

## RESEARCH ARTICLE

## 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*, *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.

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## 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 CO<sub>2</sub> 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-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  $A_{max}$  and higher  $N_{area}$ , 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<sub>3</sub> 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 ( $P_R$ ) 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 leaf 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 CO<sub>2</sub> partial pressure is important for Rubisco activity; this is because O<sub>2</sub> is a competitive inhibitor of the C assimilatory reaction of Rubisco, promoting the Rubisco oxidation reaction [23]. A significant negative correlation between C<sub>i</sub> (intercellular CO<sub>2</sub> concentration)-C<sub>c</sub> (CO<sub>2</sub> 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 ( $g_m$ ) by changing the nature of the diffusing molecule [26] and facilitating CO<sub>2</sub> diffusion through membranes [27]. Therefore, PNUE may be influenced by  $g_m$  [25]. A significant positive correlation was found between mesophyll conductance ( $g_m$ ) 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  $g_m$  and the fraction of leaf N to cell wall ( $P_{CW}$ ) 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  $P_R$  and fraction of leaf N to bioenergetics ( $P_B$ ) to be the main factors; the effects of  $g_m$  and  $P_{CW}$  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 CO<sub>2</sub> 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 CO<sub>2</sub> concentration ( $C_i$ ,  $\mu\text{mol mol}^{-1}$ ) were determined for each leaf (seven repetitions in each species): Under 380  $\mu\text{mol mol}^{-1}$  of leaf chamber CO<sub>2</sub> concentration (the average air CO<sub>2</sub> 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$  [37]. Under a saturated PPFD, the photosynthetic rates were detected using the same leaf-under leaf chamber CO<sub>2</sub> concentrations of 380, 200, 150, 100, 80, 50, 380, 600, 800, 1000, 1200, 1500, 1800 and 2000  $\mu\text{mol mol}^{-1}$  [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 ( $A_n$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), 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<sub>2</sub> concentration. Then light-saturated net CO<sub>2</sub> assimilation rate ( $A_{\text{max}}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), light-saturated day respiration rate ( $R_d$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and light- and CO<sub>2</sub>-saturated net CO<sub>2</sub> assimilation rate ( $A_{\text{max}}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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 CO<sub>2</sub> 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 [38]. We used Loreto *et al.* [39] methods to calculate the photosynthetic electron transport rate ( $J_f$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ):

$$J_f = \text{PPFD} \times \frac{\Delta F}{F_m'} \times \text{Leafreflu} \times \text{PARDistPhotosys}. \quad (1)$$

*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<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>):

$$g_m = \frac{A_{\max}'}{C_i - \left\{ \frac{\Gamma^* [J_f + 8(A_{\max}' + R_d)]}{J_f - 4(A_{\max}' + R_d)} \right\}}, \quad (2)$$

Where  $R_d$ ,  $C_i$ , and  $A_{\max}'$  were determined from gas exchange measurements. The CO<sub>2</sub> photo compensation point ( $\Gamma^*$ , μmol mol<sup>-1</sup>) 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<sub>2</sub>, we used the experience value instead (*Leafflu* = 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 $V_{c\max}$ and $J_{\max}$

The mean value of  $g_m$  calculated from three methods was used to calculate CO<sub>2</sub> concentration in chloroplasts ( $C_c$ , μmolmol<sup>-1</sup>):

$$C_c = C_i - \frac{A_{\max}'}{g_m} \quad (3)$$

Then  $C_c$  was used to fit an  $A_n$ - $C_c$  curve, followed by the maximum carboxylation rate ( $V_{c\max}$ , μmol m<sup>-2</sup> s<sup>-1</sup>) calculated according to Farquhar *et al.* [14], and the maximum electron transport rate ( $J_{\max}$ , μmol m<sup>-2</sup> s<sup>-1</sup>) 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:

$$l_s = \frac{g_{\text{tot}}/g_s \times \partial A_n / \partial C_c}{g_{\text{tot}} + \partial A_n / \partial C_c} \quad (4)$$

$$l_m = \frac{g_{\text{tot}}/g_m \times \partial A_n / \partial C_c}{g_{\text{tot}} + \partial A_n / \partial C_c} \quad (5)$$

$$l_b = \frac{g_{\text{tot}}}{g_{\text{tot}} + \partial A_n / \partial C_c} \quad (6)$$

Where  $g_s$  and  $g_m$  were used in light-saturated and atmospheric CO<sub>2</sub> concentration was 380 μmol mol<sup>-1</sup>, and  $g_m$  was the mean value of three methods. The  $g_{\text{tot}}$  is the total conductance to CO<sub>2</sub> from ambient air to chloroplasts (the sum of the inverse CO<sub>2</sub> 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<sup>-1</sup> [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<sup>-2</sup>) 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<sub>mass</sub> mg g<sup>-1</sup>). Nitrogen concentration was determined by a VELP automatic Kjeldahl N determination apparatus (UDK-139, Milano, Italy), and leaf N per mass (N<sub>mass</sub>, mg g<sup>-1</sup>) and per area (N<sub>area</sub> g m<sup>-2</sup>) values were calculated [30]. The PNUE (μmol mol<sup>-1</sup> s<sup>-1</sup>) was then calculated by the formula:

$$PNUE = \frac{A_{max}}{N_{area}} \times 14 \tag{7}$$

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<sup>-1</sup>, 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<sub>CW</sub>) 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<sub>R</sub>), bioenergetics (P<sub>B</sub>), and the light-harvesting components (P<sub>L</sub>) (g g<sup>-1</sup>) were calculated from V<sub>cmax</sub>, J<sub>max</sub> and chlorophyll contents using the method of Niinemets and Tenhunen [12], which has been widely used in recent years [15, 56–58]:

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

$$P_B = \frac{J_{max}}{8.06 \times J_{mc} \times LMA \times N_{mass}}, \tag{9}$$

$$P_L = \frac{C_{Chl}}{C_B \times N_{mass}}, \tag{10}$$

Where C<sub>Chl</sub> is the chlorophyll concentration (mmol g<sup>-1</sup>), V<sub>cr</sub> is the specific activity of Rubisco (μmol CO<sub>2</sub> g<sup>-1</sup> Rubisco s<sup>-1</sup>), J<sub>mc</sub> is the potential rate of photosynthetic electron transport (μmol electrons μmol<sup>-1</sup> Cyt f s<sup>-1</sup>), and C<sub>B</sub> is the ratio of leaf chlorophyll to leaf N during light-harvesting (mmol Chl (g N)<sup>-1</sup>). Where V<sub>cr</sub>, J<sub>mc</sub>, and C<sub>B</sub> were calculated according to

Niinemets and Tenhunen [12]. The fraction of leaf N allocated to the photosynthetic apparatus ( $P_p$ ) was calculated as the sum of  $P_R$ ,  $P_B$ , and  $P_L$ .

### 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. 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). The LMA of *C. hystrix* ( $100.13 \text{ g m}^{-2}$ ) 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).

### Photosynthetic parameters in four seedling leaves

Analysis of the quantitative limitations of photosynthesis revealed that photosynthetic capacity was mainly limited by diffusional processes ( $l_s$  and  $l_m$ ), whereas biochemical limitations ( $l_b$ ) 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  $V_{cmax}$  and  $J_{max}$  in *E. fordii* were higher than the other three species (Table 3) but the statistically significant values

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

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 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>	F
$I_s$ (%)	68.09±1.35 <sup>a</sup>	58.23±1.93 <sup>b</sup>	56.84±1.67 <sup>b</sup>	56.68±2.22 <sup>b</sup>	9.001***
$I_m$ (%)	31.54±1.35 <sup>b</sup>	41.39±1.95 <sup>a</sup>	42.82±1.64 <sup>a</sup>	42.87±2.22 <sup>a</sup>	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) 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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(F-ratios) were lower than PNUE (Table 1). Stomatal conductance ( $g_s$ , 0.100 mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) and  $C_i$  (292.88 μmol mol<sup>-1</sup>) 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<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) was higher than the other three species but  $g_{m-Ethier}$  (0.140 mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) and  $g_{m-Gu}$  (0.160 mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) 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<sup>-1</sup>) were higher than *D. odorifera* and *E. fordii* (both were 0.27 g g<sup>-1</sup>). 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<sup>-1</sup>), followed by *D. odorifera* (0.10 g g<sup>-1</sup>), *C. hystrix* (0.07 g g<sup>-1</sup>), and *E. fordii* (0.06 g g<sup>-1</sup>).

### 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 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>	F
$V_{cmax}$ (μmolm <sup>-2</sup> s <sup>-1</sup> )	78.13±4.59 <sup>b</sup>	99.83±9.37 <sup>a</sup>	72.98±1.51 <sup>b</sup>	82.78±1.47 <sup>ab</sup>	4.786**
$J_{max}$ (μmolm <sup>-2</sup> s <sup>-1</sup> )	100.71±5.80 <sup>bc</sup>	128.76±11.20 <sup>a</sup>	98.38±5.37 <sup>bc</sup>	109.27±4.05 <sup>ab</sup>	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 ( $g_m$ ), intercellular CO<sub>2</sub> concentration ( $C_i$ ), and CO<sub>2</sub> 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 <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	0.067±0.004 <sup>bc</sup>	0.046±0.002 <sup>c</sup>	0.100±0.013 <sup>a</sup>	0.074±0.004 <sup>b</sup>	11.106 <sup>***</sup>
$C_i$ (μmol mol <sup>-1</sup> )	251.54±6.44 <sup>b</sup>	235.61±6.19 <sup>b</sup>	292.88±5.94 <sup>a</sup>	256.78±5.24 <sup>b</sup>	7.348 <sup>**</sup>
$g_{m-Harley}$ (mol CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	0.114±0.013 <sup>ab</sup>	0.068±0.007 <sup>b</sup>	0.136±0.013 <sup>a</sup>	0.109±0.006 <sup>ab</sup>	15.391 <sup>***</sup>
$g_{m-Ethier}$ (mol CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	0.140±0.01 <sup>a</sup>	0.066±0.01 <sup>c</sup>	0.130±0.01 <sup>ab</sup>	0.099±0.01 <sup>bc</sup>	14.772 <sup>**</sup>
$g_{m-Gu}$ (mol CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	0.160±0.01 <sup>a</sup>	0.063±0.01 <sup>b</sup>	0.138±0.02 <sup>a</sup>	0.090±0.01 <sup>b</sup>	19.390 <sup>**</sup>
$C_{c-Harley}$ (μmol mol <sup>-1</sup> )	178.39±7.84 <sup>b</sup>	136.80±5.18 <sup>c</sup>	228.78±8.44 <sup>a</sup>	172.17±6.10 <sup>b</sup>	29.182 <sup>**</sup>
$C_{c-Ethier}$ (μmol mol <sup>-1</sup> )	192.99±7.11 <sup>b</sup>	133.56±7.95 <sup>c</sup>	224.91±9.13 <sup>a</sup>	173.93±3.73 <sup>b</sup>	27.639 <sup>**</sup>
$C_{c-Gu}$ (μmol mol <sup>-1</sup> )	200.92±6.72 <sup>a</sup>	127.42±9.56 <sup>b</sup>	225.58±12.27 <sup>a</sup>	157.86±9.56 <sup>b</sup>	20.268 <sup>**</sup>

Data of CO<sub>2</sub> conductance was measured in light-saturated and atmospheric CO<sub>2</sub> concentration was 380 μmol mol<sup>-1</sup>. 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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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).

## Discussion

The range of PNUE in these tree seedlings was 45.92–120.54 μmol mol<sup>-1</sup> s<sup>-1</sup> (Table 1) which was close to six *Fagus sylvatica* populations (68.74–122.22 μmol mol<sup>-1</sup> s<sup>-1</sup>) [59] and four *Quercus* species (approximately 60–150 μmol mol<sup>-1</sup> s<sup>-1</sup>) [20]; lower than *P. cathayana* (171.64–213.36 μmol mol<sup>-1</sup> s<sup>-1</sup>) [17] and *S. alterniflora* (171.64–213.36 μmol mol<sup>-1</sup> s<sup>-1</sup>) [15] under different N deposition. Wright *et al.* summed up PNUE in 710 species and the range was between 10 and 500 μmol mol<sup>-1</sup> s<sup>-1</sup> [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 ( $1 - P_P - P_{CW} - P_{Other}$ ) 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 <sup>-1</sup> )	0.13±0.01 <sup>b</sup>	0.16±0.01 <sup>b</sup>	0.26±0.03 <sup>a</sup>	0.30±0.01 <sup>a</sup>	22.130 <sup>***</sup>
$P_B$ (g g <sup>-1</sup> )	0.03±0.002 <sup>b</sup>	0.04±0.003 <sup>b</sup>	0.07±0.007 <sup>a</sup>	0.07±0.003 <sup>a</sup>	18.111 <sup>***</sup>
$P_L$ (g g <sup>-1</sup> )	0.10±0.01 <sup>ab</sup>	0.06±0.01 <sup>c</sup>	0.12±0.01 <sup>a</sup>	0.07±0.01 <sup>b</sup>	8.848 <sup>***</sup>
$P_P$ (g g <sup>-1</sup> )	0.27±0.02 <sup>b</sup>	0.27±0.02 <sup>b</sup>	0.44±0.04 <sup>a</sup>	0.44±0.02 <sup>a</sup>	14.796 <sup>***</sup>
$P_{CW}$ (g g <sup>-1</sup> )	0.07±0.004 <sup>c</sup>	0.05±0.002 <sup>c</sup>	0.22±0.010 <sup>b</sup>	0.27±0.011 <sup>a</sup>	182.914 <sup>***</sup>
$P_{Other}$ (g g <sup>-1</sup> )	0.66±0.02 <sup>a</sup>	0.68±0.02 <sup>a</sup>	0.34±0.04 <sup>b</sup>	0.29±0.02 <sup>b</sup>	63.830 <sup>***</sup>

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

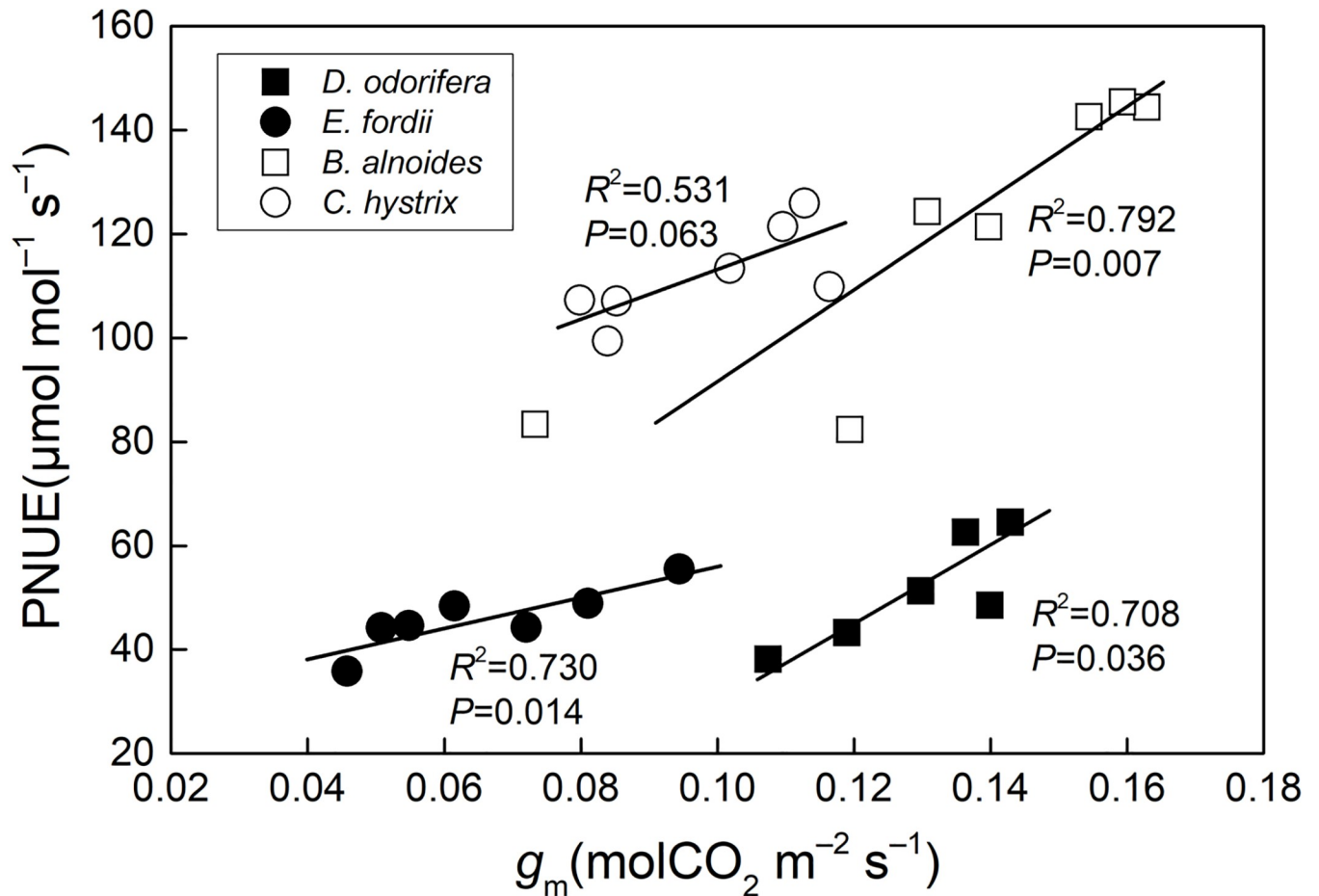
\* $P < 0.05$

\*\* $P < 0.01$

\*\*\* $P < 0.001$ .

<https://doi.org/10.1371/journal.pone.0208971.t005>



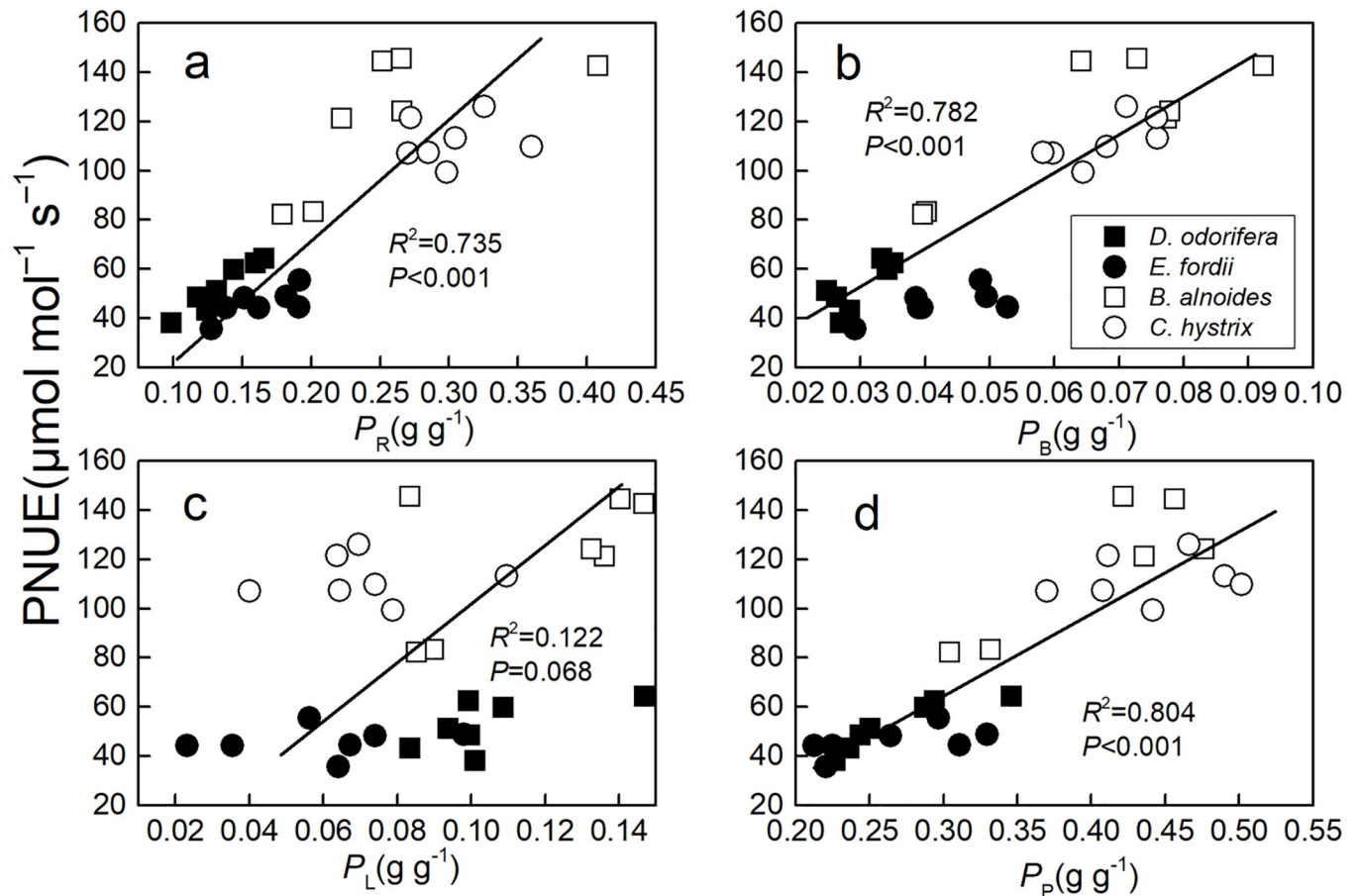


**Fig 1. Regression analysis of mesophyll conductance ( $g_m$ ) with photosynthetic-N use efficiency (PNUE) in four species seedling leaves.** The determination coefficient ( $R^2$ ) and  $P$ -value were also shown. The lines fitted separately for four species were significantly different ( $P < 0.05$ ) 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.

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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  $200 \mu\text{mol mol}^{-1} \text{s}^{-1}$ , 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.

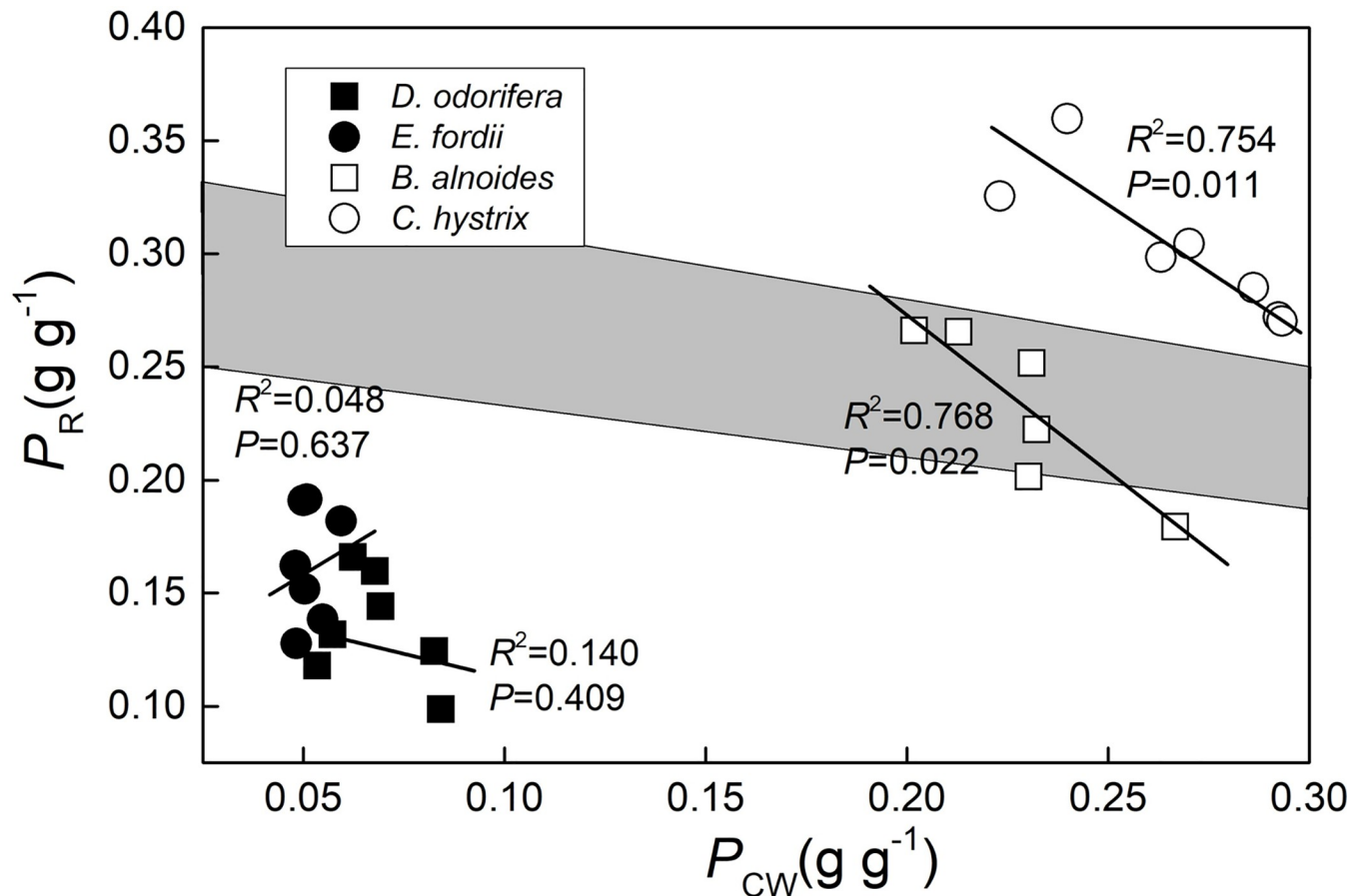


**Fig 2. Regression analysis of the fraction of leaf N allocated to (a) Rubisco ( $P_R$ ), (b) bioenergetics ( $P_B$ ), (c) light-harvesting components ( $P_L$ ), and (d) the photosynthetic apparatus ( $P_p$ ) with photosynthetic-N use efficiency (PNUE) in four species seedling leaves.** The determination 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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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 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 were also shown. The shaded zone was drawn according to this hypothesis: when  $P_{CW}$  was  $0.300 \text{ g g}^{-1}$ , the rest ( $0.700 \text{ g g}^{-1}$ ) were soluble and thylakoid protein, Rubisco represented one quarter to one-third of the N in soluble and thylakoid protein,  $P_R$  valued  $0.175\text{--}0.233 \text{ g g}^{-1}$  (right side of shaded zone). When  $P_{CW}$  was valued  $0.000 \text{ g g}^{-1}$  in limiting case (does not exist in reality), all the rest ( $1.000 \text{ g g}^{-1}$ ) were soluble and thylakoid protein,  $P_R$  valued  $0.250\text{--}0.333 \text{ g g}^{-1}$  (left side of shaded zone). For more information see Harrison *et al.* [21]. The lines fitted separately for four species were significantly different ( $P < 0.05$ ) according to the result of a one-way ANCOVA with  $P_R$  as a dependent variable, tree species as fixed factors, and  $P_{CW}$  as a covariate.

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higher  $N_{area}$  and  $A_{max}'$  [66]. The relationship of  $N_{area}$  and  $A_{max}'$  varies in different species [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 [67].

Lower  $P_p$ ,  $P_R$ , and  $P_B$  were main reasons that led to lower PNUE in N-fixing tree species (*D. odorifera* and *E. fordii*). These results agreed with previous studies [10, 11, 51] and our study on five Fagaceae tree species and five Leguminosae big tree species [30]. Rubisco catalyzes the limiting step for photosynthetic capacity [14]. A positive correlation between  $A_{max}'$  and Rubisco has been frequently reported [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  $N_{mass}$  largely affected the N allocation to the photosynthetic apparatus and  $P_{CW}$ .

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 Fagaceae 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 involved 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  $74.34 \pm 8.54 \mu\text{mol mol}^{-1} \text{s}^{-1}$  [30], smaller than its seedlings (Table 1). *C. hystrix* big tree also had higher  $P_{CW}$  ( $0.46 \text{ g g}^{-1}$ ) than seedlings, but its  $P_P$  ( $0.26 \text{ g g}^{-1}$ ),  $P_R$  ( $0.20 \text{ g g}^{-1}$ ),  $P_B$  ( $0.041 \text{ g g}^{-1}$ ) and  $P_L$  ( $0.014 \text{ g g}^{-1}$ ) [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  $N_{\text{mass}}$ , LMA,  $g_m$ ,  $C_c$ , 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  $N_{\text{mass}}$  [16]. Simultaneously, if higher LMA is a result of mesophyll cell wall thickening, it will reduce  $g_m$

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 ( $\pm$  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 \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

(DOCX)

**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 ( $\pm$  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 \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

(DOCX)

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**Visualization:** Baodi Sun.

**Writing – original draft:** Jingchao Tang.

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## References

1. Field C, Mooney HA. The photosynthesis nitrogen relationship in wild plants. In: Givnish TJ (eds) *On the Economy of Plant Form and Function*: Cambridge University Press, Cambridge.1986; pp: 25–55.
2. Balotf S, Islam S, Kavosi G, Kholdebarin B, Juhasz A, et al. How exogenous nitric oxide regulates nitrogen assimilation in wheat seedlings under different nitrogen sources and levels. *Plos One*. 2018; 13: e0190269. <https://doi.org/10.1371/journal.pone.0190269> PMID: 29320529
3. Warren CR, Adams MA. Internal conductance does not scale with photosynthetic capacity: implications for carbon isotope discrimination and the economics of water and nitrogen use in photosynthesis. *Plant Cell Environ*. 2006; 29: 192–201. PMID: 17080635
4. Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, et al. The worldwide leaf economics spectrum. *Nature*. 2004; 428: 821–827. <https://doi.org/10.1038/nature02403> PMID: 15103368
5. Hikosaka K. Interspecific difference in the photosynthesis-nitrogen relationship: patterns, physiological causes, and ecological importance. *J Plant Res*. 2004; 117: 481–494. <https://doi.org/10.1007/s10265-004-0174-2> PMID: 15583974
6. Hikosaka K. Mechanisms underlying interspecific variation in photosynthetic capacity across wild plant species. *Plant Biotechnology*. 2010; 27: 223–229.
7. Harris W, Baker MJ, Williams WM. *Population dynamics and competition*. White Clover Wallingford, UK CAB International. 1987; 205–297.
8. Chen LY, Zhao J, Zhang RY, Wang SM. Effects of nitrogen and phosphorus fertilization on legumes in *Potentilla fruticosa* shrub in alpine meadow. *Ecol Sci*, 2012; 9: 512–517.
9. Reed SC. Disentangling the complexities of how legumes and their symbionts regulate plant nitrogen access and storage. *New Phytol*. 2017; 213: 478–480. <https://doi.org/10.1111/nph.14390> PMID: 28000933
10. Novriyanti E, Watanabe M, Makoto K, Takeda T, Hashidoko Y, et al. Photosynthetic nitrogen and water use efficiency of acacia and eucalypt seedlings as afforestation species. *Photosynthetica*. 2012; 50: 273–281.
11. Zhu JT, Li XY, Zhang XM, Yu Q, Lin LS. Leaf nitrogen allocation and partitioning in three groundwater-dependent herbaceous species in a hyper-arid desert region of north-western China. *Aus J Bot*. 2012; 60: 61–67.
12. Niinemets Ü, Tenhunen JD. A model separating leaf structural and physiological effects on carbon gain along light gradients for the shade-tolerant species *Acer saccharum*. *Plant Cell Environ*. 1997; 20: 845–866.
13. Evans JR. Photosynthesis and nitrogen relationships in leaves of C<sub>3</sub> plants. *Oecologia*. 1989; 78: 9–19. <https://doi.org/10.1007/BF00377192> PMID: 28311896
14. Farquhar GD, von Caemmerer S, Berry JA. A biochemical model of photosynthetic CO<sub>2</sub> assimilation in leaves of C<sub>3</sub> species. *Planta*. 1980; 149:78–90. <https://doi.org/10.1007/BF00386231> PMID: 24306196
15. Qing H, Cai Y, Xiao Y, Yao YH, An SQ. Leaf nitrogen partition between photosynthesis and structural defense in invasive and native tall form *Spartina alterniflora* populations: effects of nitrogen treatments. *Biol Invasions*. 2012; 14: 2039–2048.
16. Hikosaka K, Shigeno A. The role of Rubisco and cell walls in the interspecific variation in photosynthetic capacity. *Oecologia*. 2009; 160: 443–451. <https://doi.org/10.1007/s00442-009-1315-z> PMID: 19288136
17. Chen L, Dong T, Duan B. Sex-specific carbon and nitrogen partitioning under n deposition in *Populus cathayana*. *Trees*. 2014; 28:793–806.

18. Onoda Y, Wright IJ, Evans JR, Hikosaka K, Kitajima K, et al. Physiological and structural tradeoffs underlying the leaf economics spectrum. *New Phytol.* 2017; 214: 1447–1463. <https://doi.org/10.1111/nph.14496> PMID: 28295374
19. Onoda Y, Hikosaka K, Hirose T. Allocation of nitrogen to cell walls decreases photosynthetic nitrogen-use efficiency. *Funct Ecol.* 2004; 18: 419–425.
20. Takashima T, Hikosaka K, Hirose T. Photosynthesis or persistence: nitrogen allocation in leaves of evergreen and deciduous *Quercus* species. *Plant Cell Environ.* 2004; 27: 1047–1054.
21. Harrison MT, Edwards EJ, Farquhar GD, Nicotra, AB, et al. Nitrogen in cell walls of sclerophyllous leaves accounts for little of the variation in photosynthetic nitrogen-use efficiency. *Plant Cell Environ.* 2009; 32: 259–270. <https://doi.org/10.1111/j.1365-3040.2008.01918.x> PMID: 19054350
22. Niinemets Ü, Flexas J, Peñuelas J. Evergreens favored by higher responsiveness to increased CO<sub>2</sub>. *Trends Ecol Evol.* 2011; 26: 136–142. <https://doi.org/10.1016/j.tree.2010.12.012> PMID: 21277042
23. Li Y, Gao Y, Xu X, Shen Q, Guo S. Light-saturated photosynthetic rate in high-nitrogen rice (*Oryza sativa* L.) leaves is related to chloroplastic CO<sub>2</sub> concentration. *J Exp Bot.* 2009; 60: 2351–2360. <https://doi.org/10.1093/jxb/erp127> PMID: 19395387
24. Xu G, Huang TF, Zhang XL, Duan BL. Significance of mesophyll conductance for photosynthetic capacity and water-use efficiency in response to alkaline stress in *Populus cathayana* seedlings. *Photosynthetica.* 2013; 51: 438–444.
25. Buckley TN, Warren CR. The role of mesophyll conductance in the economics of nitrogen and water use in photosynthesis. *Photosynth res.* 2014; 119: 77–88. <https://doi.org/10.1007/s11120-013-9825-2> PMID: 23609621
26. Flexas J, Barbour MM, Brendel O, Cabrera HM, Carriqui M, et al. Mesophyll diffusion conductance to CO<sub>2</sub>: an unappreciated central player in photosynthesis. *Plant Sci.* 2012; 193–194: 70–84. <https://doi.org/10.1016/j.plantsci.2012.05.009> PMID: 22794920
27. Nakhoul NL, Davis BA, Romero MF, Boron WF. Effect of expressing the water channel aquaporin-1 on the CO<sub>2</sub> permeability of *Xenopus oocytes*. *Am J Physiol.* 1998; 274: C543–C548. PMID: 9486145
28. Broeckx LS, Fichot R, Verlinden MS, Ceulemans R. Seasonal variations in photosynthesis, intrinsic water-use efficiency and stable isotope composition of poplar leaves in a short-rotation plantation. *Tree Physiol.* 2014; 34:701–715. <https://doi.org/10.1093/treephys/tpu057> PMID: 25074859
29. Warren CR, Adams MA. Evergreen trees do not maximize instantaneous photosynthesis. *Trends plant sci.* 2004; 9: 270–274. <https://doi.org/10.1016/j.tplants.2004.04.004> PMID: 15165557
30. Tang JC., Cheng RM, Shi ZM, Xu GX, Liu SR, et al. Fagaceae tree species allocate higher fraction of nitrogen to photosynthetic apparatus than Leguminosae in Jianfengling tropical montane rain forest, China. *Plos One.* 2018; 13:e0192040. <https://doi.org/10.1371/journal.pone.0192040> PMID: 29390007
31. Luo WY, Luo P, Liu YJ. Choice and development of the fine and valuable hardwood tree species in tropical and south subtropical regions of China. *Chinese J Tropical Agriculture.* 2010; 30: 15–21 (in Chinese).
32. Pang ZH. The study progress of *Betula alnoides* in China. *China J Guangxi Academy Sci.* 2011; 27: 243–250 (in Chinese).
33. Yang BG, Liu SL, Hao J, Pang SJ, Zhang P. Research advances on the rare tree of *Erythrophleum fordii*. *Guangxi Forestry Sci.* 2017; 46: 165–170 (in Chinese).
34. You Y, Huang X, Zhu H, Liu S, Liang H, et al. Positive interactions between *Pinus massoniana* and *Castanopsis hystrix* species in the uneven-aged mixed plantations can produce more ecosystem carbon in subtropical China. *Forest Ecol Manag.* <https://doi.org/10.1016/j.foreco.2017.08.025>
35. Wang WX, Shi ZM, Luo D, Liu SR, Lu LH. Characteristics of soil microbial biomass and community composition in three types of plantations in southern subtropical area of China. *Chinese J Applied Ecol.* 2013; 24: 1784–1792 (in Chinese).
36. Tang JC, Shi ZM, Luo D, Liu SR. Photosynthetic nitrogen-use efficiency of *Manglietia glauca* seedling leaves under different shading levels. *Acta Ecol Sinica.* 2017; 37:7493–7502 (in Chinese).
37. Zhang L, Chen X, Wen D. Interactive effects of rising CO<sub>2</sub>, and elevated nitrogen and phosphorus on nitrogen allocation in invasive weeds *Mikania micrantha* and *Chromolaena odorata*. *Biol Invasions.* 2016; 18: 1391–1407.
38. Feng QH, Cheng RM, Shi ZM, Liu SR, Wang WX, et al. Response of *Rumex dentatus* foliar nitrogen and its allocation to altitudinal gradients along Balang Mountain, Sichuan, China. *Chinese J Plant Ecol.* 2013; 37: 591–600 (in Chinese).
39. Loreto F, Di Marco G, Tricoli D, Sharkey TD. Measurements of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. *Photosynth Res.* 1994; 41: 397–403. <https://doi.org/10.1007/BF02183042> PMID: 24310154

40. Loreto F, Tsonev T, Centritto M. The impact of blue light on leaf mesophyll conductance. *J Eep Bot.* 2009; 112:1–8.
41. Harley PC, Loreto F, Di Marco G, Sharkey TD. Theoretical considerations when estimating the mesophyll conductance to CO<sub>2</sub> flux by analysis of the response of photosynthesis to CO<sub>2</sub>. *Plant Physiol.* 1992; 98: 1429–1436. PMID: 16668811
42. Li Y, Ren BB, Ding L, Shen QR, Peng SB, Guo SW. Does chloroplast size influence photosynthetic nitrogen use efficiency?. *PLoS One.* 2013; 8: e62036. <https://doi.org/10.1371/journal.pone.0062036> PMID: 23620801
43. Sorrentino G, Haworth M, Wahbi S, Mahmood T, Zuomin S, Centritto M. Abscisic acid induces rapid reductions in mesophyll conductance to carbon dioxide. *PLoS One.* 2016; 11: e0148554. <https://doi.org/10.1371/journal.pone.0148554> PMID: 26862904
44. Momayyezi M, Guy RD. Blue light differentially represses mesophyll conductance in high vs low latitude genotypes of *Populus trichocarpa* Torr. & Gray. *J Plant Physiol.* 2017; 213: 122–128. <https://doi.org/10.1016/j.jplph.2017.03.006> PMID: 28364640
45. Wang X, Du T, Huang J, Peng S, Xiong D. Leaf hydraulic vulnerability triggers the decline in stomatal and mesophyll conductance during drought in rice (*Oryza sativa*). *J Eep Bot.* 2018; 69: 4033–4045.
46. Bernacchi CJ, Singaas EL, Pimentel C, Portis JAR, Long SP. Improved temperature response functions for models of rubisco-limited photosynthesis. *Plant Cell Environ.* 2001; 24: 253–259.
47. Ethier GJ, Livingston NJ. On the need to incorporate sensitivity to CO<sub>2</sub> transfer conductance into the farquhar-von caemmerer-berry leaf photosynthesis model. *Plant Cell Environ.* 2004; 27: 137–153.
48. Gu LH, Pallardy SG, Tu K, Law BE, Wullschlegel SD. Reliable estimation of biochemical parameters from C<sub>3</sub> leaf photosynthesis-intercellular carbon dioxide response curves. *Plant Cell Environ.* 2010; 33: 1852–1874. <https://doi.org/10.1111/j.1365-3040.2010.02192.x> PMID: 20561254
49. Sharkey TD, Bernacchi CJ, Farquhar GD, Singaas EL. Fitting photosynthetic carbon dioxide response curves for C<sub>3</sub> leaves. *Plant Cell Environ.* 2007; 30: 1035–1040. <https://doi.org/10.1111/j.1365-3040.2007.01710.x> PMID: 17661745
50. Loustau D, Brahim MB, Gaudillère JP, Dreyer E. Photosynthetic responses to phosphorus nutrition in two-year-old maritime pine seedlings. *Tree Physiol.* 1999; 19: 707–715. PMID: 12651309
51. Grassi G, Magnani F. Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. *Plant Cell Environ.* 2005; 28: 834–849.
52. Tomás M, Flexas J, Copolovici L, Galmés J, Hallik L, et al. Importance of leaf anatomy in determining mesophyll diffusion conductance to CO<sub>2</sub> across species: quantitative limitations and scaling up by models. *J Eep Bot.* 2013; 64: 2269–2281.
53. Peguero-Pina JJ, Sisó S, Flexas J, Galmés J, Niinemets Ü, et al. Coordinated modifications in mesophyll conductance, photosynthetic potentials and leaf nitrogen contribute to explain the large variation in foliage net assimilation rates across *Quercus ilex* provenances. *Tree Physiol.* 2017; 37: 1084–1094. <https://doi.org/10.1093/treephys/tpx057> PMID: 28541538
54. Peguero-Pina JJ., Sisó S, Flexas J, Galmés J, García-Nogales A, et al. Cell-level anatomical characteristics explain high mesophyll conductance and photosynthetic capacity in sclerophyllous *Mediterranean oaks*. *New Phytol.* 2017; 214: 1–12.
55. Nha B, Hayes L, Scafaro AP, Atkin OK, Evans JR. Mesophyll conductance does not contribute to greater photosynthetic rate per unit nitrogen in temperate compared with tropical evergreen wet-forest tree leaves. *New Phytol.* 2018; 218: 1–13.
56. Feng YL, Wang JF, Sang WG. Biomass allocation, morphology and photosynthesis of invasive and noninvasive exotic species grown at four irradiance levels. *Acta Oecologica.* 2007; 31: 40–47.
57. Bahar NH, Ishida FY, Weerasinghe LK, Guerrieri R, O'Sullivan OS, et al. Leaf-level photosynthetic capacity in lowland Amazonian and high-elevation Andean tropical moist forests of Peru. *New Phytol.* 2016; <https://doi.org/10.1111/nph.14079> PMID: 27389684
58. Yao HS, Zhang YL, Yi XP, Zhang XJ, Fan DY, et al. Diaheliotropic leaf movement enhances leaf photosynthetic capacity and photosynthetic use efficiency of light and photosynthetic nitrogen via optimizing nitrogen partitioning among photosynthetic components in cotton (*Gossypium hirsutum* L.). *Plant Biol.* 2018; 20: 213–222. <https://doi.org/10.1111/plb.12678> PMID: 29222927
59. Sánchez-Gómez D, Robson TM, Gascó A, Gil-Pelegrín E, Aranda I. Differences in the leaf functional traits of six beech (*Fagus sylvatica*, L.) populations are reflected in their response to water limitation. *Environ Exp Bot.* 2013; 87: 110–119.
60. Wright IJ, Reich PB, Cornelissen JH, Falster DS, Garnier E, et al. Assessing the generality of global leaf trait relationships. *New phytol.* 2005; 166: 485–496. <https://doi.org/10.1111/j.1469-8137.2005.01349.x> PMID: 15819912



61. Reich PB, Oleksyn J, Wright IJ. Leaf phosphorus influences the photosynthesis-nitrogen relation: a cross-biome analysis of 314 species. *Oecologia*. 2009; 160: 207–212. <https://doi.org/10.1007/s00442-009-1291-3> PMID: 19212782
62. Hikosaka K, Hirose T. Photosynthetic nitrogen-use efficiency in evergreen broad-leaved woody species coexisting in a warm-temperate forest. *Tree Physiol*. 2000; 20: 1249–1254. PMID: 12651488
63. Ellsworth DS, Reich PB. Photosynthesis and leaf nitrogen in five Amazonian tree species during early secondary succession. *Ecology*. 1996; 77: 581–594.
64. Kikuzawa K. A cost-benefit analysis of leaf habit and leaf longevity of trees and their geographical pattern. *Am Nat*. 1991; 138: 1250–1263.
65. Robinson DE, Wagner RG, Bell FW, Swanton CJ. Photosynthesis, nitrogen-use efficiency, and water-use efficiency of jack pine seedlings in competition with four boreal forest plant species. *Can J Forest Res*. 2001; 31: 2014–2025.
66. Moon M, Kang KS, Park IK, Kim T, Kim HS. Effects of leaf nitrogen allocation on the photosynthetic nitrogen-use efficiency of seedlings of three tropical species in Indonesia. *J Korean Soc Appl Bi*. 2015; 58: 511–519.
67. Qin RM, Zheng YL, Valiente-Banuet A, Callaway RM, Barclay GF, et al. The evolution of increased competitive ability, innate competitive advantages, and novel biochemical weapons act in concert for a tropical invader. *New Phytol*. 2013; 197: 979–988. <https://doi.org/10.1111/nph.12071> PMID: 23252450
68. Ruan J, Haerdter R, Gerendás J. Impact of nitrogen supply on carbon/nitrogen allocation: a case study on amino acids and catechins in green tea [*Camellia sinensis* (L.) O. Kuntze] plants. *Plant Biology*. 2010; 12: 724–734. <https://doi.org/10.1111/j.1438-8677.2009.00288.x> PMID: 20701695
69. Funk JL, Glenwinkel LA, Sack L. Differential allocation to photosynthetic and non-photosynthetic nitrogen fractions among native and invasive species. *PloS one*. 2013; 8: e64502. <https://doi.org/10.1371/journal.pone.0064502> PMID: 23700483
70. Stitt M., Schulze ED. Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant Cell Environ*. 1994; 17: 465–487.
71. Warren CR, Adams MA. Distribution of N, Rubisco and photosynthesis in *Pinus pinaster* and acclimation to light. *Plant Cell Environ*. 2001; 24: 597–609.
72. Carmo-Silva E, Scales JC, Madgwick PJ, Parry MAJ. Optimizing Rubisco and its regulation for greater resource use efficiency. *Plant Cell Environ*. 2015; 38: 1817–1832. <https://doi.org/10.1111/pce.12425> PMID: 25123951
73. Wright IJ, Cannon K. Relationships between leaf lifespan and structural defences in a low-nutrient, sclerophyll flora. *Funct Ecol*. 2001; 15: 351–359.
74. Onoda Y, Schieving F, Anten NP. Effects of light and nutrient availability on leaf mechanical properties of *Plantago major*: a conceptual approach. *Ann Bot*. 2008; 101: 727–736. <https://doi.org/10.1093/aob/mcn013> PMID: 18272529
75. Warren CR. Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO<sub>2</sub> transfer. *J Exp Bot*. 2008; 59: 1475–1487. <https://doi.org/10.1093/jxb/erm245> PMID: 17975206
76. Scafaro AP, Von CS, Evans JR, Atwell BJ. Temperature response of mesophyll conductance in cultivated and wild *Oryza* species with contrasting mesophyll cell wall thickness. *Plant Cell Environ*. 2011; 34: 1999–2008. <https://doi.org/10.1111/j.1365-3040.2011.02398.x> PMID: 21752031
77. Piel C, Frak E, Le Roux X, Genty B. Effect of local irradiance on CO<sub>2</sub> transfer conductance of mesophyll in walnut. *J Exp Bot*. 2002; 53: 2423–2430. PMID: 12432034
78. Tosens T, Niinemets U, Vislap V, Eichelmann H, Díez PC. Developmental changes in mesophyll diffusion conductance and photosynthetic capacity under different light and water availabilities in *Populus tremula*: how structure constrains function. *Plant Cell Environ*. 2012; 35: 839–856. <https://doi.org/10.1111/j.1365-3040.2011.02457.x> PMID: 22070625