that N-fixing trees had both

Fig 2. Regression analysis of the fraction of leaf N allocated to (a) Rubisco ($P_R$), (b) bioenergetics ($P_B$), (c) light-harvesting components ($P_L$), and (d) the photosynthetic apparatus ($P_P$) with photosynthetic-N use efficiency (PNUE) in four species seedling leaves. The determination coefficient ($R^2$) and $P$-value were also shown. Only one line was fitted for four species, because there was no significant difference ($P > 0.05$) according to the result of a one-way ANCOVA with PNUE as a dependent variable, tree species as fixed factors, and $P_R$, $P_B$, $P_L$ or $P_P$ as a covariate.

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higher $N_{\text{area}}$ and $A_{\text{max}}$\cite{66}. The relationship of $N_{\text{area}}$ and $A_{\text{max}}$ varies in different species \cite{60}, because different species have their own N allocation patterns. The N allocation in photosynthesis was more important than the total leaf N for photosynthesis \cite{67}.

Lower $P_{\text{P}}$, $P_{\text{R}}$, and $P_{\text{B}}$ were main reasons that led to lower PNUE in N-fixing tree species ($D.\ odorifera$ and $E.\ fordii$). These results agreed with previous studies \cite{10, 11, 51} and our study on five Fagaceae tree species and five Leguminosae big tree species \cite{30}. Rubisco catalyzes the limiting step for photosynthetic capacity \cite{14}. A positive correlation between $A_{\text{max}}$ and Rubisco has been frequently reported \cite{16, 19}. An improved fraction of leaf N allocated to Rubisco could maximize the use of leaf N in photosynthesis. It should be noted that although there was a significant difference in N allocation proportion between N-fixing trees and non-N-fixing trees, there were smaller differences in N allocation quantity in Rubisco, bioenergetics, photosynthetic apparatus, cell wall, and other parts in the four species seedling leaves (mass and area, see S2 Table). The $N_{\text{mass}}$ largely affected the N allocation to the photosynthetic apparatus and $P_{\text{CW}}$. 

Fig 3. Regression analysis of the fraction of leaf N allocated to the cell wall ($P_{\text{CW}}$) with leaf N allocated to Rubisco ($P_{\text{R}}$) in four species seedling leaves. The determination coefficient ($R^2$) and $P$-value were also shown. The shaded zone was drawn according to this hypothesis: when $P_{\text{CW}}$ was 0.300 g g$^{-1}$, the rest (0.700 g g$^{-1}$) were soluble and thylakoid protein, Rubisco represented one quarter to one-third of the N in soluble and thylakoid protein, $P_{\text{R}}$ valued 0.175–0.233 g g$^{-1}$ (right side of shaded zone). When $P_{\text{CW}}$ was valued 0.000 g g$^{-1}$ in limiting case (does not exist in reality), all the rest (1.000 g g$^{-1}$) were soluble and thylakoid protein, $P_{\text{R}}$ valued 0.250–0.333 g g$^{-1}$ (left side of shaded zone). For more information see Harrison et al. \cite{21}. The lines fitted separately for four species were significantly different ($P < 0.05$) according to the result of a one-way ANCOVA with $P_{\text{R}}$ as a dependent variable, tree species as fixed factors, and $P_{\text{CW}}$ as a covariate.

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The $g_m$ could also influence the variation in PNUE through N allocation [25]. There was a significant positive relationship between $g_m$ and PNUE in *D. odorifera*, *E. fordii*, and *B. alnoides*, but the effect of $g_m$ to PNUE was not consistent between species (Fig 1). Broeckx et al. [28] also found this relationship in six poplar (*Populus*) genotypes, and Nha et al. [55] found $g_m$ does not contribute to greater PNUE in temperate forest. We also found $g_m$ of ten Fabaceae and Leguminosae species big trees was not significantly related to the PNUE. The effect of $g_m$ on PNUE may also age-related.

A significant negative correlation between $P_{CW}$ and $P_R$ in *B. alnoides* and *C. hystrix* ($P < 0.05$) suggested a trade-off between N allocation to Rubisco and cell walls, whereas no trade-off was detected in *D. odorifera* and *E. fordii* (Fig 3). A similar trade-off was found in *Polygonum cuspidatum* [19], *Quercus* species [20], *Mikania micrantha* and *Chromolaena odorata* [37]; but this relationship does not exist in some other trees [16]. Some researchers believed that the main influencing factors were whether leaf N could meet the needs of both cell wall N and Rubisco N [15, 21]. We used the method described by Harrison et al. [21] to determine whether leaf N could meet these two needs: the regression analysis of $P_{CW}$ with $P_R$ in *B. alnoides* seedling leaves was within the shaded zone (the shaded zone represents the distribution area of $P_{CW}$ and $P_R$ when a trade-off exists), *C. hystrix* was on the shaded zone which means that *B. alnoides* and *C. hystrix* had high $P_{CW}$ and $P_R$ and therefore leaf N could not meet both needs, these two factors may affect each other. We believe the high $P_{Other}$ (Table 5, possibly composed of free amino acids [68] and inorganic N (NO$_3^-$, NH$_4^+$) [69]) weakens the correlation between Rubisco and cell wall N. It must be noted that *C. hystrix* showed a unique relationship between $P_{CW}$ and $P_R$ (on the shaded zone), which means higher $P_{CW}$ and $P_R$ than the results of Harrison et al. [21]. More trees need to be studied to determine the distribution area of $P_{CW}$ and $P_R$ when a trade-off exists.

Excessive storage of N in N-fixing tree species may reduce their PNUE but may be useful for future physiological processes such as reproduction [17]. Storage of N could buffer changes in other N pools such as cell wall N [19, 20, 37] (Fig 3). Evergreen tree leaves with low PNUE have multiple roles in nutrient conservation, nutrient storage, stress tolerance, herbivore deterrence, and photosynthesis [3]. We should consider that some Rubisco can also function as N storage and may not be involve in photosynthesis [70, 71]. This type of Rubisco might lead to greater rates of photosynthesis under suboptimal conditions [3]. Therefore, Rubisco N calculated by the model of Farquhar et al. [14] might be N in activated Rubisco. Using chemical methods to extract and determine Rubisco N content could be useful [20, 72].

We used to do experiment with *C. hystrix* big trees, and its PNUE was 74.34±8.54 μmol mol$^{-1}$ s$^{-1}$ [30], smaller than its seedlings (Table 1). *C. hystrix* big tree also had higher $P_{CW}$ (0.46 g g$^{-1}$) than seedlings, but its $P_P$ (0.26 g g$^{-1}$), $P_R$ (0.20 g g$^{-1}$), $P_{h}$ (0.041 g g$^{-1}$) and $P_{l}$ (0.014 g g$^{-1}$) [30] were lower than seedlings (Table 1). Seedlings with high PNUE could grow fast, reach the canopy earlier and increased ability of competition for light [5]. Big trees with higher $P_{CW}$ could better resist the environmental stress in canopy, such as typhoon, insect attack and diseases [19]. We suspect that trade-offs for N allocation to photosynthesis versus cell walls may also exist at different stages of a tree’s growth, in order to meet the N demand in different growth stages.

Although both the *B. alnoides* and *C. hystrix* are non-N-fixing broadleaf plants, and have some similar functional traits, there were significant differences showed in $N_{mass}$, LMA, $g_m$, $C_v$, and $P_{CW}$ (Tables 1, 4 and 5). *B. alnoides* is a deciduous broad-leaved plant and *C. hystrix* is an evergreen broad-leaf plant. In order to contribute to a longer leaf life span, evergreen broad-leaf plants should improve leaf tolerance to environmental disturbance [73,74], reflected in higher LMA [60], and $P_{CW}$ [16]. Higher defensive investment could also reduce $N_{mass}$ [16]. Simultaneously, if higher LMA is a result of mesophyll cell wall thickening, it will reduce $g_m$. 

## Table 1.

| Species        | $P_{CW}$ (g g$^{-1}$) | $P_R$ (g g$^{-1}$) | $P_{h}$ (g g$^{-1}$) | $P_{l}$ (g g$^{-1}$) | $N_{mass}$ (g g$^{-1}$) | LMA (g cm$^{-2}$) | $g_m$ (mol m$^{-2}$ s$^{-1}$) | $C_v$ (%) | $P_{Leaf}$ (g g$^{-1}$) |
|----------------|-----------------------|-------------------|----------------------|----------------------|------------------------|-------------------|-----------------------------|----------|-------------------------|
and $C_c$ [75, 76], and variations in LMA are often inversely correlated with $g_m$ and $C_c$ [77, 78], consistent with the results of those two species.

Conclusions
This study indicated that PNUE was significantly lower in two N-fixing trees ($D. odorifera$ and $E. fordii$) than that in two non-N-fixing trees ($B. alnoides$ and $C. hystrix$). This finding was mainly attributed to lower $P_R$ and $P_B$. $B. alnoides$ and $C. hystrix$ optimized their leaf N allocation to photosynthesis. Although $g_m$ had a significant positive correlation with PNUE in $D. odorifera$, $E. fordii$, and $B. alnoides$, the effect of $g_m$ on PNUE was different between species. $P_{CW}$ had a significant negative correlation with $P_R$ in $B. alnoides$ and $C. hystrix$ seedling leaves, but there was no significant correlation between $P_{CW}$ and $P_R$ in $D. odorifera$ and $E. fordii$ seedling leaves, which may indicate that $B. alnoides$ and $C. hystrix$ seedling leaves did not have enough N to satisfy the demand from both the cell wall and Rubisco. $B. alnoides$ and $C. hystrix$ with higher PNUE may have a higher competitive ability in natural ecosystems with fertile soil. Our results indicate that mixing these non-N-fixing and N-fixing trees for afforestation is useful for improving soil N utilization efficiency in the tropical forests.

Supporting information
S1 Table. Chlorophyll contents (chlorophyll a, chlorophyll b, chlorophyll a+b and Chla/b) in four species seedling leaves. Mean values (± SD) were shown (n = 7). Different letters indicated significant differences between species (Tukey’s test, $P<0.05$). Statistically significant $F$-ratios were denoted by $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

S2 Table. Quantity of leaf N (per area and per mass) allocated to Rubisco ($Q_{Rarea}$, $Q_{Rmass}$), bioenergetics ($Q_{Barea}$, $Q_{Bmass}$), light-harvesting components ($Q_{Larea}$, $Q_{Lmass}$), photosynthetic apparatus ($Q_{Parea}$, $Q_{Pmass}$), cell wall ($Q_{CWarea}$, $Q_{CWmass}$), and other parts ($Q_{Other-area}$, $Q_{Other-mass}$) in four species seedling leaves. Mean values (± SD) were shown (n = 7). Different letters indicated significant differences between species (Tukey’s test, $P<0.05$). Statistically significant $F$-ratios were denoted by $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

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