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# Leaf nitrogen assimilation and partitioning differ among subtropical forest plants in response to canopy addition of nitrogen treatments

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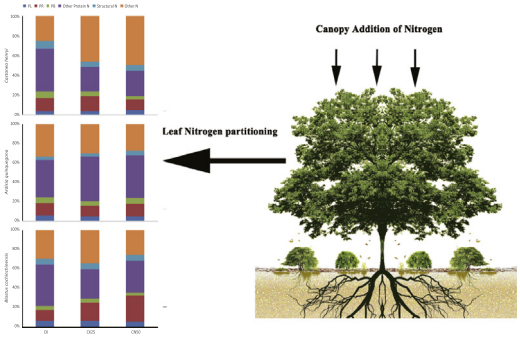
## HIGHLIGHTS

- N addition decreased photosynthesis and metabolic protein allocation of a canopy tree.
- Both understory plants could acclimate to N addition but by different mechanisms.
- Dominant forest plants with large canopies may be susceptible to N deposition.
- Specific differences in nitrogen metabolism may explain subtropical forest degradation.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Global increases in nitrogen deposition may alter forest structure and function by interfering with plant nitrogen metabolism (e.g., assimilation and partitioning) and subsequent carbon assimilation, but it is unclear how these responses to nitrogen deposition differ among species. In this study, we conducted a 2-year experiment to investigate the effects of canopy addition of nitrogen (CAN) on leaf nitrogen assimilation and partitioning in three subtropical forest plants (*Castanea henryi*, *Ardisia quinquegona*, and *Blastus cochinchinensis*). We hypothesized that responses of leaf nitrogen assimilation and partitioning to CAN differ among subtropical forest plants. CAN increased leaf nitrate reductase (NR) activity, and leaf nitrogen and chlorophyll contents but reduced leaf maximum photosynthetic rate ($A_{max}$), photosynthetic nitrogen use efficiency (PNUE), ribulose-1,5-bisphosphate carboxylase (Rubisco) activity, and metabolic protein content of an overstory tree species *C. henryi*. In an understory tree *A. quinquegona*, CAN increased NR activity and glutamine synthetase activity and therefore increased metabolic protein synthesis (e.g., Rubisco) in leaves. In the shrub *B. cochinchinensis*, CAN increased $A_{max}$, PNUE, Rubisco content, metabolic protein content, and Rubisco activity in leaves. Leaf nitrogen assimilation and partitioning results indicated that *A. quinquegona* and *B. cochinchinensis* may better acclimate to CAN than *C. henryi* and that the acclimation mechanism differs among the species. Results from this study suggest that long-term elevated atmospheric nitrogen deposition has contributed to the ongoing transformation of subtropical forests into communities dominated by small trees and shrubs.



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## 1. Introduction

Global atmospheric nitrogen deposition has increased dramatically due to increases in the combustion of fossil fuels, in the use of nitrogen in agriculture, and in industrial activities (Galloway et al., 2008). As estimated by Galloway et al. (2004), the reactive nitrogen deposited on the earth's surface increased from 34 Tg N $year^{-1}$ in 1860 to 100 Tg N $year^{-1}$ in 1995, and is projected to be 200 Tg N $year^{-1}$ by 2050. China has experienced substantial nitrogen deposition over the past decades, especially in its rapidly developing central and southeastern regions, and nitrogen deposition in China is predicted to increase dramatically in the future (Liu et al., 2011; Liu et al., 2013; Jia et al., 2014). As a driver of global change, nitrogen deposition has the potential to affect forest ecosystems, and researchers have attempted to understand its long-term effects on forest structure and function (Magill et al., 2004; Galloway et al., 2008; Lu et al., 2010; Talhelm et al., 2013). To date, studies on the effects of nitrogen deposition on forest ecosystems have mainly concentrated on deciduous broad-leaved forests and coniferous forests in the temperate zone (Takashima et al., 2004; Gradowski and Thomas, 2006; Högberg, 2007; Lehmann and Johansson, 2010), and few studies have focused on tropical and subtropical evergreen broad-leaved forests, especially in China (Lu et al., 2010, 2014).

As natural nitrogen passes through the forest canopy layer in the process of being deposited, water soluble $NH_4^+$, $NO_3^-$, and gaseous nitrogen ($NH_3$ and $HNO_3$) become immediately available to trees (Rose, 1996; Wortman et al., 2012). Canopy leaves, shoots, and branches may intercept and retain from 8 to 70% of the natural nitrogen deposition (Gaige et al., 2007; Staelens et al., 2008; Dail et al., 2009). Most experiments that have simulated nitrogen deposition in forests have sprayed nitrogen on the understory or applied it to the soil (Warren et al., 2003; Högberg, 2007; Lu et al., 2014). These experiments exclude the effects of the canopy and thus may not indicate the true effects of nitrogen deposition on forest ecosystems (Zhang et al., 2015).

Plants require nitrogen for the synthesis of amino acids, proteins, chlorophylls, nucleic acids, lipids, and a variety of other metabolites (Kusano et al., 2011). Nitrate ($NO_3^-$) and ammonium ($NH_4^+$) are the main sources of inorganic nitrogen for plant growth, and plants integrate several potential signals of internal nitrogen status to regulate nitrogen uptake and assimilation in order to match plant demand. Following its uptake, $NO_3^-$ is reduced and incorporated into cells. In the first step, $NO_3^-$ is converted into nitrite ($NO_2^-$) by nitrate reductase (NR) in the cytosol. $NO_2^-$ can be further reduced to $NH_4^+$ via the enzyme nitrite reductase (NiR) in chloroplasts or plastids. $NH_4^+$, derived either from $NO_3^-$ reduction or from direct uptake, is first converted to glutamine (Gln) by glutamine synthetase (GS) and then to glutamate (Glu) by glutamate synthase (GOGAT) (Sanchez-Rodriguez et al., 2011; Liu et al., 2014).

The increased deposition of nitrogen has caused a series of ecological problems in forests, including an imbalance in the partitioning of nitrogen within forest plants (Warren et al., 2003). Some forest studies have indicated that the addition of nitrogen increased leaf biomass and ribulose-1,5-bisphosphate carboxylase (Rubisco, the photosynthesizing enzyme) content and thereby increased $CO_2$ assimilation and photosynthesis per unit area of forest (Warren et al., 2003; Högberg, 2007). According to other studies, the increased input of nitrogen may drive chlorophyll degradation (Shi et al., 2017), disorder cellular carbon metabolism (Bauer et al., 2004), increase free amino acid content (Strengbom et al., 2003), decrease forest viability, and increase tree death (Liu et al., 2011). As a result, nitrogen deposition could significantly change the species composition and structure of forests (Nordin et al., 2005; Lu et al., 2010; Gilliam et al., 2006). It is still unclear, however, how nitrogen deposition interferes with the nitrogen metabolism of different tree species and how intensified atmospheric nitrogen deposition may affect the species composition of diverse forest types.

In this study, we determined the effects of canopy addition of nitrogen (CAN) on nitrogen assimilation and partitioning in three woody species (an overstory tree, an understory tree, and a shrub) in a subtropical forest. We tested the following two hypotheses: (1) Overstory trees with large canopies are more sensitive than understory trees to CAN in terms of leaf nitrogen metabolism; and (2) Changes in leaf nitrogen assimilation and allocation in response to CAN differ among woody plants with different ecological characteristics. Finally, we consider the potential effects of CAN on the species composition of subtropical forests.

## 2. Materials and methods

### 2.1. Study site

CAN was used to realistically simulate natural nitrogen deposition in the current study. The CAN experiment was conducted at the Shimentai forest site, which is located in Shimentai National Nature Reserve (24°22′-24°31′ N, 113°05′-113°31′ E), Guangdong Province, South China. The site has a subtropical monsoon climate with a wet season (April–September) and dry season (November–March). The annual rainfall is 2364 mm, and the mean annual temperature is 20.8 °C. The site has a latosolic red soil with pH from 5.0 to 5.5. The average total soil phosphorus content was 0.37 g/kg, and while the total soil nitrogen content was 1.92 g/kg; the system is phosphorus limited. The study site is covered by a broad-leaved evergreen forest. The main overstory tree is *Castanea henryi* (Skam) Rehd; the main understory tree is *Ardisia quinquegona* Blume; and the main shrubs are *Blastus cochinchinensis* Lour., *Lasianthus chinensis* (Champ.) Benth, and *Symplocos ramosissima* Wall. ex G. Don.

Nitrogen was applied with a canopy spraying system located in the center of each CAN treatment plot (Fig. S1). The system was composed of a tank for nitrogen solution storage, connecting pipes, a supporting tower, four sprinklers, and a central computer controller. A nitrogen solution ($NH_4NO_3$) of the designated concentration was made by mixing the salt with surface lake water. Each application of nitrogen solution was equivalent to 3 mm of rainfall; approximated 7–10% of the precipitation was intercepted by the forest canopy and the rest penetrated through. The treatments were applied monthly from April to October (seven times per year) in 2013, 2014, and 2015. Nitrogen addition events were completed within 1 h and were conducted in the morning or evening on days when sunlight radiation was minimal and wind speed was <1 m $s^{-1}$. The total solution addition was 21 mm per year, which accounted for <1% of the total annual precipitation of the forest site such that, the confounding effect of water addition was negligible. Our previous study showed that the nitrogen deposition in rainfall in Shimentai National Nature Reserve was 34.1 kg N $ha^{-1}$ $year^{-1}$ (Zhang et al., 2015). Thus, 25 kg N $ha^{-1}$ $year^{-1}$ was selected as the medium level, and 50 kg N $ha^{-1}$ $year^{-1}$ was selected as the high level of nitrogen addition. The experiment had a full factorial design with two levels of nitrogen application, including 50 kg N $ha^{-1}$ $year^{-1}$ (CN50) and 25 kg N $ha^{-1}$ $year^{-1}$ (CN25), and the control (CK, 0 kg N $ha^{-1}$ $year^{-1}$). There were four blocks, and the three treatments were randomly assigned to three circular plots within each block. Each circular plot had a radius of 17 m, an area of 907 $m^2$, and a central area of 400 $m^2$ for plant and soil sampling. Between-plot nitrogen contamination was minimized by a 20-m buffer zone and the placement of polyvinylchloride boards between adjacent plots.

### 2.2. Plant species

Three native and dominant woody species of subtropical broad-leaved forest were selected for this study. *Castanea henryi* is a fast-growing tree with a straight, symmetrical trunk. It is a light-preferring pioneer species that can grow up to 30 m tall. *Ardisia quinquegona* is a shade-tolerant species. It is a non-pioneer native tree that can attain a height of 6 m. *Blastus cochinchinensis* is a mesophytic small tree or

shrub with heights ranging from 0.6 to 3.0 m (Flora of China, http://foc.eflora.cn). At the study site, the importance values were 0.3197 for *C. henryi*, 0.2219 for *A. quinquegona*, and 0.1128 for *B. cochinchinensis* and the three species are the most abundant woody plants in this site. In October 2014 (2 years after the CAN treatments were initiated) and October 2015 (3 years after the CAN treatments were initiated), the leaves from 3 to 4 individuals per species per plot were selected on outer branches of the canopy. These were used for photosynthetic measurement while they were still attached to the branch and were then collected for determination of other properties.

### 2.3. Gas exchange measurements

Photosynthesis was measured with a portable photosynthesis system (LI-6400, Li-Cor, USA) equipped with a fluorometer leaf chamber (6400-40). Branches of each sampled tree were excised and quickly placed in a water-containing bottle and acclimated under an iodine-tungsten lamp with a photosynthetic photon flux density (PPFD) within 1200 to 1600 μmol $m^{-2}$ $s^{-1}$ at a fixed distance for 15–20 min. Before the leaf photosynthetic responses to varying substomatal $CO_2$ concentrations (A-Ci curve) were measured, the light-saturated net photosynthetic rate ($A_{max}$) of leaves was obtained at 1500 μmol $m^{-2}$ $s^{-1}$ PPFD. Each A-Ci curve included nine steps, starting at 400 and decreasing to 200, 100, and 50 μmol $m^{-2}$ $s^{-1}$, and then increasing to 300, 500, 700, 1000, and 1400 μmol $m^{-2}$ $s^{-1}$. Before data were logged, leaves were allowed to equilibrate for 3 min at each step (Misson et al., 2010).

The photochemical efficiency of photosystem II ($\Phi PSII = (Fm' - Fs') / (Fm')$) was measured at each step of the A-Ci curve, and the photosynthetic electron transport rate ($J_{ETR} = 0.5\Phi PSII \cdot \alpha \cdot PPFD$) was then calculated. Fm′ is the maximum fluorescence; Fs is the steady-state fluorescence during a light-saturating pulse; 0.5 is the factor accounting for the distribution of light between the two photosystems; and α is leaf absorbance (taken as 0.93) following Niinemets et al. (2005).

The internal mesophyll diffusion conductance of the substomatal cavities to the chloroplasts ($g_m$) was estimated based on $J_{ETR}$ using the following equation (Harley et al., 1992):

$$g_m = \frac{A}{C_i - \frac{\Gamma\,[J_{ETR} + 8(A + R_d)]}{J_{ETR} - 4(A + R_d)}}.$$

where Γ was obtained from Bernacchi and Long (2002), and $R_d$ was taken as half of the rate of respiration measured in the dark (Niinemets et al., 2005). Mesophyll conductance values were then transferred from A-Ci curves to A-Cc curves, where Cc is the $CO_2$ concentration in the chloroplast stroma:

$$Cc = Ci - \frac{A}{g_m}$$

The maximum electron transport rate ($J_{max}$) and the maximum carboxylation rate ($V_{cmax}$) were estimated by using A-Cc curves and by fitting the equations of the Farquhar model (Farquhar et al., 1980). Kc and Ko, the Michaelis-Menten constants for $CO_2$ and $O_2$, were taken from Bernacchi and Long (2002) for fitting A-Cc curves.

### 2.4. Specific leaf area, chlorophyll, and nitrogen measurements

After gas exchange was measured, leaves were removed from branches, and leaf areas were measured using a portable area meter (LI-3000, LI-Cor, USA). A group of leaves (5 to 10 leaves per species per plot) was dried for 72 h at 65 °C and then weighed to calculate leaf area per dry mass (specific leaf area, SLA). Leaf nitrogen was determined on the same dried leaves with the Kjeldahl method, and photosynthetic nitrogen use efficiency (PNUE) was estimated as the ratio of $A_{max}$ to leaf nitrogen content. Another set of fresh leaves (5 to 10 leaves per species per plot) was frozen in liquid nitrogen and stored at −80 °C. For determination of leaf chlorophyll content, chlorophyll was extracted from a 0.1-g fresh leaf sample with 10 ml of 80% acetone, and the extracts were measured with a spectrophotometer at 663 and 645 nm (Lin et al., 1984).

### 2.5. Leaf protein determination and estimation

Rubisco concentration was determined from frozen leaf samples. A 0.5-g quantity of leaf sample was ground to a powder in liquid nitrogen and homogenized in 3 ml of 1 M Tris–HCl buffer (pH 6.8) with 20% sodium dodecyl sulfate (SDS), 20% glycerin, 0.5 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 3% mercaptoethanol, and 1% polyvinylpolypyrrolidone. The homogenate was centrifuged, and the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Brilliant Blue R-250. The bands of the large and small subunits of Rubisco were separated and extracted with formamide for spectrophotometric determination of Rubisco (Hikosaka et al., 1998; Guan and Wen, 2011).

Leaf proteins were divided into water-soluble, detergent-soluble, and detergent-insoluble fractions (Takashima et al., 2004). The frozen leaf samples were homogenized in 1 ml of phosphate buffer (pH 7.0) and then centrifuged at 15,000*g* for 30 min; the supernatant was regarded as the water-soluble fraction. Phosphate buffer containing 3% (w/v) SDS was then added to the pellet, which was heated and centrifuged again (repeated three times). All of the resulting supernatants were collected as the SDS-soluble fraction. The final pellet was considered to be the SDS-insoluble fraction. Each protein fraction was determined after hydrolysis to amino acids with 0.316 mmol $Ba(OH)_2$ in an autoclave (120 °C, 0.12 MPa) for 30 min. Bovine serum albumin was used to make a calibration curve (Guan and Wen, 2011). The quantities of nitrogen in metabolic nitrogen (water-soluble and SDS-soluble fractions) and structural nitrogen (SDS-insoluble fraction) were estimated assuming 16% nitrogen in proteins.

The in vivo specific activity of Rubisco was estimated as the $V_{cmax}$/Rubisco content. Nitrogen factions in Rubisco ($P_R$) were calculated assuming a nitrogen concentration in Rubisco of 16% (Hikosaka and Terashima, 1995). Nitrogen in bioenergetics ($P_B$, other Calvin cycle enzymes, ATP synthase, and electron carriers) was assumed to be proportional to $J_{max}$, where the ratio of $J_{max}$ to the cytochrome *f* content is 156 mmol $mol^{-1}$ $s^{-1}$, and the nitrogen in bioenergetics per unit cytochrome *f* is 8.06 mol $mmol^{-1}$ (Niinemets et al., 2005). Nitrogen in the light-harvesting complex ($P_L$) was calculated assuming 37.1 mol $mol^{-1}$ Chl (Evans and Seemann, 1989). Other protein nitrogen was calculated as the nitrogen partitioned into metabolic protein minus the nitrogen partitioned into photosynthesis ($P_R + P_L + P_B$). Other nitrogen was estimated as the difference between the leaf nitrogen and the nitrogen partitioned into protein.

### 2.6. Nitrogen assimilation enzyme activity determination

NR activity was determined as described by Liu et al. (2014) with slight modification. A 0.1-g quantity of frozen leaves was ground in a chilled mortar in phosphate buffer (pH 8.7) containing 10 mM cysteine and 1 mM EDTA. The homogenate was then centrifuged at 12,000*g* for 15 min at 4 °C, and the supernatant was mixed with 20 mM $KNO_3$ and 2% (w/v) NADH. After 30 min at 25 °C, the reaction was terminated by addition of 1% (w/v) sulfanilamide and 0.02% (w/v) *N*(1-naphthyl) ethylene diamine dihydrochloride. Absorbance at 540 nm was measured with a spectrophotometer after 15 min of color reflection.

To analyze NiR activity, a 0.1-g quantity of frozen leaves was extracted in Tris-HCl (pH 7.8) containing 1 mM EDTA, 15% (v/v) glycerol, 14 mM 2-mercaptoethanol, and 0.1% (v/v) Triton X-100. The homogenate was centrifuged twice at 10,000*g* for 10 min at 4 °C. NiR activity was measured by determining the decrease in $NO_2^-$ in the reaction medium (100 mM phosphate buffer at pH 6.5, 100 mM NaCl, 100 mM $NaNO_2$ and 100 mM methyl viologen and 100 mM $Na_2S_2O_4$) at 520 nm (Lillo, 1984).

GS activity was determined by detecting the formation of glutamyl hydroxamate in the extract at 540 nm after reaction with acidified ferric chloride (Kaiser and Lewis, 1984). The reaction mixture contained 100 mM Tris-HCl (pH 7.4) and 10 mM ATP. GOGAT activity was determined by measuring the oxidation of NADH in the supernatant at 340 nm (Tang, 1999). The reaction medium contained 25 mM Tris–HCl (pH 7.8), 20 mM oxoglutarate, 10 mM KCl, and 2 mM NADH.

### 2.7. Statistical analyses

Two-way repeated-measures ANOVA was performed to compare the effects of treatment (CK, CN25, and CN50), species, and their interaction on $A_{max}$, leaf nitrogen content, PNUE, chlorophyll content, Rubisco content, $V_{cmax}$/Rubisco, metabolic nitrogen content, NR, NiR, GS, and GOGAT. Levene's test was applied to test for homogeneity of variance. When necessary, variables were log-transformed to meet model assumptions. The relationships between paired variables were tested by Pearson correlation analysis (two-tailed) and logistic regression. Statistical significance was determined at $P < 0.05$. Data analyses were carried out by R version 3.4.1 (R Core Team, 2017) and SPSS 17.0 (SPSS Inc.).

## 3. Results

### 3.1. Photosynthetic performance

Relative to the control (CK), the $A_{max}$ of *C. henryi* decreased but that of *A. quinquegona* and *B. cochinchinensis* increased in response to CN25 and CN50 in both 2014 and 2015 (Fig. 1A, B). $A_{max}$ did not differ between CN25 and CN50 except for *C. henryi* in 2014, and the interaction effect between CAN and species was significant on $A_{max}$. The canopy leaf nitrogen content in *C. henryi* significantly increased in response to CN25 and CN50, and the nitrogen contents in the understory species *A. quinquegona* and *B. cochinchinensis*, however, were not changed by nitrogen addition in either 2014 or 2015 (Fig. 1C, D). As a result, in response to CN25 and CN50 in both 2014 and 2015, PNUE decreased for *C. henryi* but not for *A. quinquegona* or *B. cochinchinensis*, and all of the interaction effects were significant on PNUE (Fig. 1E, F).

### 3.2. Chlorophyll, Rubisco content, and carboxylation activity

In both 2014 and 2015, leaf chlorophyll content was elevated in *C. henryi* but not in *A. quinquegona* or *B. cochinchinensis* in CN25 and CN50 plots, and the interaction effect between species and year was significant on chlorophyll content (Fig. 2A, B). In 2014, Rubisco content in *A. quinquegona* but not in the other two species was elevated in response to CN25 and CN50 (Fig. 2C). In 2015, Rubisco content was significantly elevated only in *A. quinquegona* treated with CN25 (Fig. 2D). And the interaction effect among CAN, species, and year was significant on Rubisco content. In both years, the in vivo specific activity of Rubisco ($V_{cmax}$/Rubisco) decreased in *C. henryi* and *A. quinquegona* but increased in *B. cochinchinensis* in response to CN25 and CN50, and the interaction effect among CAN, species, and year was significant on $V_{cmax}$/Rubisco (Fig. 2E, F).

### 3.3. Leaf metabolic protein content

For *C. henryi*, metabolic protein content was significantly decreased by CN25 and CN50 in both years (Fig. 3A, B). For *A. quinquegona*,

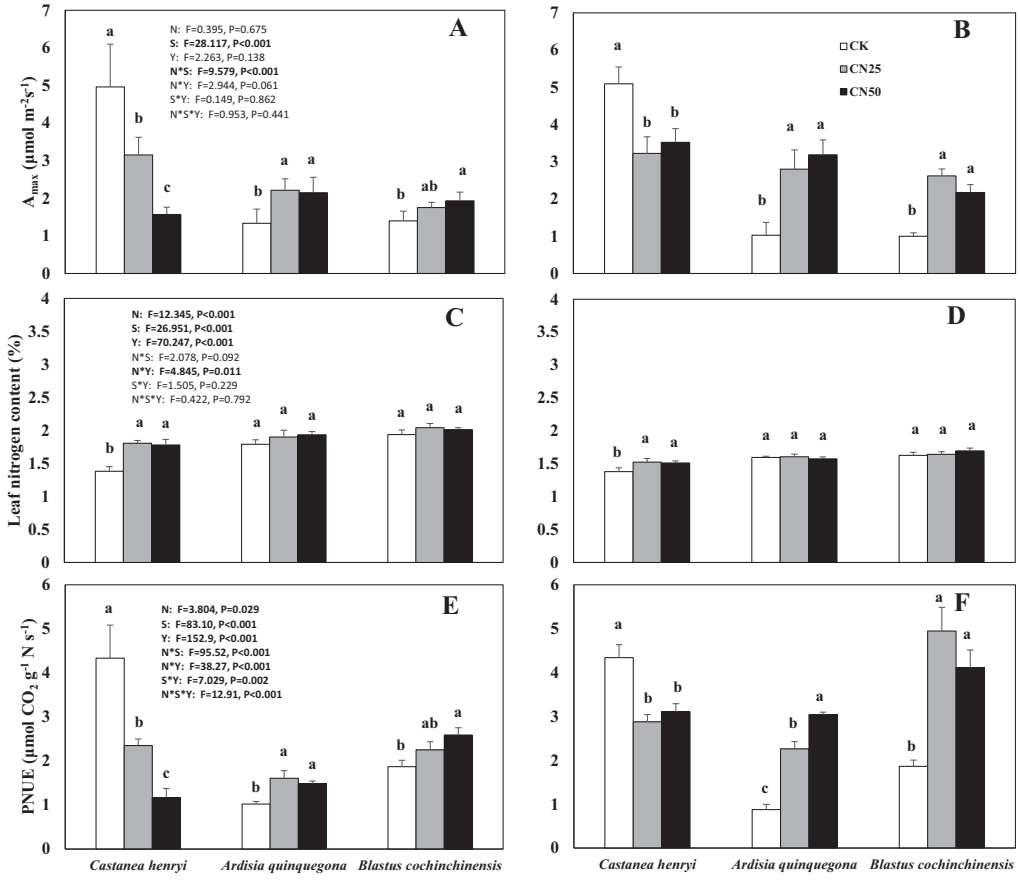


**Fig. 1.** Maximum photosynthetic rate ($A_{max}$), leaf nitrogen content, and photosynthetic nitrogen use efficiency (PNUE) of *Castanea henryi*, *Ardisia quinquegona*, and *Blastus cochinchinensis* leaves as affected by three levels of canopy nitrogen addition including 50 kg N $ha^{-1}$ $year^{-1}$ (CN50) and 25 kg N $ha^{-1}$ $year^{-1}$ (CN25), and the control (CK, 0 kg N $ha^{-1}$ $year^{-1}$) in 2014 (left panels) and 2015 (right panels). Values are means + SE. N: canopy nitrogen addition treatment; S: species; Y: year. For each groups of three bars, different lowercase letters indicate significant differences among the CAN treatments ($P < 0.05$).

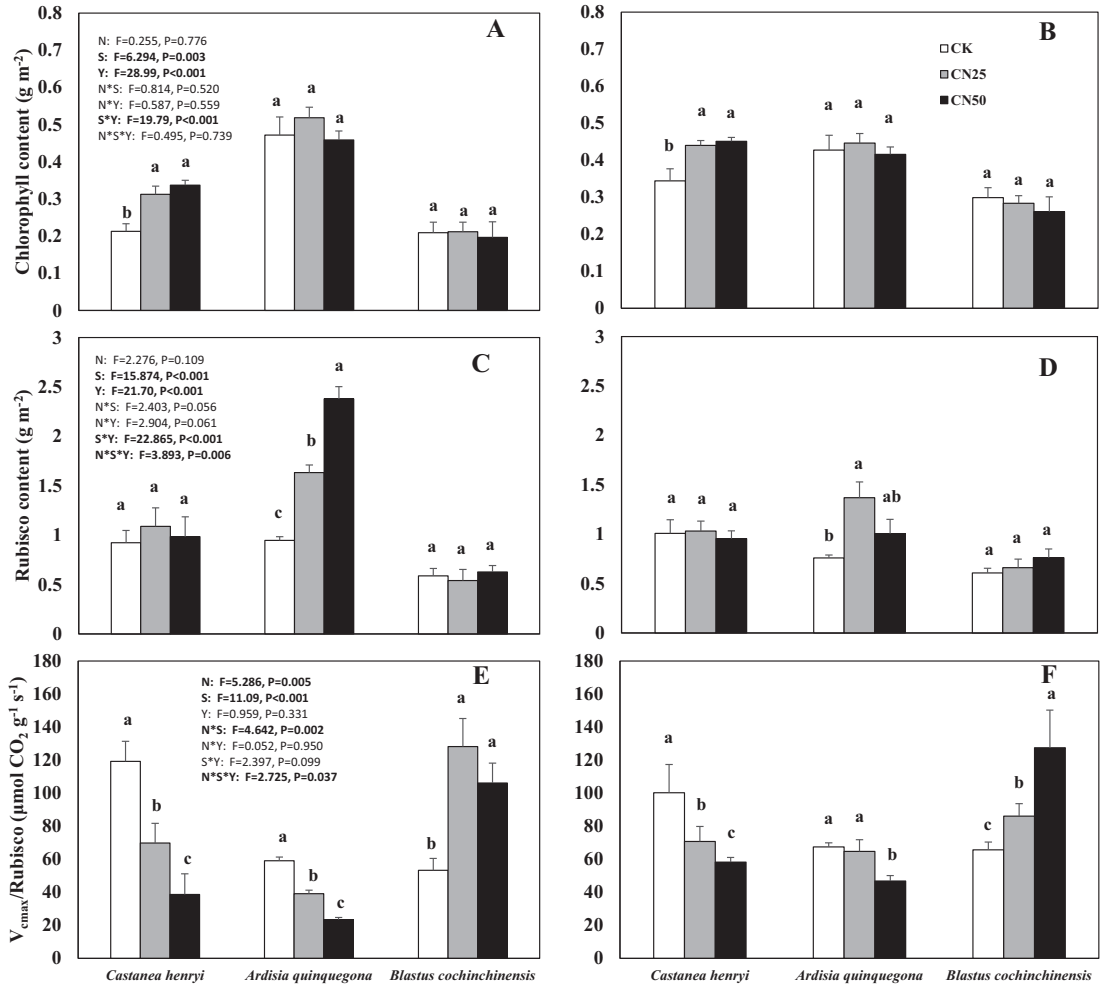


**Fig. 2.** Chlorophyll content, Rubisco content, and $V_{cmax}$/Rubisco of *Castanea henryi*, *Ardisia quinquegona*, and *Blastus cochinchinensis* leaves as affected by three levels of canopy nitrogen addition including 50 kg N $ha^{-1}$ $year^{-1}$ (CN50) and 25 kg N $ha^{-1}$ $year^{-1}$ (CN25), and the control (CK, 0 kg N $ha^{-1}$ $year^{-1}$) in 2014 (left panels) and 2015 (right panels). Values are means + SE. N: canopy nitrogen addition treatment; S: species; Y: year. For each groups of three bars, different lowercase letters indicate significant differences among the CAN treatments ($P < 0.05$).

metabolic protein content was significantly increased by CN25 in 2015 and by CN50 in both years. For *B. cochinchinensis*, metabolic protein content was significantly increased by CN25 and CN50 in 2015. In general, there were significant interaction effects between CAN and species, or between species and year on metabolic protein content.

### 3.4. Nitrogen assimilation enzyme activities

NR activity was significantly increased in *C. henryi* by CN25 and CN50 in both 2014 and 2015, but the changes were not significant in the other two species, resulting in significant interaction effects

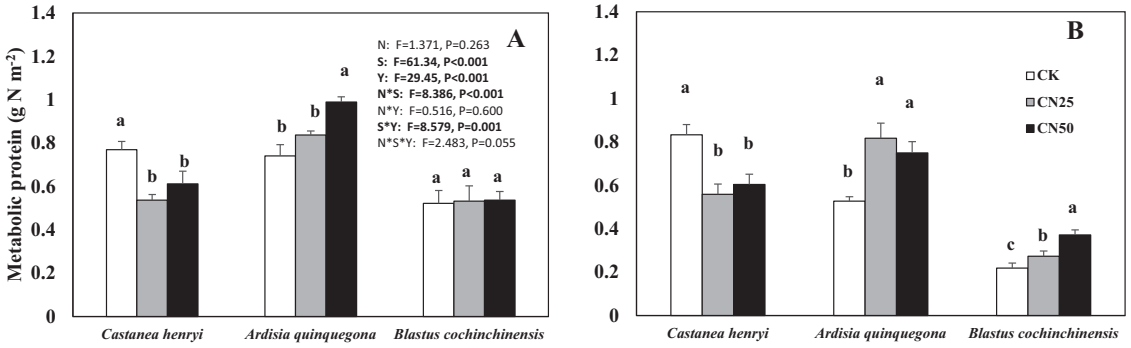


**Fig. 3.** Metabolic protein content of *Castanea henryi*, *Ardisia quinquegona*, and *Blastus cochinchinensis* leaves as affected by three levels of canopy nitrogen addition including 50 kg N $ha^{-1}$ $year^{-1}$ (CN50) and 25 kg N $ha^{-1}$ $year^{-1}$ (CN25), and the control (CK, 0 kg N $ha^{-1}$ $year^{-1}$) in 2014 (A) and 2015 (B). Values are means + SE. N: canopy nitrogen addition treatment; S: species; Y: year. For each groups of three bars, different lowercase letters indicate significant differences among the CAN treatments ($P < 0.05$).

between species and year, or between CAN and species on NR activity (Fig. 4A, B). Leaf NiR activities were mostly increased by CN25 and CN50 for *A. quinquegona* but not for *C. henryi* or *B. cochinchinensis* (Fig. 4C, D). GS activity in the leaves of *A. quinquegona* was significantly elevated by CN25 and CN50 in both years, and the interaction effect among CAN, species, and year was significant on GS activity (Fig. 4E, F). GOGAT activity did not significantly differ among the treatments for any of the three species in 2014 or 2015 (Fig. 4G, H).

### 3.5. Relationships between leaf biochemistry and nitrogen partitioning

$P_B$ was negatively correlated with leaf nitrogen content in each species (Fig. 5A, B). $V_{cmax}$/Rubisco was negatively correlated with Rubisco content in each species and across all three species, i.e., Rubisco relative activity decreased as the Rubisco content in leaves increased (Fig. 5C, D). PNUE was positively correlated with $P_B$ for each species in both 2014 and 2015 (Fig. 5E, F), indicating that a high partitioning of nitrogen to bioenergetics (other Calvin cycle enzymes, ATP synthase, and electron carriers) leads to high PNUE in the leaves of all three species.

## 4. Discussion

Information on nitrogen retention by the forest canopy is required to understand the effects of nitrogen deposition on forest ecosystems (Wortman et al., 2012; Zhang et al., 2015). The nitrogen retained by the canopy may be absorbed by canopy leaves, twigs, barks, epiphytes, and microorganisms (Sparks, 2009). It may also be immobilized by decaying leaves or dead organic matter in the canopy (Matson et al.,

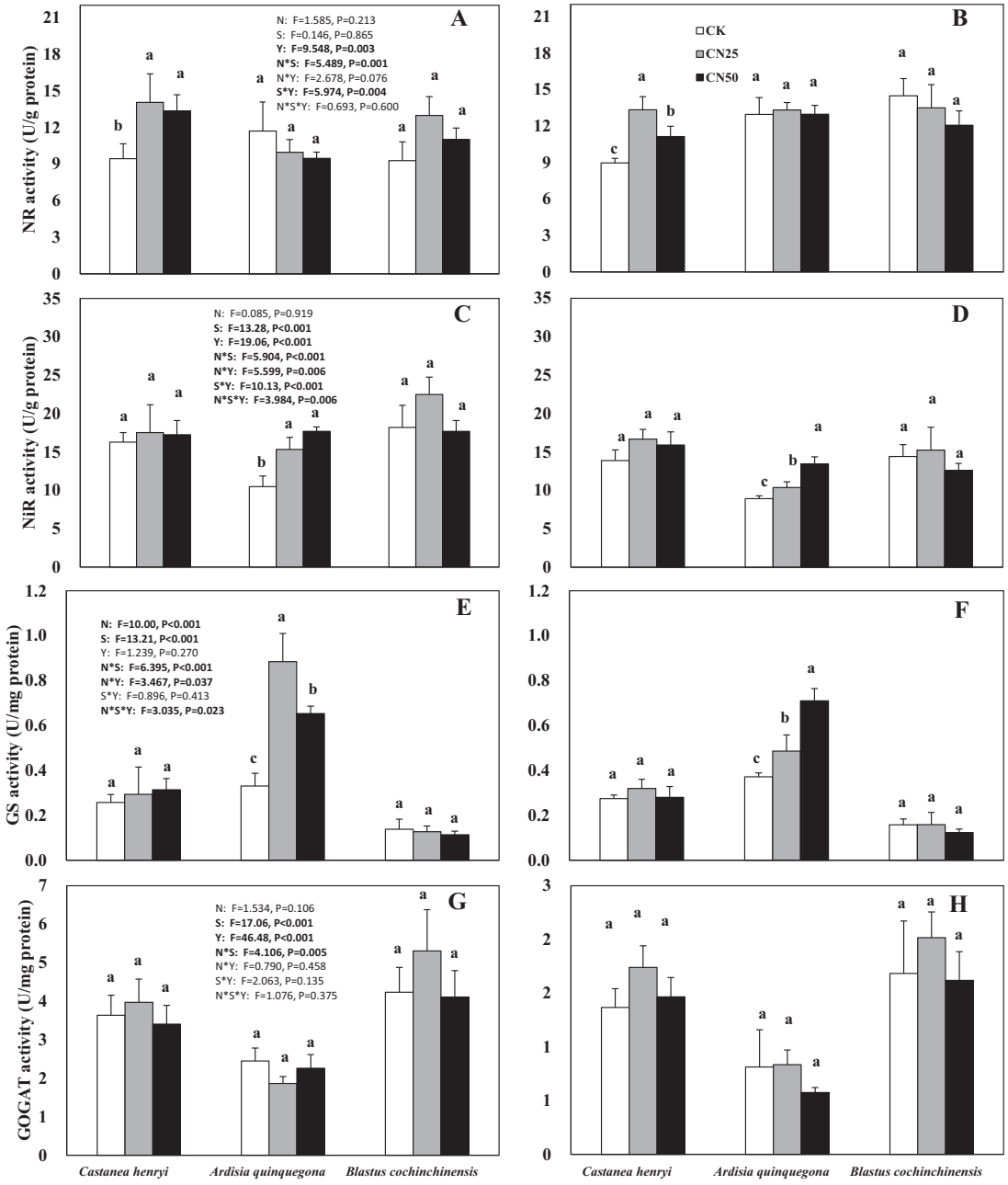


**Fig. 4.** Nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT) activities in leaves of *Castanea henryi*, *Ardisia quinquegona*, and *Blastus cochinchinensis* as affected by three levels of canopy nitrogen addition including 50 kg N $ha^{-1}$ $year^{-1}$ (CN50) and 25 kg N $ha^{-1}$ $year^{-1}$ (CN25), and the control (CK, 0 kg N $ha^{-1}$ $year^{-1}$) in 2014 (left panels) and 2015 (right panels). Values are means + SE. N: canopy nitrogen addition treatment; S: species; Y: year. For each groups of three bars, different lowercase letters indicate significant differences among the CAN treatments ($P < 0.05$).

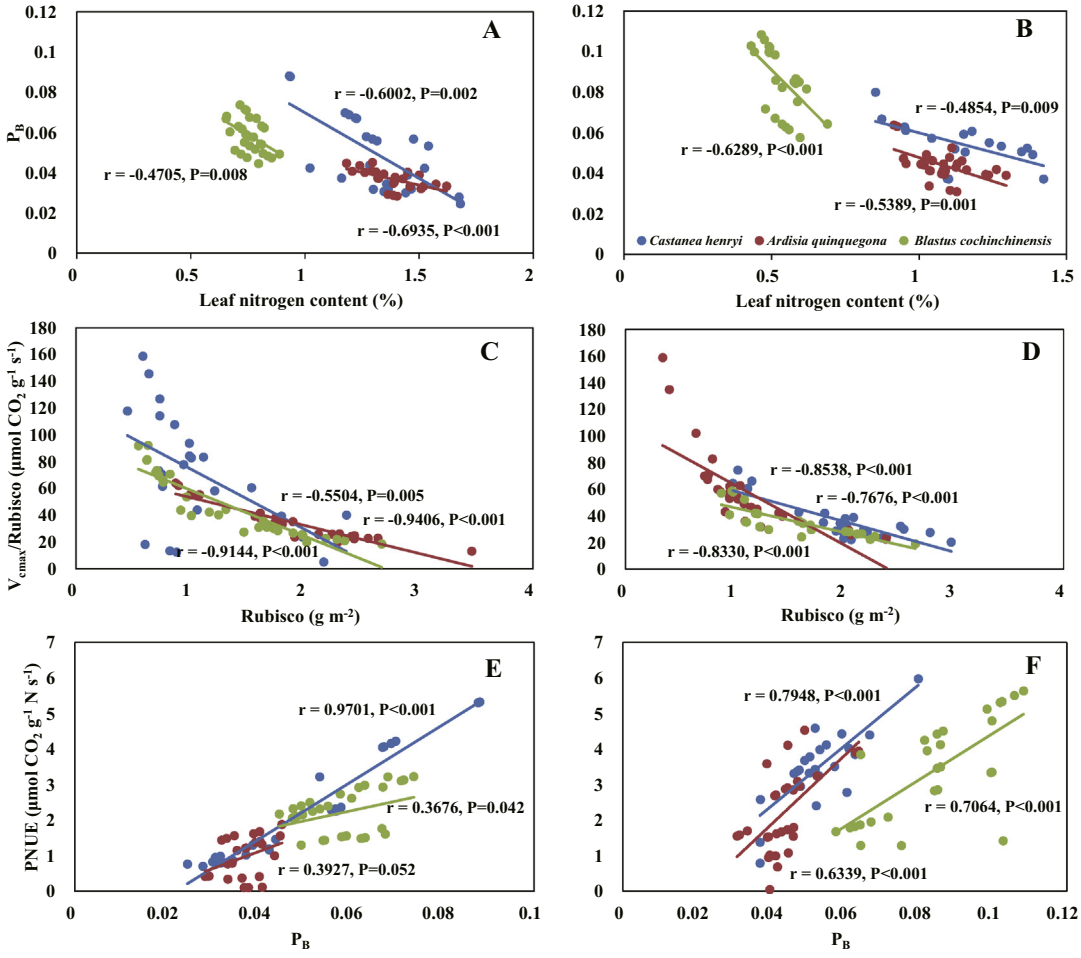


**Fig. 5.** Correlations between nitrogen partitioned into bioenergetics ($P_B$) vs. leaf nitrogen content (A, B), Rubisco activity ($V_{cmax}$/Rubisco) vs. Rubisco content (C, D), and photosynthetic nitrogen use efficiency (PNUE) vs. $P_B$ (E, F) in 2014 (left panels) and 2015 (right panels) for *Castanea henryi* (blue dots), *Ardisia quinquegona* (red dots), and *Blastus cochinchinensis* (green dots). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2014) or volatilized (Hanson and Lindberg, 1991). The proportion of nitrogen retained by forest canopies varied in different studies. For example, the canopy uptake of $NO_x$ and $NH_3$ could range from 8 to 14% and from 11 to 22%, respectively, for a 30-year-old spruce forest in Fichtelgebirge, the Czech Republic (Harrison et al., 2000). In another study, which was conducted in the Howland Integrated Forest (Maine, USA), 70% of the added nitrogen was retained in the canopy, with 10–25% of the nitrogen retained in twigs and branches, and only 3–6% in live foliage and bole wood (Gaige et al., 2007; Dail et al., 2009). At our study site, about 30–50% of nitrogen is retained by the canopies of *C. henryi* and other dominant tree species (unpublished data), and the CAN treatment increased the canopy leaf nitrogen content of *C. henryi* by 20% in 2014 and by only 5% in 2015. A significant percentage of the added nitrogen in the current study was retained by the dominant tree species, i.e., *C. henryi*, but not by the understory tree *A. quinquegona* or the shrub *B. cochinchinensis*. Perhaps because subtropical evergreen forests are nitrogen-saturated ecosystems (Mo et al., 2003; Lu et al., 2010, 2014), the input of additional nitrogen through the canopy of the dominant species may have had little effect on nitrogen absorption by leaves of the understory species *A. quinquegona* and *B. cochinchinensis*.

The increased leaf nitrogen content resulted in increased leaf chlorophyll contents in the canopy leaves of *C. henryi* in response to CN25 and CN50 treatments in both years. This agrees with many studies that found that nitrogen addition increases the light harvesting complex and photosystem components, causing 0.4- to 4.0-fold increases in total chlorophyll content (Warren et al., 2003; Ji et al., 2015). In our study, however, a higher leaf nitrogen content and elevated chlorophyll content resulted in a reduced $A_{max}$ as well as a reduced PNUE and $V_{cmax}$/Rubisco in *C. henryi* leaves treated with CN25 and CN50. Although many studies have reported a positive correlation between leaf nitrogen content and $A_{max}$ in naturally growing plants (Hikosaka et al., 1998; Kitao et al., 2018), this correlation may change in nitrogen-saturated plants. The combination of chronic and increasing nitrogen deposition can reduce Rubisco activation and decrease $A_{max}$, because the Rubisco catalytic function might be suppressed, leading to a reduced $CO_2$ assimilation efficiency (Bauer et al., 2004; Manter et al., 2005; Guan and Wen, 2011). Our finding that CN25 and CN50 treatments reduced $A_{max}$ as well as PNUE and $V_{cmax}$/Rubisco in *C. henryi* leaves but not in *A. quinquegona* or *B. cochinchinensis* leaves is consistent with our hypothesis that leaf nitrogen metabolism would be more sensitive to CAN in an overstory tree with a large canopy than in two understory trees.

Because of the retention of nitrogen by the canopy of *C. henryi* in our study, the CN25 and CN50 treatments did not change the leaf nitrogen content but increased $A_{max}$, Rubisco content, and $V_{cmax}$/Rubisco, and thus elevated PNUE for the two understory tree species, *A. quinquegona* and *B. cochinchinensis*. Therefore, the effects of CAN on leaf nitrogen allocation and carbon fixation depended on species; their positive effects only occurred in the two understory species. This indicates that the effects of CAN on plant photosynthesis differed depending on the intrinsic characteristics of the species. The increase in $A_{max}$ was also in consistent with the increased distribution of nitrogen to the photosynthetic apparatus ($P_L + P_R + P_B$) in the understory species (data not shown). In *C. henryi*, the decrease in $A_{max}$ in response to the CN25 and CN50

treatments was related to the reduced distribution of nitrogen to the photosynthetic apparatus. This suggests that, to cope with an increase in nitrogen supply, tree species may differ in how they allocate the assimilated nitrogen to photophosphorylation, ATP and NADPH syntheses, and $CO_2$ fixation during photosynthesis. This finding is consistent with our second hypothesis that leaf nitrogen assimilation and allocation of different species change in different ways in response to CAN.

Rubisco (EC.4.1.1.39), the most important enzyme in photosynthetic $CO_2$ assimilation, catalyzes the first step of the Calvin-Benson cycle, which fixes $CO_2$ through the carboxylation of ribulose-1,5-bisphosphate (Prins et al., 2016). This enzyme, however, also catalyzes additional oxygen, which leads to the loss of fixed carbon and energy through photorespiration. Rubisco is therefore considered an inefficient enzyme in carbon assimilation (Parry et al., 2007). Consistent with this view, our study revealed negative correlations between Rubisco content and carboxylation rate ($V_{cmax}$/Rubisco) for all the three species in the CK or in the CN25 and CN50 treatments. Specifically, we found that a large increase in Rubisco content did not result in increases in $V_{cmax}$/Rubisco in *A. quinquegona* leaves in the CN25 and CN50 treatments. Warren et al. (2003) reported that Rubisco, in addition to functioning catalytically, can function as a storage protein in response to nitrogen supply. In the CN25 and CN50 treatments, therefore, *A. quinquegona* stored more nitrogen in Rubisco, and the ratio of Rubisco to leaf total nitrogen ($P_R$) increased, suggesting that this species efficiently utilized the added nitrogen to produce metabolic protein components. In the other understory species, *B. cochinchinensis*, Rubisco content did not change but $V_{cmax}$ nevertheless increased, resulting in higher potentials in carbon and nitrogen assimilation in the CN25 and CN50 treatments. As shown in previous studies, different species or cultivars show large variations in carboxylase activities (Bernacchi and Long, 2002; Parry et al., 2007). Among the three subtropical woody species in the current study, *B. cochinchinensis* had the highest Rubisco carboxylation efficiency, and the two understory species benefitted more than the dominant tree *C. henryi* from the CN25 and CN50 treatments. It means that the effects of CAN on leaf carboxylase activities were different among species, and this difference showed distinct tendencies between 2014 and 2015. This also suggests that the detection of statistically significant effects may require the addition of large amounts of nitrogen in forests that are already nitrogen-saturated.

Nitrate ($NO_3^-$), which can be taken up by plant roots or leaves, is reduced to nitrite ($NO_2^-$) in the cytosol by nitrate reductase (NR), and this is the rate limiting step for nitrogen assimilation (Sanchez-Rodriguez et al., 2011; Liu et al., 2014). In the current study, NR activity in the canopy leaves of *C. henryi* was higher in the CN25 and CN50 treatments than in the CK treatment in both 2014 and 2015, indicating positive responses to $NO_3^-$ addition. This finding differs from that of a previous study in which the overstory foliar NR activities of tree species were unaffected by nitrogen enrichment in a northeastern US forest (Tang et al., 2012). We suspect that this difference may partly reflect the species-specific responses to nitrogen addition. In contrast to the leaves of the evergreen tree *C. henryi* in the current study, the leaves of deciduous trees increase their nitrogen resorption efficiency rather than their NR activity in response to nitrogen enrichment (Lovett and Mitchell, 2004). As a pioneer fast-growing species, *C. henryi*, increased its NR activity under simulated nitrogen deposition as a positive response to additional $NO_3^-$ uptake.

For the two understory species, *A. quinquegona* and *B. cochinchinensis*, NR activities were unaffected in the CN25 and CN50 treatments. In chloroplasts or plastids, $NO_2^-$ reduced from $NO_3^-$ is converted into ammonium ($NH_4^+$) by nitrite reductase (NiR). Excessive $NH_4^+$ resulting from both $NO_3^-$ reduction and direct absorption is toxic to plant cells and must be assimilated quickly through the GS/GOGAT cycle (Thomas and Hilker, 2000). In our study, NiR and GS activities of *A. quinquegona* were significantly elevated in the CN25 and CN50 treatments, which partially agrees with a previous study conducted on moss species under conditions of nitrogen addition (Liu et al., 2016). Perhaps *A. quinquegona* has a substantial capacity to acclimate to additional nitrogen supply through $NH_4^+$ assimilation mainly via the GS/GOGAT cycle; such assimilation could enable *A. quinquegona* to compose more metabolic proteins. Pioneer trees are generally sensitive to $NH_4^+$ and they prefer higher $NO_3^-$ (Oliveira et al., 2017). Our preliminary data (unpublished) also show that *C. henryi* may maximize its leaf $NO_3^-$ uptake by quickly increasing its leaf $NO_3^-$ concentration under nitrogen addition. We believe that the pioneer tree, *C. henryi*, may be stressed by the toxic effects of $NH_4^+$ accumulation as a result of the elevated $NH_4NO_3$ uptake and increased NR activity (although GS/GOGAT activity did not change). The toxic effects could reduce photosynthetic performance and the production of metabolic protein, thus repressing carbon assimilation and biomass accumulation when *C. henryi* is subjected to foliar nitrogen deposition.

## 5. Conclusion

Our results show that, in subtropical evergreen broad-leaved forests, an increase in the quantity of nitrogen delivered to the canopy may increase the nitrogen assimilation and utilization of small trees and shrubs (e.g., *A. quinquegona* and *B. cochinchinensis*) but inhibit the nitrogen metabolism of dominant trees with large canopies (e.g., *C. henryi*). Although we did not examine all tree species in the studied forest, the three species studied are the most abundant woody plants in this forest, and their responses to nitrogen addition should indicate the true effect of nitrogen deposition on the forest. We believe that the increased nitrogen deposition over the past several decades may be responsible for the degradation of subtropical forests, i.e., for their transformation into woodlands, as previously suggested (Zhou et al., 2014). Finally, alterations in nitrogen metabolism of different plants could potentially result in changes in carbon sequestration and biomass accumulation of subtropical forests under elevated nitrogen deposition in the near future.

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*Conflicts of interest*

None.

*Author contributions*

N.L., S.W. and J.W., analyzed the data and wrote the paper. Q.G. and N.L. designed the study, proposed the scientific hypothesis and supervised the project. N.L., S.W., J.W., and C.C. carried out the experiments. N.L. and S.W. collected and processed samples. N.L., S.W., and Q.G. contributed to the interpretation of the work. All authors discussed the results and reviewed the manuscript.

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