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# Alterations in leaf nitrogen metabolism indicated the structural changes of subtropical forest by canopy addition of nitrogen

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ABSTRACT

Globally, nitrogen deposition increment has caused forest structural changes due to imbalanced plant nitrogen metabolism and subsequent carbon assimilation. Here, a 2 consecutive-year experiment was conducted to reveal the effects of canopy addition of nitrogen (CAN) on nitrogen absorption, assimilation, and allocation in leaves of three subtropical forest woody species (*Castanea henryi*, *Ardisia quinquegona*, and *Blastus cochinchinensis*). We hypothesized that CAN altered leaf nitrogen absorption, assimilation and partitioning of different plants in different ways in subtropical forest. It shows that CAN increased maximum photosynthetic rate ($A_{max}$), photosynthetic nitrogen use efficiency (PNUE), and metabolic protein content of the two understory species *A. quinquegona* and *B. cochinchinensis*. By contrary, for the overstory species, *C. henryi*, $A_{max}$, PNUE, and metabolic protein content were significantly reduced in response to CAN. We found that changes in leaf nitrogen metabolism were mainly due to the differences in enzyme (e.g. Ribulose-1,5-bisphosphate carboxylase, nitrate reductase, nitrite reductase and glutamine synthetase) activities under CAN treatment. Our results indicated that *C. henryi* may be more susceptible to CAN treatment, and both *A. quinquegona* and *B. cochinchinensis* could better adapt to CAN treatment but in different ways. Our findings may partially explain the ongoing degradation of subtropical forest into a community dominated by small trees and shrubs in recent decades. It is possible that persistent high levels of atmospheric nitrogen deposition will lead to the steady replacement of dominant woody species in this subtropical forest.

## 1. Introduction

Forests account for 80% of Earth's plant biomass and 75% of the gross primary productivity of the Earth's biosphere (Pan et al., 2013). However, the world's forests declined from 4128 million ha in 1990 to 3999 million ha in 2015, and about 3.3 million ha of forests have been converted to degraded land or other land uses annually between 2010 and 2015 (FAO, 2015). Such huge forest declines have caused a series of ecological and environmental issues, and resulted in alterations of forest stand structure (Cohen et al., 2016), loss of biodiversity (Lu et al., 2010), enhanced soil erosion and land degradation (Ren et al., 2007). Apart from direct deforestation, global climate change has also had a disruptive influence in many ecosystems and contributed to forest decline (Anderegg et al., 2015; Cohen et al., 2016; Chen et al., 2018). Changes in precipitation and temperature regimes (Zhou et al., 2014), frequent extreme climatic events (Lloret et al., 2012), as well as increased rates of nitrogen deposition (Lu et al., 2010) continue to impact forest ecosystems in multiple ways.

Globally, nitrogen deposition has increased drastically due to human activities, such as increases in fossil fuel combustion, agricultural fertilization, and industrial pollution (Galloway et al., 2008). The reactive nitrogen deposited on the Earth's surface has increased from 34 Tg N $yr^{-1}$ in 1860 to 100 Tg N $yr^{-1}$ in 1995, and is going to reach 200 Tg N $yr^{-1}$ by 2050 (Galloway et al., 2004). As a driver of global change, nitrogen deposition has been proved to cause forest degradation, soil acidification and land degradation, but much of the

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long-term effects on forest structure and function (Magill et al., 2004; Galloway et al., 2008; Lu et al., 2010, 2014; Talhelm et al., 2013) remains to be investigated.

Nitrogen is largely required by plants in the synthesis of amino acids, proteins, chlorophylls, nucleic acids, lipids, and other metabolites, and plants adjust internal nitrogen status to regulate nitrogen uptake and assimilation in order to match plant demand (Kusano et al., 2011). Thus, the increased deposition of nitrogen may be a disruptive influence in natural ecosystems, causing an imbalance in the absorption, assimilation and allocation of nitrogen within plants (Warren et al., 2003). Some studies have found that the nitrogen addition could increase leaf biomass and ribulose-1,5-bisphosphate carboxylase (Rubisco, the key enzyme in photosynthesis) content so as to increase carbon assimilation forest (Warren et al., 2003; Högberg, 2007). However others have found that the nitrogen addition may cause the degradation of chlorophyll (Shi et al., 2017), disorder of carbon metabolism (Bauer et al., 2004), increase in free amino acid content (Strengbom et al., 2003), decrease in forest viability (Liu et al., 2011). As a result, nitrogen deposition was found to change the species composition and function of forests (Nordin et al., 2005; Lu et al., 2010; Gilliam et al., 2006).

China has experienced substantial nitrogen deposition, especially in its rapidly developing central and southeastern regions, and the rates of nitrogen deposition are predicted to increase dramatically in the future (Liu et al., 2011, 2013; Jia et al., 2014). Till date however, studies on the effects of nitrogen deposition on forest have mainly focused on coniferous or deciduous broad-leaved forests in the temperate zone (Takashima et al., 2004; Gradowski and Thomas, 2006; Högberg, 2007; Janssens et al., 2010), and few studies have concentrated on tropical and subtropical evergreen broad-leaved forests (Lu et al., 2010, 2014).

Worldwide, there are only a few nitrogen addition experiments, and even they have resorted to spraying nitrogen on understory plants (Warren et al., 2003; Högberg, 2007; Lu et al., 2014). These experiments on understory plants do not therefore account for the effects of nitrogen deposition on canopy-associated biota and processes, which may not reflect the full range of effects of nitrogen deposition in forest ecosystems (Zhang et al., 2015; Guerrieri et al., 2015).

In this study, we determined the effects of canopy addition of nitrogen (CAN) on nitrogen absorption, assimilation and partitioning in three woody species in a subtropical forest. We focused on the potential effects of CAN on species composition of subtropical forest and the modifications to leaf nitrogen metabolism as a driver of forest degradation. We tested the hypotheses that CAN changes nitrogen absorption, assimilation, and partitioning in canopy leaves, and that these changes would differ among tree species. Finally, we consider tree species selection in forest restoration and development.

## 2. Materials and methods

### 2.1. Study site

For the realistic simulation of natural nitrogen deposition in the forest ecosystems, the CAN experiment was conducted at the Shimentai Experimental Station, which is located in Shimentai National Nature Reserve (24°22'–24°31' N, 113°05'–113°31' E), Guangdong Province, China. The study site is covered by broad-leaved evergreen forest. The CAN experiment is a full factorial design with 3 different levels of treatments (Fig. 1), including 25 kg N $ha^{-1}$ $yr^{-1}$ (CN25), 50 kg N $ha^{-1}$ $yr^{-1}$ (CN50), and 0 kg N $ha^{-1}$ $yr^{-1}$ (CK). Four blocks was set up in the forest site and each treatment was replicated once at each of the four blocks. Three treated plots were randomly assigned in each block and a total of 12 plots were established corresponding to three treatments with four replications. Nitrogen was applied with a canopy spraying system located in the center of each CAN treatment plot. Each circular plot has a 17 m semi diameter with an area of 907 $m^2$, leaving the central core area of 400 $m^2$ for plant sampling (3–4 individuals per species per plot). Contamination of nitrogen solution between each plot was minimal as they were separated by at least 20 m buffer zone, and polyvinylchloride boards were inserted between two adjacent plots when necessary. 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, with 30–40% of the precipitation intercepted by the forest canopy and the rest penetrating through. The treatments were applied monthly from April to October (seven times per year) from year 2013 to 2016. The total solution addition was 21 mm per year, accounting for less than 1% of total annual precipitation of the forest site, so the confounding effect caused by water addition was negligible (Zhang et al., 2015).

### 2.2. Plant species

Three native woody species were chosen for this study. *Castanea henryi* (Skan) Rehd. et Wils. is the representative tree species of subtropical broad-leaved forests. The tree can grow up to 30 m tall with a straight, symmetrical trunk. *Ardisia quinquegona* Bl. is a tree in the Myrsinaceae family and can attain a height of 6 m. *Blastus cochinchinensis* Lour. is a small tree or shrub with heights ranging from 0.6 to 3.0 m (Ren et al., 2010). At this study site, the importance values for *C. henryi*, *A. quinquegona*, and *B. cochinchinensis* were 0.3197, 0.2219, and 0.1128, respectively. In June 2015 and 2016, the current-year leaves were collected from the outer branches of each species at similar heights (2–3 m) in each plot. Leaves were taken for photosynthetic measurements while they were still attached to the branch. After that, they were collected for determination of other properties in lab.

### 2.3. Measurement on gas exchange

Leaf photosynthesis was measured using a portable photosynthesis system (LI-6400, Li-Cor, USA) equipped with a fluorometer leaf chamber (6400-40). The light-saturated net photosynthetic rate ($A_{max}$) of leaves was obtained at 1500 μmol $m^{-2}$ $s^{-1}$ PPFD, before leaf photosynthetic responses to varying substomatal $CO_2$ concentrations (A-Ci curve) were measured. Each A-Ci curve was measured in nine steps, starting from 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 equilibrated for at least 3 min at each step (Misson et al., 2010). The maximum carboxylation rate ($V_{cmax}$) and the maximum electron transport rate ($J_{max}$) were estimated by A-Cc curves (transferred from A-Ci curves). The curves were fitted by the equation of the Farquhar model (Farquhar et al., 1980).

### 2.4. Measurements on specific leaf area, chlorophyll, and nitrogen content

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

### 2.5. Determination in Rubisco and leaf protein

Rubisco concentration was assayed from frozen leaf samples. A 0.5-g leaf sample was ground in liquid nitrogen and homogenized. The homogenate was then centrifuged, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the gels