

EFFECTS OF AN EIGHT-YEAR NITROGEN AND PHOSPHOROUS ADDITION ON LEAF PHOTOSYNTHESIS AND CHEMISTRY OF MATURE *CASTANOPSIS SCLEROPHYLLA* TREES IN SUBTROPICAL CHINA

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Abstract. How long-term nitrogen (N) deposition affects plant growth in N-rich subtropical forest ecosystems, and whether there are interactive effects between N and phosphorous (P) addition on physiological performances remain unclear. To address these questions, an eight-year nutrient addition experiment was conducted to examine the chronic effects on trees in a secondary *Castanopsis sclerophylla* forest in subtropical China. Our key results showed that (1) Long-term N deposition could stimulate mature *C. sclerophylla* growth mainly by increasing the net photosynthetic rate (*Pn*), soluble sugars (*SS*) and total non-structural carbohydrates (*NSCs*). Furthermore, there were significant interactive effects on growth between N and P addition. (2) Leaf chlorophyll a + b, *SS* and total *NSCs* under N, P and NP addition were all increased in two years (except the chlorophyll a + b in 2020 and total *NSCs* in 2019). The positive impacts of P or NP addition on leaf chlorophyll a + b, *SS* and total *NSCs* were stronger compared to N addition. (3) Compared to N addition, P addition had a positive stimulation on leaf *Pn*. These conclusions demonstrated that P addition either alone or together with the addition of N have positive effects on the physiological performances of mature *C. sclerophylla* trees.

Keywords: *nitrogen deposition, photosynthesis, soluble protein, nonstructural carbohydrate, nitrogen and phosphorus interaction*

Introduction

Due to intensive human activities in recent decades (e.g., agricultural intensification, fuel combustion, automobile exhaust emissions), the global nitrogen (N) deposition rate has increased dramatically and is expected to double from the current level by 2050 (Yu et al., 2019; Liu et al., 2019, 2016). Considering that N is the limiting nutrient for plant growth, development and photosynthesis, increased N deposition can provide a new source of fertilizer for plant growth (Güsewell, 2004; Li et al., 2016; Luo et al., 2015), but conversely, can modify biogeochemical cycles (Lu et al., 2014), change ecosystem structures and functions (Cao et al., 2019), and even lead to local extinction of plant species (Gotelli and Ellison, 2002; McClean et al., 2011). Phosphorous (P) is another limiting nutrient affecting plant growth. In contrast to deposited N which has multiple pathway and exhibits high mobility, P originates from fewer sources and has less mobility (Mahowald et al., 2008). Plant N: P stoichiometry can be used to determine

plant adaptation and feedback in response to environmental change. In N-saturated ecosystems, mainly in subtropical and tropical forests, excess N inputs may decrease soil labile P fractions and cause ecological N:P stoichiometry imbalance (Hui et al., 2020; Chen et al., 2018). Although the increasing recognition of soil P availability in regulating N deposition effects on subtropical and tropical forests, the direct experiment of how tree growth and physiology affected by P addition interact with N deposition is still insufficient.

N influences the carboxylation capacity and electron transport rates in photosynthesis (Evans, 1989). Thus, the effects of N addition on plant photosynthesis have been well recognized. However, the results have been inconsistent in different tests, giving both positive (Liang et al., 2020; Liu et al., 2019), neutral (Talhelm et al., 2011) and negative responses (Mao et al., 2017). In N-limited ecosystems, for temperate forests in particular, N deposition can increase photosynthetic capacity and simulate plant growth (Shi et al., 2020). In addition to the universal concept that leaf P is an essential element related to photosynthesis (Crous et al., 2017), P deficiency can affect light-use efficiency (Conroy et al., 1986). However, it remains unclear whether P addition can alleviate the effect of N-induced P limitation on plant photosynthesis.

As necessary components of proteins, amino acids and chlorophylls, N and P are involved in synthesizing various metabolites (Liu et al., 2018a). Thus, either N or P addition can influence leaf biochemical composition and metabolic processes. According to previous studies, excess N input can stimulate the formation of chlorophyll, soluble protein, and free amino acids (FAA) in plant tissues (Fritz et al., 2012), which can disorder N metabolism subsequently. Non-structural carbohydrates (NSCs) are the main substrates for both primary and secondary plant metabolism, can provide a buffer when plant photosynthesis is insufficient (Hartmann and Trumbore, 2016). N and P addition can affect plant photosynthetic processes and the concentration of NSCs including soluble sugar (SS) and starch (ST) (Liu et al., 2016; Mo et al., 2020). At present, numerous experiments have investigated the effects of increased nutrients supply on tree physiological traits, including leaf gas exchange, biochemical components concentration and carbon (C): N: P stoichiometry. These experiments usually use pulse additions on seedlings or understory shrubs over a relative short timescale (Wang et al., 2019; Mao et al., 2017), which may not indicate the true chronic effects on mature trees.

Castanopsis sclerophylla, a dominant species in evergreen broad-leaved forests, is widely distributed in subtropical areas of eastern Asia and has high ecological and economic value (Shi et al., 2011). This species plays a crucial role in maintaining the function and stability of local ecosystems (Zhang et al., 2007). Over the past few decades, the subtropical areas of eastern China have been affected by human activities, including enhanced N deposition that clearly affects many aspects of its ecosystems. In this study, changes in plant growth, leaf gas change, nutrient status, and chemical traits in a *C. sclerophylla* secondary forest with 8-year N deposition and P addition were investigated. Given the status of local soil as P-limitation (Teng et al., 2018; Han et al., 2005), our objectives were to address the following questions: (1) How does long-term N addition affect the growth, leaf photosynthesis and chemistry of the mature *C. sclerophylla*? (2) whether or not there are interactive effects between N and P addition on physiological performances.

Material and methods

Study site

The study site (30°01'47"N, 117°21'23"E) was located in Chizhou, south Anhui Province, China. It has a humid subtropical monsoon climate with an average temperature of 16 °C, and an average rainfall of 1521 mm. The soil is thin (between 70 and 100 cm depth) and has a clay-loam texture. The original vegetation was severely destroyed before the 1960s and restored by planting *Castanopsis sclerophylla* (Lindl.) Schott around 1965. This forest is now dominated by *C. sclerophylla* with a diverse sub-canopy of various tree species.

Experimental setup

In June 2012, twelve permanent plots were established (15 m × 15 m, 10-m wide buffer zone). The diameter at breast height (*DBH*) in 2012 showed no significant difference between plots. The experiment included four treatments with three replicates in a randomized block design. The treatments were: control (CK), N addition (100 kg N ha⁻¹ year⁻¹), P addition (50 kg P ha⁻¹ year⁻¹) and NP addition (100 kg N ha⁻¹ year⁻¹ + 50 kg P ha⁻¹ year⁻¹). N was applied as NH₄NO₃, and P as Ca(H₂PO₄)₂. Nutrients were dissolved in 20 L water and evenly sprayed into the corresponding plots near the soil surface using a backpack sprayer every 2 months from June 2012. Control plots only received 20 L water. The buffer zone of 10-m surrounding the plots was also fertilized.

Growth and photosynthesis measurements

In January 2019 (T₁) and December 2019 (T₂), we measured *DBH* of all individuals using a diameter ruler to calculate the breast growth rate (*GR_{DBH}*):

$$GR_{DBH} = (DBH_{T_2} - DBH_{T_1}) / DBH_{T_1} \quad (\text{Eq.1})$$

In July 2019 and July 2020, while constrained by the health status of suitable leaves, at least four trees were randomly selected from each plot for the photosynthesis measurements. We used a 20m-long sea fishing rod with a blade attached to the end (CF140-20, Gaoding, Co., Ltd., China) to collect the fully-extended branches in the canopy (>15 m). During measurements, branches were put into big bucket full of water to avoid water loss. The light response curve measurements were conducted on fully expanded healthy leaves using a Li-6400 portable photosynthesis system (Li-Cor, USA). The chamber CO₂ concentration was 380 μmol mol⁻¹, light intensity was 2000 μmol m⁻² s⁻¹, and leaf temperature was consistent with atmospheric temperature. After acclimating to the cuvette environment, leaf photosynthetic light response curve was measured in fifteen steps of light intensity, starting at 2000 and decreasing to 1800, 1500, 1300, 1100, 900, 700, 500, 400, 300, 200, 150, 100, 50 and 0 μmol m⁻² s⁻¹. Leaf photosynthesis was monitored to ensure reaching a stable state at each light intensity before data were recorded. The light response curves were fitted using the equation of the collect nonrectangular hyperbolic model (Ye, 2007).

The tendency of respiration rate (*R_d*) is given by:

$$R_d = -\alpha LCP \quad (\text{Eq.2})$$

The light compensation point (*LCP*) is given by:

$$LCP = \frac{-(\gamma R_d - \alpha) - \sqrt{(\gamma R_d - \alpha)^2 - 4\alpha\beta R_d}}{2\alpha\beta} \quad (\text{Eq.3})$$

The light saturation point (*LSP*) is given by:

$$LSP = \frac{\sqrt{(\beta + \gamma)\beta} - 1}{\gamma} \quad (\text{Eq.4})$$

The apparent quantum yield (*AQE*) is given by:

$$AQE = \alpha \frac{1 + (\gamma - \beta)LCP - \beta\gamma LCP^2}{(1 + \gamma LCP)^2} \quad (\text{Eq.5})$$

where α , β , and γ are coefficients which are independent of light intensity.

In this study, the light-saturated net photosynthetic rate (*Pn*), transpiration rate (*E*), instantaneous water use efficiency (*WUE_i*) and stomatal conductance (*g_s*) were measured at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. The leaf colour (*SPAD*) was measured with a chlorophyll meter (*SPAD-502P*, Yunnong, Co., Ltd., China).

Leaf pigments, soluble protein and free amino acid concentrations

After the leaf *Pn* measurement, leaves were picked off and immediately put into zip-lock bags with ice packs in a plastic bucket, and then stored at -20 °C freezer until leaf soluble protein (*SP*) and *FAA* measurements were conducted. The collected samples were cleaned with deionized water, and the surface water was sucked dry with filter paper. About 0.20 g fresh leaves were homogenized with a small amount of CaCO_3 and quartz sand, containing 3 ml 95% (v/v) ethanol, filter and constant volume to 25 ml. The Chlorophyll a (*Chl a*) and Chlorophyll b (*Chl b*) were determined at 665 nm and 649 nm using a microplate reader (Epoch 2, BioTek Instruments, Inc., USA).

SP and *FAA* were extracted according to Xu and Zhou (2006). Fresh leaves were homogenized with 10 ml of 50 mM sodium phosphate (pH = 7.8), containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 80 mM L-Ascorbic acid. The supernatants were collected for *SP* and *FAA* by centrifugation for 20 min at 15000 r min^{-1} . The *SP* was determined according to Bradford (1976). The *FAA* was measured with an amino acid auto analyzer (Hitachi 835-50, Hitachi Ltd., Japan) according to Barnett and Naylor (1966).

Leaf structural traits, nutrient status and NSCs

Leaf area was measured using a Li-3100A leaf area instrument (Li-Cor, USA). Specific leaf area (*SLA*) was calculated as leaf area divided by leaf dry mass. After the leaf pigments, *SP* and *FAA* measurements, leaf samples were deactivated at 105 °C for 30 min, and then oven-dried at 60 °C to determine their dry mass. Oven-dried leaves were pulverized with a Mill (BO-500S1, Boou, Co., Ltd., China), then finally passed through a 100-mesh sieve. N and P concentration per leaf dry mass (*N_{mass}* and *P_{mass}*)

were measured using a sulfuric acid/hydrogen peroxide digest. The leaf powder samples (0.100 g) were put into a 50 ml conical flask, where 3 ml distilled water and 5 ml sulfuric acid were added to dissolve the liquid at 240 °C about 3 h using a Graphite heating plate (CB-2, Keheng, Co., Ltd., China). The leaf N and P concentrations were analyzed with an element analyzer (CleverchemAnn, Germany). Leaf N concentration per leaf area (*Narea*) was calculated as *Nmass* divided by *SLA*. Photosynthetic N-use efficiency (*PNUE*) was calculated as follows:

$$PNUE = Pn/Narea \times 10 \quad (\text{Eq.6})$$

The total *NSCs* was the sum of *SS* and *ST*. Leaf *NSCs* was analyzed by standard anthrone-colourimetric method. *SS* were extracted from 50.0 mg powder sample with 4 ml 80% (v/v) ethanol. The extraction was incubated in a water bath at 80 °C for 30 min. The supernatants were collected for *SS* concentration by centrifugation for 5 min at 3000 r·min⁻¹. The residue was extracted two more times as described above. To measure *ST*, briefly, after cooling to room temperature, the residue of the ethanol extraction was extracted two times with 2 ml HClO₄ followed by colourimetric analysis with anthrone/sulfuric acid. The *SS* and *ST* were analyzed at 625 nm using a microplate reader (Epoch 2, BioTek Instruments, Inc., USA).

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (SPSS, Inc., USA). Before analysis, the data were tested for normal distribution and homogeneity of variances. Multiple comparisons were conducted using Duncan's test to evaluate the differences in variables between four treatments. We conducted two-way ANOVA to evaluate the interacting effects between N and P addition. A *p* value of less than 0.05 was considered statistically significant.

Results

Tree growth, specific leaf area and leaf nutrient

The *GR_{DBH}* under N, P and NP addition were promoted (25.35%, 19.25% and 32.86% when compared with CK, respectively; *Fig. 1a*). We did not detect significant effect of N, P addition and their interaction on *SLA* in both 2019 and 2020.

N addition had no significant effect on *Nmass* and leaf *N/P* in 2019, while it increased *Nmass* in 2020 (*Fig. 2a*). P and NP addition increased *Pmass* and decreased leaf *N/P* (14.67 and 10.68 when compared with 27.06 for the CK, respectively) in 2020, but they had no significant effect in 2019 (*Fig. 2c*). Two-way ANOVA analysis found that no significant interactive effect between N and P on *Nmass*, *Pmass* and *N/P* in 2019 (*Table 1*).

Leaf pigments and photosynthesis

P and NP addition all increased *Chl a* and *Chl b* in 2020, and N addition increased *Chl b* in 2019. Furthermore, *C. sclerophylla* possessed a lower *Chl b* under N addition when compared to P and NP addition in 2019. Meanwhile, both *Chl a* and *Chl b* under all treatments were much higher in 2020 than in 2019. Thus, a similar pattern was found

for leaf *Chl a + b* (Fig. 3). Two-way ANOVA analysis found that significant interactive effects between N and P on leaf *Chl b* in both two years and *Chl a + b* in 2020 ($p < 0.05$), whereas it had no effect on leaf *Chl a* (Table 1).

Table 1. Results (*F* and *P* values) from two-way ANOVA analysis for the effects of N addition, P addition and their interactions on foliar chemical traits

Source of variation	Time	N addition		P addition		NP addition	
		F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
<i>SLA</i>	July 2019	0.078	0.787	6.502	0.034	0.034	0.858
	July 2020	2.543	0.149	1.989	0.196	0.488	0.504
<i>Nmass</i>	July 2019	0.526	0.489	2.397	0.160	1.489	0.257
	July 2020	0.008	0.929	6.043	0.039	31.408	0.001
<i>Pmass</i>	July 2019	0.159	0.700	5.360	0.049	2.465	0.155
	July 2020	5.785	0.043	83.537	0.000	0.019	0.894
<i>N/P</i>	July 2019	1.227	0.300	6.129	0.037	3.886	0.084
	July 2020	2.537	0.150	43.659	0.000	0.162	0.697
<i>Chl a</i>	July 2019	1.559	0.247	9.195	0.016	0.065	0.805
	July 2020	15.655	0.004	56.718	0.000	2.212	0.175
<i>Chl b</i>	July 2019	74.324	0.000	183.414	0.000	9.247	0.016
	July 2020	27.186	0.001	60.350	0.000	15.856	0.004
<i>Chl a + b</i>	July 2019	29.279	0.001	84.669	0.000	3.193	0.112
	July 2020	20.420	0.002	62.228	0.000	5.509	0.047
<i>SS</i>	July 2019	18.557	0.003	14.349	0.005	1.050	0.336
	July 2020	1.421	0.267	108.209	0.000	23.988	0.001
<i>ST</i>	July 2019	0.130	0.727	4.813	0.060	2.004	0.195
	July 2020	0.003	0.957	3.859	0.085	0.117	0.741
<i>NSCs</i>	July 2019	8.308	0.020	11.300	0.010	0.061	0.811
	July 2020	1.211	0.303	109.157	0.000	20.082	0.002

SLA: specific leaf area; *Nmass*: nitrogen content per leaf dry mass; *Pmass*: phosphorus content per leaf dry mass; *Chl a*: chlorophyll a; *Chl b*: chlorophyll b; *SS*: soluble sugar; *ST*: starch; *NSCs*: nonstructural carbohydrates. Significant differences ($p < 0.05$) are shown in bold

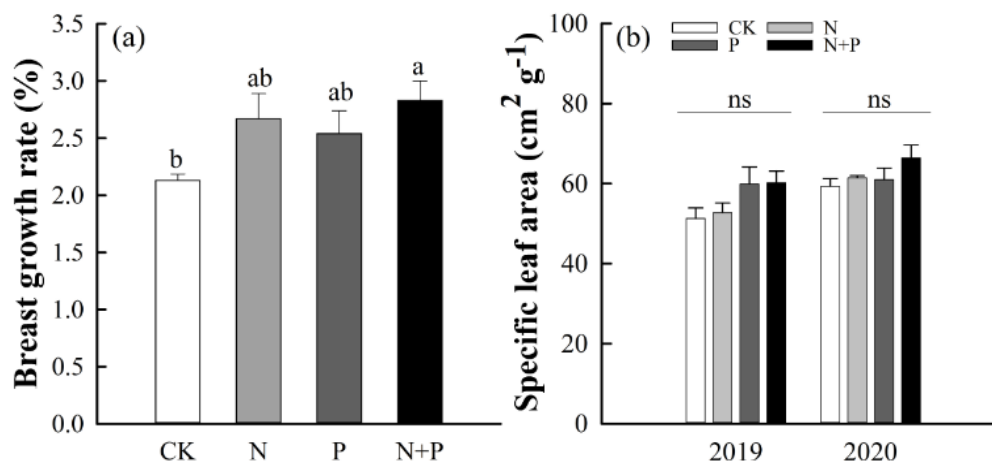


Figure 1. Effects of N and P addition on breast growth rate (a), specific leaf area (b) of mature *Castanopsis sclerophylla* trees. Values are mean \pm SE ($n = 3$). Lower case letters indicate significant differences among treatments at the $p < 0.05$ level in each year

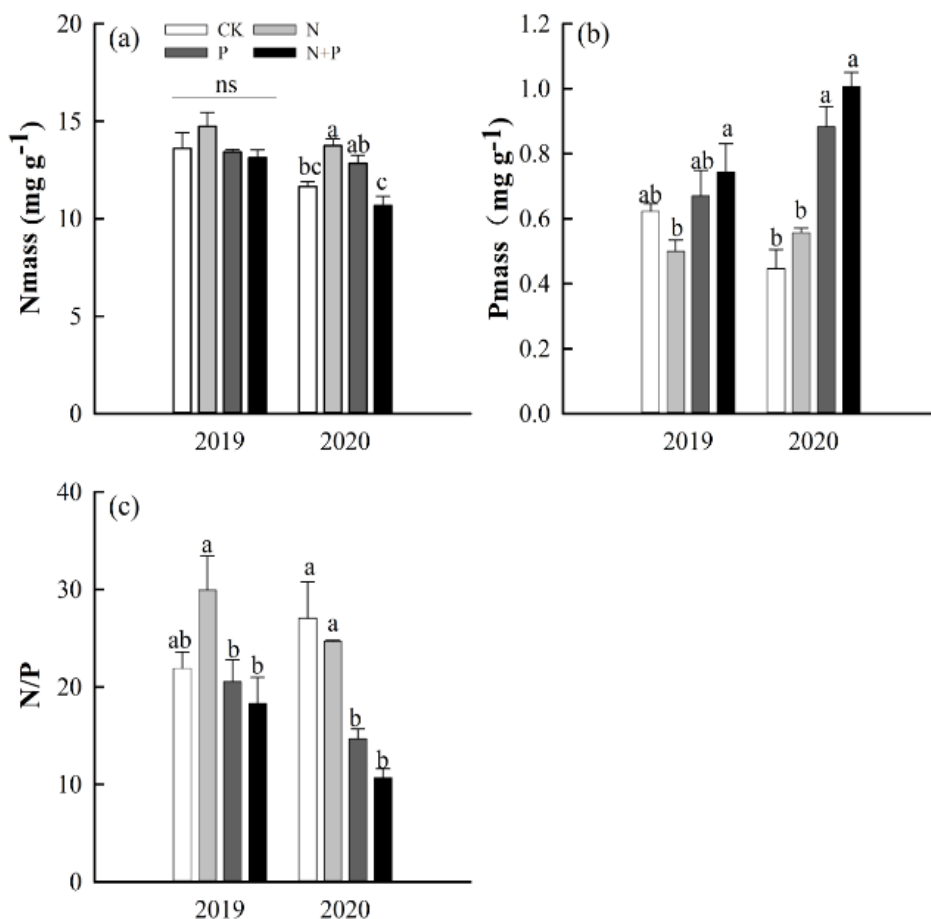


Figure 2. Effects of N and P addition on leaf N content per unit leaf dry mass (a), leaf P content per unit leaf dry mass (b) and leaf N:P ratio (c) of mature *Castanopsis sclerophylla* trees. Values are mean \pm SE ($n = 3$). Lower case letters indicate significant differences among treatments at the $p < 0.05$ level in each year

There was no significant difference in LSP , LCP , AQE and R_d under N, P or NP addition when compared to the control ($p > 0.05$) (Table 2). However, N, P and NP addition increased leaf Pn , g_s and $SPAD$ ($p < 0.05$). P and NP addition increased leaf $PNUE$ and E ($p < 0.05$). The WUE_i increased with N and P addition ($p < 0.05$), but slightly decreased with NP addition ($p > 0.05$) (Table 2).

Table 2. Comparisons in parameters from light-response curves between treatments

Treatment	LSP $\mu\text{mol m}^{-2}\text{s}^{-1}$	LCP $\mu\text{mol m}^{-2}\text{s}^{-1}$	AQE mol mol^{-1}	R_d $\mu\text{mol m}^{-2}\text{s}^{-1}$	Pn $\mu\text{mol m}^{-2}\text{s}^{-1}$	$PNUE$ $\mu\text{mol g}^{-1}\text{s}^{-1}$	E $\text{mol m}^{-2}\text{s}^{-1}$	WUE_i $\mu\text{mol mol}^{-1}$	g_s $\text{mol m}^{-2}\text{s}^{-1}$	$SPAD$ mg g^{-1}
CK	724.0 (166.6)	81.3 (17.6)	0.032 (0.005)	2.40 (0.21)	8.03c (0.21)	4.09b (0.15)	2.67b (0.18)	3.03c (0.14)	0.112c (0.004)	37.54c (0.18)
N addition	676.0 (93.2)	41.3 (10.4)	0.055 (0.014)	2.07 (0.15)	11.66b (0.22)	5.22b (0.24)	2.88b (0.04)	4.05a (0.03)	0.138b (0.003)	44.85b (1.37)
P addition	804.0 (48.2)	49.3 (8.1)	0.048 (0.008)	2.47 (0.66)	14.38a (0.13)	6.86a (0.50)	4.37a (0.03)	3.29b (0.01)	0.185a (0.002)	55.60a (1.86)
NP addition	1070.7 (187.0)	66.7 (16.7)	0.032 (0.004)	1.97 (0.18)	12.22b (0.14)	7.68a (0.76)	4.06a (0.04)	3.01c (0.01)	0.182a (0.004)	45.84b (0.75)

Values are the mean \pm (SE); $n = 3$. Lower cases letters indicate significant differences among treatments at the $p < 0.05$ level

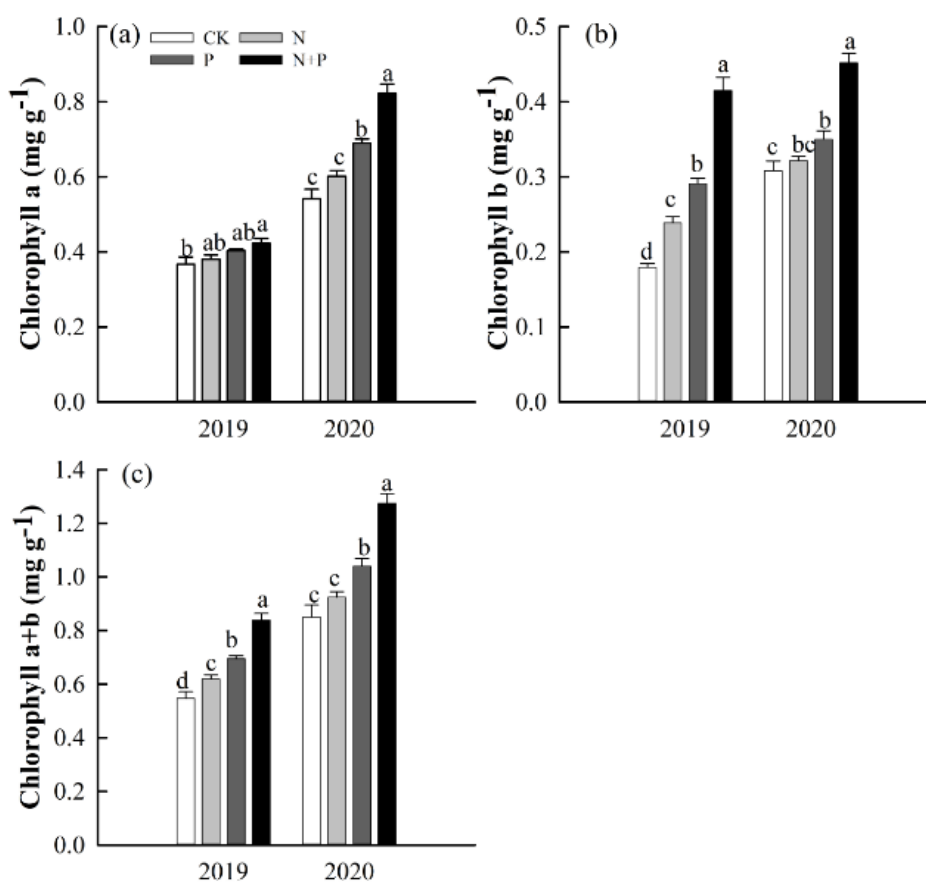


Figure 3. Effects of N and P addition on concentration of chlorophyll a (a), chlorophyll b (b) and chlorophyll a + b (c) in leaves of mature *Castanopsis sclerophylla* trees. Values are mean \pm SE ($n = 3$). Lower case letters indicate significant differences among treatments at the $p < 0.05$ level in each year

Leaf soluble protein, free amino acid and non-structural carbohydrates

In 2020, N and P addition greatly increased leaf SP, while P addition decreased leaf FAA of *C. sclerophylla* ($p < 0.05$). NP addition had no influence on leaf SP but increased the FAA of *C. sclerophylla* leaves ($p < 0.05$). Two-way ANOVA analysis found that significant interactive effect between N and P on leaf FAA (Table 3; Fig. 4).

N, P and NP addition increased leaf SS and total NSCs during the experiment, with the exception of N addition that had no influence on total NSCs in 2019. There was no detectable change in leaf ST in both two years. Two-way ANOVA analysis found that significant interactive effects between N and P on leaf SS and NSCs in 2020, whereas it had no effect on leaf ST (Table 1; Fig. 5).

Discussion

Response of growth, specific leaf area and leaf nutrient

In this study, the GR_{DBH} under N addition was 25.35% higher than control ($p = 0.059$, Fig. 1a). Interestingly, Tian et al. (2017) found 3.4 years N deposition declined growth of small *Castanopsis eyrei* in a subtropical forest, but it had no significant effect on median

and large trees. In a meta-analysis, Yue et al. (2017) found that the plant growth could be suppressed by N-induced P limitation. N addition can enhance the availability of N in soil. P addition could relieve soil P deficiency, and consequently promoted the plant growth. Moreover, we found that NP addition posed a positive effect on plant growth. Therefore, it is not surprising that the GR_{DBH} was increased under long-term nutrient addition. No significant difference of SLA was found under nutrient addition in two years (Fig. 1b). This result was consistent with the findings of Mao et al. (2017), which found long-term N addition has no significant difference of SLA .

Table 3. Results from two-way ANOVA analysis for the effects of N addition, P addition and their interactions on tree growth and physiological traits

Source of variation	Time	N addition		P addition		NP addition	
		F	Sig.	F	Sig.	F	Sig.
GR_{DBH}	July 2020	5.717	0.044	2.696	0.139	0.519	0.492
Protein	July 2020	0.039	0.849	0.247	0.633	20.351	0.002
FAA	July 2020	43.442	0.000	6.591	0.033	31.957	0.000
SPAD	July 2020	1.452	0.263	58.169	0.000	51.940	0.000
LSP	July 2020	0.649	0.444	3.056	0.119	1.343	0.280
LCP	July 2020	0.672	0.436	0.058	0.816	4.300	0.072
AQE	July 2020	0.149	0.710	0.149	0.710	5.357	0.049
R_d	July 2020	1.319	0.284	0.002	0.964	0.053	0.824
Pn	July 2020	16.898	0.003	373.241	0.000	262.392	0.000
PNUE	July 2020	4.178	0.075	29.913	0.001	0.108	0.751
E	July 2020	0.258	0.625	218.283	0.000	6.852	0.031
WUE_i	July 2020	25.858	0.001	28.983	0.001	81.092	0.000
g_s	July 2020	12.097	0.008	321.954	0.000	21.011	0.002

GR_{DBH} : the breast growth rate; FAA: free amino acid; SPAD: leaf color value; LSP: light saturation point; LCP: light compensation point; AQY: apparent quantum yield; R_d : respiration rate; Pn: light-saturated net photosynthetic rate; PNUE: photosynthetic nitrogen use efficiency; E: transpiration rate; WUE_i : instantaneous water use efficiency; g_s : stomatal conductance. Significant differences ($p < 0.05$) are shown in bold

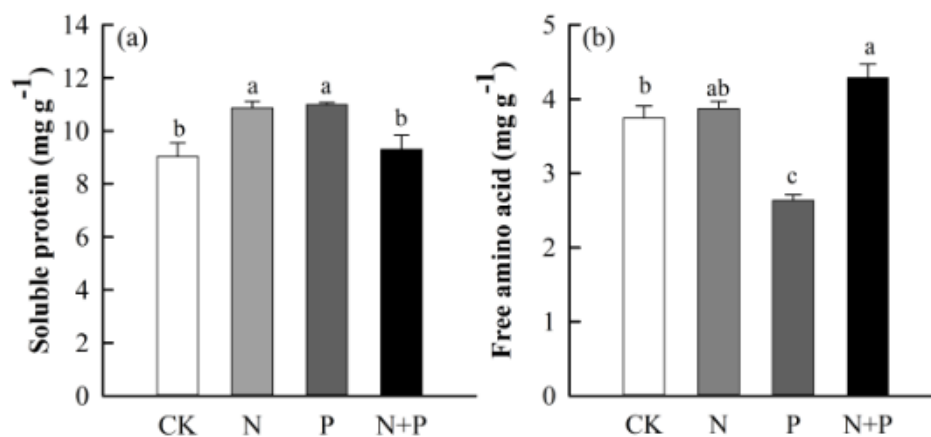


Figure 4. Effects of N and P addition on concentration of soluble protein (a) and free amino acids (b) in leaves of mature *Castanopsis sclerophylla* trees. Values are mean \pm SE ($n = 3$). Lower case letters indicate significant differences among treatments at the $p < 0.05$ level in 2020

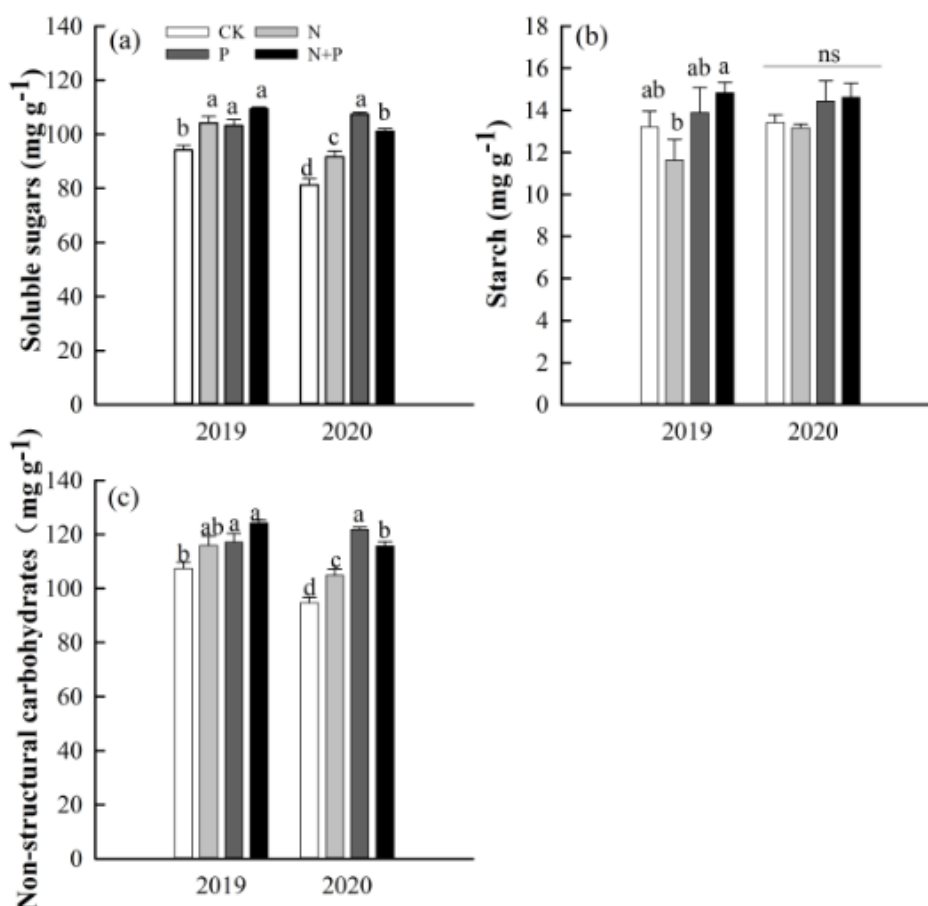


Figure 5. Effects of N and P addition on concentration of soluble sugars (a), starch (b) and non-structural carbohydrates (c) in leaves of mature *Castanopsis sclerophylla* trees. Values are mean \pm SE ($n = 3$). Lower case letters indicate significant differences among treatments at the $p < 0.05$ level in each year

Plant N/P can indicate the limitation between N and P. Previous study found the $N/P < 14$ and $N/P > 16$ may amount to N-limited and P-limited (Koerselman and Meuleman, 1996). According to Reich and Oleksyn (2004), the leaf N/P in temperate tree species was around 19.9. Our study found that this forest had a relatively higher leaf N/P (21.91 and 27.06 at 2019 and 2020, respectively; Fig. 2b) compared with temperate tree species. One of the most consistent responses of plants to high N deposition is higher leaf N contents, indicating that N accumulates in plants when excess atmospheric N is absorbed (Talhelm et al., 2011; Mao et al., 2018). In the present study, N_{mass} was increased under N addition in this forest in 2020 (Fig. 2a). The increases of N_{mass} prove that plants in N-rich ecosystems can still take up the excessive N supply (Mao et al., 2018; Wang et al., 2019; Gurmessa et al., 2016). Under prolonged nutrient addition, based on the responses of P_{mass} to N addition in both two years, N addition likely did not inhibit P resorption in mature *C. sclerophylla* in this subtropical forest. In addition, P and NP addition significantly increased P_{mass} in 2020. Meanwhile, P_{mass} was much higher in 2020 than in 2019 (Fig. 2c). It could be attributed to plant leaves continue to absorb the external phosphorus to diminish the long-term P limitation (Mayor et al., 2014). The leaf N/P was significantly or

marginally decreased under P and NP addition, indicating that P input may alleviate the soil P deficiency and leaf *N/P* imbalance, which induced by excessive N. Furthermore, plant leaves can be affected by environmental changes, the divergent responses of *Nmass*, *Pmass* and leaf *N/P* under N, P addition and their interaction between two years might be attributed to completely different precipitation in July 2019 (160.31 mm) and July 2020 (797.72 mm). The total monthly precipitation was provided by the Shitai Meteorological Bureau.

Response of leaf photosynthesis

In most cases, the *LSP*, *LCP*, *AQE* and *R_d* were unaffected by nutrient addition (Table 3). However, the *Pn* of *C. sclerophylla* was significantly increased under nutrient addition. There are several possible reasons for the increases leaf photosynthesis in this N-rich subtropical forest ecosystems. First, as the critical photosynthetic pigment, leaf *Chl* concentrations were greatly enhanced under P and NP addition. Furthermore, there is often a positive relationship between *SPAD* and *Chlorophyll* (Samdur et al., 2000). We found the leaf *SPAD* was also significantly increased under nutrient addition, and leaf *Chl* was also increased. Therefore, nutrient addition increased leaf *Chl* and *SPAD*, indicating an enhanced photosynthetic capacity. Second, we found that *Pn* increased in line with *g_s* and *E*. A significant increase of *g_s* under nutrient addition may positively affect the CO₂ uptake and CO₂ photosynthetic assimilation efficiency, which were significantly correlated with the leaf *Pn* (Li et al., 2018). Third, we also observed increased *Nmass* and *Pmass* under N, P addition or their interaction. Previous studies found that leaf N and P is essential to photosynthetic machinery and have strong influences on the Rubisco carboxylation and electron transport (Evans, 1989; Conroy et al., 1986). Consequently, increases in *Nmass* and *Pmass* were always accompanied by increases in photosynthesis.

Furthermore, we found a relatively stronger stimulation on leaf *Pn* by P addition compared to N addition. This could be attributed to higher leaf *g_s*, *SPAD* and photosynthetic enzymes (soluble protein in this paper). *C. sclerophylla* exhibited higher *PNUE* under P and NP addition than CK, which can reflect nutrition-related physiological traits of leaves. There is a previous evidence that increased N and P availability can improve *PNUE* (Hidaka and Kitayama, 2009). Such phenomenon could mainly be ascribed to increase of the *Nmass*, *Pmass* and *Pn* under long-term N and P addition.

Response of leaf soluble protein, free amino acid and non-structural carbohydrates

In this subtropical forest, similar to the change of *Nmass*, leaf N allocated to *SP* or *FAA* enhanced consistently with N addition in 2020 (Fig. 5), indicating that long-term N addition may result in increases in leaf N assimilation. Increases in leaf N owing to excessive N input are always accompanied by increases in protein, total *FAA*, and/or chlorophyll in previous studies (Bubier et al., 2011; Mao et al., 2018). Besides, the highest *SP* under P addition may be associated with enhanced photosynthetic enzyme activities, given that the *Pn* with P addition was the highest compared with N and NP addition. Compared to N and P addition, however, leaf *SP* under NP addition was lower (Fig. 5a). The decline in *SP* might result from the observed significant increases in *FAA* (Fig. 5b). Until now, the information about N metabolism in subtropical forests is still insufficient. Our results were consistent with findings in temperate forests. Furthermore,

the consistent increase of N compounds in leaves indicates that the increased leaf N might be reserved as pigments and *SP* or *FAA* of *C. Sclerophylla* in this N-rich subtropical forest.

As the immediate products of plant photosynthesis, *NSCs* variations can indicate the regulation of plants C metabolism to environmental stress (Liu et al., 2018b; Xiao et al., 2017). Our long-term experiment indicated that nutrient addition significantly increased the leaf *SS* and total *NSCs* (Fig. 4a,c). Such a phenomenon may be attributed to higher photosynthesis, which accelerated the C assimilation (Liu et al., 2019). However, our study found that N addition posed a slight negative effect on leaf *ST* in both two years (Fig. 4b). As a substance for osmotic adjustment and respiration, *SS* are the primary substrates for plant growth (Hartmann and Trumbore, 2016). It is noteworthy that July was the fast-growing season of *C. sclerophylla*, when the leaf *Pn* cannot maintain high respiration and fast-growing, the leaf *ST* may be degraded to leaf *SS*. Moreover, the higher *SS*, *ST* and total *NSCs* under P and NP addition compared to N addition might indicate that P input can promote the C assimilation ability of *C. sclerophylla* by a higher leaf *Pn*.

Conclusions

This study investigated the response of plant growth, leaf nutrient status, N metabolites and *NSCs* under long-term N and P addition in a secondary *Castanopsis sclerophylla* forest. Results showed that long-term N deposition can promote leaf *Pn*, *SS* and *NSCs* to stimulate mature *C. sclerophylla* growth (*GR_{DBH}*). Significant interactive effects between N and P were detected in *GR_{DBH}*, leaf chlorophyll a + b, *SS* and total *NSCs* in both two years, but not in *SLA*, *N_{mass}* and leaf *ST*. Furthermore, despite the leaf chlorophyll a + b, *Pn*, *SS* and total *NSCs* were increased under N, P and NP addition (except the chlorophyll a + b in 2020 and total *NSCs* in 2019), the positive impacts of P or NP addition on these parameters were stronger compared to N addition. These conclusions indicated that N and P addition have positive interactive effects on the physiological performances of mature *C. sclerophylla*.

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