Seedling leaves allocate lower fractions of nitrogen to photosynthetic apparatus in nitrogen fixing trees (*Dalbergia odorifera* and *Erythrophleum fordii*) than in non-nitrogen fixing trees (*Betula alnoides* and *Castanopsis hystrix*) in subtropical China

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Abstract

Photosynthetic-nitrogen use efficiency (PNUE) is a useful trait to characterize leaf economics, physiology, and strategy. 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). Seedlings of the four species were cultured in pots and received the same nutrient solution, water volume, and light. LiCor-6400 was used to determine fluorescence yield, photosynthetic response to light, and intercellular CO₂ concentration (C_i). N allocation fractions in the photosynthetic apparatus were calculated according to Niinemets and Tenhunen method; g_m was calculated according to variable J , EDO, and $A-C_i$ curve fitting methods. PNUE of *D. odorifera* and *E. fordii* were significantly lower than those of *B. alnoides* and *C. hystrix* 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*. The fraction of leaf N to cell wall (P_{CW}) had a significant negative correlation with P_R in *B. alnoides* and *C. hystrix*. We conclude that *B. alnoides* and *C. hystrix* optimized their leaf N allocation toward photosynthesis, with the trade-off being N allocation to the cell wall and Rubisco. Thus, these two species may have a higher competitive ability in natural ecosystems with fertile soil.

Introduction

Nitrogen (N) is a major constituent of proteins, nucleic acids, amino acids, and chlorophylls [1,2] and is important for plant survival and growth. In leaves, N is mainly used for photosynthesis [3], and therefore, there is a clear relationship between leaf N contents and photosynthetic capacity. Many researchers use photosynthetic-N use efficiency (PNUE, the ratio of light-saturated net CO₂ assimilation rate ($A_{\max}$) to leaf N content per area (N_{area}) [4]) to show how efficiently N resources are used during photosynthesis, and studies have been conducted on a variety of species [3,5,6].

N involved in photosynthesis has always been an important factor that influences PNUE [7]. Rubisco is the most abundant enzyme in C₃ plants [8], and it is the key factor in carbon assimilation [9]. Many researchers have found a positive correlation between leaf N fraction in Rubisco (P_R) and PNUE in various plants [10-12]. Apart from photosynthetic machinery, N in leaves is also involved in other machineries, such as structure, storage, and respiration [13]. Cell walls contain proteins that have functions in plant defense, growth, development, signaling, intercellular communication, environmental sensing, and as selective exchange interfaces [14, 15]. Trade-offs may occur between N allocation to cell walls, Rubisco (when N is needed to maintain cellular functions), seed development, and immune response [15-17]. However, some studies have shown that these trade-offs only exist in individuals of the same species [12] or species lacking N in leaves [17, 18].

CO₂ is an important raw material for photosynthesis [19], 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 [20]. A significant negative correlation between C_i (intercellular CO₂ concentration)- C_c (CO₂ concentration at carboxylation site) and PNUE was found in *Populus cathayana* [21]. N is also involved in carbonic anhydrases and aquaporins [22]. These proteins play a role in mesophyll conductance (g_m) by changing the nature of the diffusing molecule [23] and facilitating CO₂ diffusion through membranes [24].

Therefore, PNUE may be influenced by g_m [22]. A significant positive correlation was found between mesophyll conductance (g_m) and PNUE in six *Populus* genotypes [25].

Species with a high N_{area} usually have a high A_{max}' according to the worldwide leaf economic spectrum [4]. However, there are some differences in N-fixing plants. N-fixing species could convert N from the air through legume bacteria, and this could meet more than half of the plant's N demand. Several studies have shown that N-fixing species always have enough N in leaves [26-28]. One study showed that N-fixing trees had higher A_{max}' and N_{area} , and there was no significant difference in PNUE or the proportion of leaf N allocated to Rubisco (P_R) between N-fixing and non-N-fixing trees [29]. However, other studies have shown that N-fixing trees had lower A_{max}' and higher N_{area} , which resulted in a lower PNUE [30, 31]. These contradicting results may imply that some N-fixing species use a different strategy to utilize N compared to non-N-fixing species. One possible explanation is that the percentage of N in the photosynthetic apparatus is lower in the N-fixing trees [30, 31]. However, these studies neglect that g_m and P_{CW} could also influence PNUE [15, 16, 32]. We studied the factors that affect PNUE in both N-fixing and non-N-fixing large trees and found P_R and P_B to be the main factors; the effects of g_m and P_{CW} were relatively small [33], 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 [34-37]. *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 and photosynthetic capacity, leaf N allocation, and diffusional conductances to CO_2 , and 3) determine the relationship between N in Rubisco and the cell wall, and the relationship between PNUE and g_m 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 [38,39].

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 LiCor-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 (*C_i*, μmol mol⁻¹) were determined for each leaf (seven repetitions in each species) according to Broeckx *et al.* 2014 [25] and Zhang *et al.* 2016 [40]. 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 (A_n , $\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s , $\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$), and C_i of each sampled leaf were recorded ten times after 200 s under each PPFD and CO_2 concentration. Then light-saturated net CO_2 assimilation rate ($A_{\max}'$, $\mu\text{mol m}^{-2} \text{s}^{-1}$), light-saturated day respiration rate (R_d , $\mu\text{mol m}^{-2} \text{s}^{-1}$) and light- and CO_2 -saturated net CO_2 assimilation rate ($A_{\max}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$) were measured or calculated. For further details see Tang *et al.* [33].

Determination of chlorophyll fluorescence and mesophyll conductance

Fluorescence yield was measured with a LiCor-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 CO_2 concentration was set to 380 $\mu\text{mol mol}^{-1}$. PPFD was set to light saturation point. Constant values of fluorescence yield ($\Delta F/F_m'$) of each leaf sample were recorded 10 times after 200 s [41]. We used Loreto *et al.* [42] methods to calculate the photosynthetic electron transport rate (J_f , $\mu\text{mol m}^{-2} \text{s}^{-1}$):

$$J_f = PPFD \times \frac{\Delta F}{F_m'} \times Leafreflu \times PARDistPhotosys.$$

Leafreflu (leaf absorptance valued) and *PARDistPhotosys* (the fraction of quanta absorbed by photosystem II) were 0.85 [43] and 0.5 [42], respectively. We used the variable J method described by Harley *et al.* [44], which has been used in recent years [45–48] to calculate mesophyll conductance (g_m , $\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$):

$$g_m = \frac{A_{\max}'}{C_i - \left\{ \frac{I^* [I_f + 8(A_{\max}' + R_d)]}{J_f - 4(A_{\max}' + R_d)} \right\}},$$

R_d , C_i , and $A_{\max}'$ were determined from gas exchange measurements. The CO_2 photo compensation point (I^* , $\mu\text{mol mol}^{-1}$) value was 54.76 at 30°C according to Bernacchi *et al.* [49].

Because the Harley method should calibrate the ETR using Chl fluorescence and gas exchange under low O_2 , we used the experience value instead (*Leafreflu* = 0.85) [33]. We also used Ethier and Livingston [50] and the exhaustive dual optimization (EDO) method [51] to calculate g_m . We used software based on the Ethier and

Livingston method developed by Sharkey *et al.* [52] 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_{cmax} and J_{max}

The mean value of g_m calculated from three methods was used to calculate CO_2 concentration in chloroplasts (C_c , $\mu\text{mol mol}^{-1}$):

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

Then C_c was used to fit an A_n-C_c curve, followed by the maximum carboxylation rate (V_{cmax} , $\mu\text{mol m}^{-2} \text{s}^{-1}$) calculated according to Farquhar *et al.* [9], and the maximum electron transport rate (J_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$) calculated according to Loustau *et al.* [53]. The fitting model used *in vivo* Rubisco kinetics parameters (K_o , K_c , and their activation energy) measured by Niinemets and Tenhunen [7].

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 [54] as applied in Tomás *et al* [55], Peguero-Pina *et al.* [56, 57] and Nha *et al.* [58]. Different fractional limitations, l_s , l_m , and l_b ($l_s + l_m + l_b = 1$) were calculated as:

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

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

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

Where g_s and g_m were used in light-saturated and atmospheric CO_2 concentration was $380 \mu\text{mol mol}^{-1}$, and g_m was the mean value of three methods. g_{tot} is the total conductance to CO_2 from ambient air to chloroplasts (the

sum of the inverse CO₂ serial conductances g_s and g_m). $\partial A_n / \partial C_c$ was calculated as the slope of $A_n - C_c$ response curves over a C_c range of 50–100 $\mu\text{mol mol}^{-1}$ [56, 57].

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_{mass} mg g^{-1}). N concentration was determined by a VELP automatic Kjeldahl N determination apparatus (UDK-139, Milano, Italy), and leaf N per mass (N_{mass} , mg g^{-1}) and per area (N_{area} g m^{-2}) values were calculated [33]. PNUE ($\mu\text{mol} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$) 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.* [15]: 1 g of leaves were powdered in liquid nitrogen 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,

165 and oven dried (75°C) for 2 days. N in the final pellet was determined using an automatic Kjeldahl apparatus
166 (VELP Scientifica, Usmate, Italy). The fraction of leaf N allocated to cell walls (P_{CW}) represents the ratio of cell
167 wall N content to the total N content.

168 Calculation of N Allocation in the Photosynthetic Apparatus

169 The fraction of leaf N allocated to Rubisco (P_R), bioenergetics (P_B), and the light-harvesting components (P_L)
170 (g g^{-1}) were calculated from V_{cmax} , J_{max} and chlorophyll contents using the method of Niinemets and Tenhunen [7],
171 which has been widely used in recent years [10, 59-61]:

$$172 \quad P_R = \frac{V_{\text{cmax}}}{6.25 \times V_{\text{cr}} \times \text{LMA} \times N_{\text{mass}}},$$

$$173 \quad P_B = \frac{J_{\text{max}}}{8.06 \times J_{\text{mc}} \times \text{LMA} \times N_{\text{mass}}},$$

$$174 \quad P_L = \frac{C_{\text{Chl}}}{C_B \times N_{\text{mass}}},$$

175 where C_{Chl} is the chlorophyll concentration (mmol g^{-1}), V_{cr} is the specific activity of Rubisco ($\mu\text{mol CO}_2 \text{ g}^{-1}$
176 Rubisco s^{-1}), J_{mc} is the potential rate of photosynthetic electron transport ($\mu\text{mol electrons } \mu\text{mol}^{-1} \text{ Cyt f s}^{-1}$), and C_B
177 is the ratio of leaf chlorophyll to leaf N during light-harvesting ($\text{mmol Chl (g N)}^{-1}$). V_{cr} , J_{mc} , and C_B were
178 calculated according to Niinemets and Tenhunen [7]. The fraction of leaf N allocated to the photosynthetic
179 apparatus (P_P) was calculated as the sum of P_R , P_B , and P_L .

180 Statistical Analysis

181 Differences between the seedling leaves were analyzed using one-way analysis of variance (ANOVA), and a
182 post hoc test (Tukey's test) was conducted if the differences were significant. The significance of the correlation
183 between each pair of variables was tested with a Pearson correlation (two-tailed). All analyses were carried out
184 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. fordii*, which was mainly attributed to their lower N_{area} and N_{mass} values. The highest PNUE in *B. alnoides* ($120.54 \mu\text{mol mol}^{-1} \text{s}^{-1}$) was 2.6 times the lowest, found in *E. fordii* ($45.92 \mu\text{mol mol}^{-1} \text{s}^{-1}$). However, N_{area} and N_{mass} in *B. alnoides* were 48.75% and 45.21% lower than in *E. fordii*, respectively (Table 1). There were no significant differences between *B. alnoides*, *C. hystrix*, and *D. odorifera* seedling leaves in A_{max}' and the value in *E. fordii* ($6.60 \mu\text{mol m}^{-2} \text{s}^{-1}$) was the smallest (Table 1). LMA of *C. hystrix* (100.13 g m^{-2}) was the highest (Table 1). *E. fordii* and *B. alnoides* seedling leaves had higher C_{mass} than *D. odorifera* and *C. hystrix*, but C/N was higher in *B. alnoides* and *C. hystrix* seedling leaves than *D. odorifera* and *E. fordii* (Table 1).

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

Leaf traits	<i>D. odorifera</i>	<i>E. fordii</i>	<i>B. alnoides</i>	<i>C. hystrix</i>	<i>F</i>
A_{max}' ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	$8.04 \pm 0.46^{\text{ab}}$	$6.60 \pm 0.50^{\text{b}}$	$8.55 \pm 1.60^{\text{a}}$	$8.16 \pm 0.18^{\text{ab}}$	3.441*
N_{area} (g m^{-2})	$2.19 \pm 0.13^{\text{a}}$	$2.01 \pm 0.12^{\text{a}}$	$1.03 \pm 0.25^{\text{b}}$	$1.02 \pm 0.06^{\text{b}}$	36.314***
N_{mass} (mg g^{-1})	$31.70 \pm 0.76^{\text{a}}$	$28.09 \pm 1.49^{\text{a}}$	$15.36 \pm 1.04^{\text{b}}$	$10.22 \pm 0.18^{\text{c}}$	106.219***
C_{mass} (mg g^{-1})	$449.50 \pm 8.86^{\text{b}}$	$516.65 \pm 13.98^{\text{a}}$	$493.63 \pm 5.40^{\text{a}}$	$479.65 \pm 4.66^{\text{b}}$	9.713***
C/N (g g^{-1})	$14.24 \pm 0.48^{\text{c}}$	$18.70 \pm 1.05^{\text{c}}$	$32.98 \pm 2.15^{\text{b}}$	$47.02 \pm 1.09^{\text{a}}$	123.492***
LMA (g m^{-2})	$68.97 \pm 3.90^{\text{b}}$	$71.35 \pm 0.89^{\text{b}}$	$67.60 \pm 5.45^{\text{b}}$	$100.13 \pm 2.60^{\text{a}}$	18.272***
PNUE ($\mu\text{mol mol}^{-1} \text{s}^{-1}$)	$52.64 \pm 3.78^{\text{b}}$	$45.92 \pm 2.24^{\text{b}}$	$120.54 \pm 5.18^{\text{a}}$	$112.01 \pm 4.62^{\text{a}}$	30.833***

Mean values ($\pm$ SD) are shown ($n = 7$). Different letters indicate significant differences between species (Tukey's test, $P < 0.05$). Statistically significant F -ratios were denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Photosynthetic Parameters in Four Seedling Leaves

Analysis of the quantitative limitations of photosynthesis revealed that photosynthetic capacity was mainly limited by diffusional processes (I_s and I_m), whereas biochemical limitations (I_b) were only between 0.33% and 0.45% of the total for all studied species (Table 2).

Table 2. Relative stomatal (I_s), mesophyll (I_m) and biochemical (I_b) photosynthesis limitations in four species seedling leaves.

Leaf traits	<i>D. odorifera</i>	<i>E. fordii</i>	<i>B. alnoides</i>	<i>C. hystrix</i>	<i>F</i>
I_s (%)	68.09±1.35 ^a	58.23±1.93 ^b	56.84±1.67 ^b	56.68±2.22 ^b	9.001 ^{***}
I_m (%)	31.54±1.35 ^b	41.39±1.95 ^a	42.82±1.64 ^a	42.87±2.22 ^a	8.957 ^{***}
I_b (%)	0.36±0.05	0.37±0.07	0.33±0.04	0.45±0.03	0.964

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

Photosynthetic parameters are shown in Table 3 and Table 4. The V_{cmax} and J_{max} in *E. fordii* were higher than the other three species (Table 3) but the statistically significant values (F -ratios) were lower than PNUE (Table 1). Stomatal conductance (g_s , 0.100 mol CO₂ 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, $g_{\text{m-Harley}}$ in *B. alnoides* (0.136 mol CO₂ m⁻² s⁻¹) was higher than the other three species but $g_{\text{m-Ethier}}$ (0.140 mol CO₂ m⁻² s⁻¹) and $g_{\text{m-Gu}}$ (0.160 mol CO₂ 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).

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

Leaf traits	<i>D. odorifera</i>	<i>E. fordii</i>	<i>B. alnoides</i>	<i>C. hystrix</i>	<i>F</i>
V_{cmax} (μmol m ⁻² s ⁻¹)	78.13±4.59 ^b	99.83±9.37 ^a	72.98±1.51 ^b	82.78±1.47 ^{ab}	4.786 ^{**}
J_{max} (μmol m ⁻² s ⁻¹)	100.71±5.80 ^{bc}	128.76±11.20 ^a	98.38±5.37 ^{bc}	109.27±4.05 ^{ab}	3.822 [*]

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

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

Leaf traits	<i>D. odorifera</i>	<i>E. fordii</i>	<i>B. alnoides</i>	<i>C. hystrix</i>	<i>F</i>
g_s (mol CO ₂ m ⁻² s ⁻¹)	0.067±0.004 ^{bc}	0.046±0.002 ^c	0.100±0.013 ^a	0.074±0.004 ^b	11.106 ^{***}
C_i (μmol mol ⁻¹)	251.54±6.44 ^b	235.61±6.19 ^b	292.88±5.94 ^a	256.78±5.24 ^b	7.348 ^{**}
$g_{m-Harley}$ (mol CO ₂ m ⁻² s ⁻¹)	0.114±0.013 ^{ab}	0.068±0.007 ^b	0.136±0.013 ^a	0.109±0.006 ^{ab}	15.391 ^{***}
$g_{m-Ethier}$ (mol CO ₂ m ⁻² s ⁻¹)	0.140±0.01 ^a	0.066±0.01 ^c	0.130±0.01 ^{ab}	0.099±0.01 ^{bc}	14.772 ^{***}
g_{m-Gu} (mol CO ₂ m ⁻² s ⁻¹)	0.160±0.01 ^a	0.063±0.01 ^b	0.138±0.02 ^a	0.090±0.01 ^b	19.390 ^{***}
$C_{c-Harley}$ (μmol mol ⁻¹)	178.39±7.84 ^b	136.80±5.18 ^c	228.78±8.44 ^a	172.17±6.10 ^b	29.182 ^{***}
$C_{c-Ethier}$ (μmol mol ⁻¹)	192.99±7.11 ^b	133.56±7.95 ^c	224.91±9.13 ^a	173.93±3.73 ^b	27.639 ^{***}
C_{c-Gu} (μmol mol ⁻¹)	200.92±6.72 ^a	127.42±9.56 ^b	225.58±12.27 ^a	157.86±9.56 ^b	20.268 ^{***}

Data of CO₂ conductance was measured in light-saturated and atmospheric CO₂ concentration was 380 μmol mol⁻¹. Mean values (± SD) are shown (n = 7). Different letters indicate significant differences between species (Tukey's test, $P < 0.05$). Statistically significant F -ratios were denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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). 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*. $P_R > P_L > P_B$ 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).

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 ($1-P_p-P_{CW}$, P_{Other}) in four species seedling leaves.

Leaf traits	<i>D. odorifera</i>	<i>E. fordii</i>	<i>B. alnoides</i>	<i>C. hystrix</i>	<i>F</i>
P_R (g g ⁻¹)	0.13±0.01 ^b	0.16±0.01 ^b	0.26±0.03 ^a	0.30±0.01 ^a	22.130***
P_B (g g ⁻¹)	0.03±0.002 ^b	0.04±0.003 ^b	0.07±0.007 ^a	0.07±0.003 ^a	18.111***
P_L (g g ⁻¹)	0.10±0.01 ^{ab}	0.06±0.01 ^c	0.12±0.01 ^a	0.07±0.01 ^b	8.848***
P_P (g g ⁻¹)	0.27±0.02 ^b	0.27±0.02 ^b	0.44±0.04 ^a	0.44±0.02 ^a	14.796***
P_{CW} (g g ⁻¹)	0.07±0.004 ^c	0.05±0.002 ^c	0.22±0.010 ^b	0.27±0.011 ^a	182.914***
P_{Other} (g g ⁻¹)	0.66±0.02 ^a	0.68±0.02 ^a	0.34±0.04 ^b	0.29±0.02 ^b	63.830***

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

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).

The relationship between P_{CW} and P_R 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 P_{CW} with P_R 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).

Figure 1. Regression analysis of mesophyll conductance (g_m) with photosynthetic-N use efficiency (PNUE) in four species seedling leaves. The determination coefficient (R^2) and P -value are also shown. The lines fitted separately for four species were significantly different ($P < 0.05$) according to the result of a one-way ANCOVA with PNUE as a dependent variable, tree species as fixed factors, and g_m as a covariate.

Figure 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 are also shown. Only one

line is 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.

257

Figure 3. Regression analysis of the fraction of leaf N allocated to the cell wall (P_{CW}) with leaf N allocated to Rubisco (P_R) in four species seedling leaves. The determination coefficient (R^2) and P -value are also shown. The shaded zone represents the distribution area of P_{CW} and P_R when a trade-off exists, for more information see Harrison *et al.* [18]. The lines fitted separately for four species were significantly different ($P < 0.05$) according to the result of a one-way ANCOVA with P_R as a dependent variable, tree species as fixed factors, and P_{CW} as a covariate.

Discussion

The range of PNUE in these tree seedlings was 45.92–120.54 $\mu\text{mol}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ (Table 1) which was close to six *Fagus sylvatica* populations (68.74–122.22 $\mu\text{mol}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$) [62] and four *Quercus* species (approximately 60–150 $\mu\text{mol}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$) [16]; lower than *P. cathayana* (171.64–213.36 $\mu\text{mol}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$) [10] and *S. alterniflora* (171.64–213.36 $\mu\text{mol}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$) [11] under different N deposition. Wright *et al.* summed up PNUE in 710 species and the range was between 10 and 500 $\mu\text{mol}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ [63]; therefore, our results seem reasonable. Shrubs and trees usually have a low PNUE and grasses usually have high value [63]. Fast growing herbaceous species may have a PNUE higher than 200 $\mu\text{mol}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$, whereas values for evergreen woody species can be lower than 50 [3]. Our value is within the medium range.

The overall result highlights a substantial difference between N-fixing and non-N-fixing tree seedling leaves in PNUE. PNUE in *B. alnoides* and *C. hystrix* were significantly higher than that in *D. odorifera* and *E. fordii* (Table 1). The variation of PNUE may be attributable to plant evolution and natural selection [64]. Low PNUE

species compensate for their low productivity with a long leaf life-span [16]; stress-tolerant species [65] and late successional species [66] 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 [67]. Higher PNUE species such as *B. alnoides* and *C. hystrix* could grow faster [16] and have a stronger competitive ability in ecosystems with fertile soil [68].

As PNUE is the ratio of $A_{\max}'$ and N_{area} , changes of $A_{\max}'$ and N_{area} affect PNUE. We found no significant differences in $A_{\max}'$ between the four species' seedling leaves ($F = 3.441$). Biochemical (l_b) photosynthesis limitations were not significantly different between species ($F = 0.964$, Table 2) and V_{cmax} values were not significantly different between species ($F \leq 4.786$, Table 3). Stomatal (l_s) and mesophyll (l_m) photosynthesis limitations were higher in these trees (Table 2). *D. odorifera* had higher l_s and lower l_m values than other species (Table 2) but had lower g_s and higher g_m than other species (Table 4). Therefore, limitations to CO₂ diffusion did not change PNUE. The g_m could also influence the variation in PNUE through N allocation [22]. There was a significant positive relationship between g_m and PNUE but the effect of g_m to PNUE was not consistent between species (Fig 1). Broeckx *et al.* [25] also found this relationship in six poplar (*Populus*) genotypes, and Nha *et al.* [58] found g_m does not contribute to greater PNUE in temperate forest. The effect of g_m on PNUE was different between species.

N-fixing trees *D. odorifera* and *E. fordii* had significantly higher N_{area} than *B. alnoides* and *C. hystrix* (Table 1). Because $N_{\text{area}} = N_{\text{mass}} \times \text{LMA}$, N_{area} may also be affected by LMA besides N content N_{mass} . The difference of LMA between species was far lower than the difference of N_{mass} (Table 1). Therefore, the significantly higher N_{mass} , caused the significantly higher N_{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 [30, 31] and our study in five Fagaceae and five Leguminosae tree species [33]. However, one study reported that N-fixing trees had both higher N_{area} and $A_{\max}'$ [29]. The relationship of N_{area} and $A_{\max}'$ varies in different species [63], because different

species have their own N allocation patterns. The N allocation in photosynthesis was more important than the total leaf N for photosynthesis [69].

D. odorifera and *E. fordii* allocated a lower percentage of N to photosynthetic apparatus than *B. alnoides* and *C. hystrix*, especially to Rubisco and bioenergetics (Table 5). P_P , P_R , and P_B had significant positive correlations with PNUE in these trees ($R^2 \geq 0.735$) (Fig 2) that led to lower PNUE in N-fixing tree species (*D. odorifera* and *E. fordii*). These results agreed with previous studies [30, 31, 33, 54]. Rubisco catalyzes the limiting step for photosynthetic capacity [9]. A positive correlation between $A_{\max}'$ and Rubisco has been frequently reported [12, 15]. 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). N_{mass} largely affected the N allocation to the photosynthetic apparatus and P_{CW} .

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* [15], *Quercus* species [16], *Mikania micrantha* and *Chromolaena odorata* [40]; but this relationship does not exist in some other trees [12]. Some researchers believe that the main influencing factors were whether leaf N could meet the needs of both cell wall N and Rubisco N [11, 18]. We used the method described by Harrison *et al.* [18] 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 [70] and inorganic N (NO_3^- , NH_4^+) [71]) weakens the correlation between Rubisco and cell

322 wall N. It must be noted that *C. hystrix* showed a unique relationship between P_{CW} and P_R (on the shaded zone),
323 which means higher P_{CW} and P_R than the results of Harrison *et al.* [18]. More trees need to be studied to determine
324 the distribution area of P_{CW} and P_R when a trade-off exists.

325 Although both the *B. alnoides* and *C. hystrix* are non-N-fixing broadleaf plants, and had some similar
326 functional traits, there were significant differences showed in N_{mass} , LMA, g_m , C_c , and P_{CW} (Table 1, 4 and 5). *B.*
327 *alnoides* is a deciduous broad-leaved plant and *C. hystrix* is an evergreen broad-leaf plant. In order to contribute to
328 a longer leaf life span, evergreen broad-leaf plants should improve leaf tolerance to environmental disturbance
329 [72,73], reflected in higher LMA [63], and P_{CW} [12]. Higher defensive investment could also reduce N_{mass} [12].
330 Therefore, it can be concluded that the performance of these two species is consistent with leaf economics
331 spectrum [4, 63]. Simultaneously, if higher LMA is a result of mesophyll cell wall thickening, it will reduce g_m
332 and C_c [74, 75], and variations in LMA are often inversely correlated with g_m and C_c [76, 77], consistent with the
333 results of those two species.

334 In conclusion, four tree species seedlings have a similar photosynthetic capacity. Leaf N content and
335 percentage of N in Rubisco and bioenergetics caused the major difference in PNUE in these tree species.
336 Excessive storage of N in N-fixing tree species may reduce their PNUE but may be useful for future physiological
337 processes such as reproduction [10]. Storage of N could buffer changes in other N pools such as cell wall N [15,
338 16, 40] (Fig 3). Evergreen tree leaves with low PNUE have multiple roles in nutrient conservation, nutrient
339 storage, stress tolerance, herbivore deterrence, and photosynthesis [1]. We should consider that some Rubisco can
340 also function as N storage and may not involve in photosynthesis [78, 79]. This type of Rubisco might lead to
341 greater rates of photosynthesis under suboptimal conditions [1]. Therefore, Rubisco N calculated by the model of
342 Farquhar *et al.* [9] might be N in activated Rubisco. Using chemical methods to extract and determine Rubisco N
343 content could be useful [16, 80].

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 these trees, 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.

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521

Figure 1

Figure 2

Figure 3