Received: 29 April 2020 | Accepted: 10 August 2020

DOI: 10.1111/1365-2745.13496

RESEARCH ARTICLE

Journal of Ecology BRITISH ECOLOGICAL SOCIETY

Addition of nitrogen to canopy versus understorey has different effects on leaf traits of understorey plants in a subtropical evergreen broad-leaved forest

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Funding information
the Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory, Grant/Award Number: GML2019ZD0408; the National Natural Science Foundation of China, Grant/Award Number: 31570401, 41471443 and 41771522

Handling Editor: Frank Gilliam

Abstract

1. Atmospheric nitrogen (N) deposition has substantial effects on forest ecosystems. The effects of N deposition on understorey plants have been simulated by spraying N on the forest floor. Such understorey addition of N (UAN) might simulate atmospheric N deposition in a biased manner, because it bypasses the canopy.
2. We compared the effects of UAN and canopy addition of N (CAN) at 0, 25 and 50 kg N ha^{-1} year^{-1} on SLA, leaf construction costs (CC), concentrations of leaf carbon ([C]), nitrogen ([N]), phosphorus ([P]), minerals ([Mineral]), nitrate ([NO_3^-]), lignin ([Lignin]), lipids ([Lipid]), organic acids ([OA]), soluble phenolics ([SP]), total non-structural carbohydrates ([TNC]) and total structural carbohydrates ([TSC]) in six dominant understorey species in a subtropical evergreen forest after 5 years of N treatments.
3. We found that leaf CC, [C], [Lignin], [OA], [TNC] and [TSC] were significantly affected by N addition approach and rate, but leaf [P] and [Lipid] were affected by N addition approach and N addition rate respectively; leaf CC, [C], [P], [OA] and [TNC] were significantly lower under UAN than under CAN, but leaf [TSC] and [Lignin] were significantly higher and lower, respectively, under UAN than under CAN at 50 kg N ha^{-1} year^{-1}; the decline of leaf [C] and [Lignin] contributed to the significantly lower leaf CC under UAN than under CAN.
4. *Synthesis*. We show that canopy and understorey N addition exerted significantly different effects on leaf traits of understorey plants. The results indicate that understorey plants in subtropical forest respond differently to UAN from those to atmospheric deposition of N. Further studies are warranted to

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Songbo Tang and Lingling Zhang equally contributed to this work.

evaluate the unbiased ecological processes and functions of forest ecosystem responding to atmospheric N deposition via both CAN and UAN experiments over a longer term.

KEYWORDS

canopy addition of nitrogen, defence, global change ecology, growth, leaf construction costs, leaf functional traits, understorey addition of nitrogen, understorey species

1 | INTRODUCTION

Human activities have dramatically increased nitrogen (N) deposition regionally and globally over the past century (Stevens, 2019). In southern China, atmospheric N deposition ranges from 30 to 73 kg N ha^{-1} year^{-1} and is expected to increase (Liu et al., 2011). Global atmospheric N deposition has increased from 31.6 Tg N year^{-1} to 100 Tg N year^{-1} over the past century, and may reach 196 Tg N year^{-1} in 2050 (Galloway et al., 2008; Han et al., 2020). The increase in N deposition has major effects on growth (Bartels, Gendreau-Berthiaume, & Macdonald, 2019; Gilliam, Welch, et al., 2016), reproduction (Zheng & Ma, 2018) and biodiversity (Gilliam, Welch, et al., 2016; Humbert, Dwyer, Andrey, & Arlettaz, 2016; Lu, Mo, Gilliam, Zhou, & Fang, 2010) of forest species, as demonstrated by many experiments simulating N deposition via application of N to the forest understorey (Bartels et al., 2019; Fang et al., 2011; Humbert et al., 2016; Lu et al., 2010) or spraying N on the forest canopy (Gilliam, Billmyer, Walter, & Peterjohn, 2016; Gilliam, Welch, et al., 2016).

Leaf functional traits can reflect resource allocation associated with growth and defence of plant species, and the complex correlations among leaf traits reveal the diversity of plant strategies under different nutrient-supply conditions (Niinemets, 2015; Sakschewski et al., 2015; Santiago & Wright, 2007). Leaf construction costs (CC), an important trait that is correlated with carbon (C) allocation, reflect the resource-use efficiency and adaptation of plants to diverse habitats (Cavatte et al., 2012; Liu, Guo, Ren, & Sun, 2016). Concentrations of leaf nutrients and chemical compounds play crucial roles in growth (e.g. phosphorus ([P]) and [N]) or in defence (e.g. total non-structural carbohydrates ([TNC]), lignin ([Lignin] and soluble phenolics ([SP]; Dietze et al., 2014; Lambers & Poorter, 1992; Liu, Wu, et al., 2016; Sala, Woodruff, & Meinzer, 2012). Typically, plants with a high growth capacity are characterized by a high SLA and high [Protein], [N], [P] and [Mineral] in their leaves (Lambers & Poorter, 1992; Onoda et al., 2017). In addition, many plant species enhance defence via allocating more C to leaf lignin and SP under biotic and abiotic stresses (Aspinwall, King, Booker, & McKeand, 2011; Karabourniotis et al., 2014; Lambers & Poorter, 1992; Shan, Song, Zhang, & Ren, 2018). The changes in leaf traits reflect the plants' capacity for growth or defence (De Long, Sundqvist, Gundale, Giesler, & Wardle, 2016).

Leaf traits respond specifically to N addition, depending on plant species and N addition rates. For instance, different responses of foliar [TNC] to N addition were observed in different studies (Griffin, Winner, & Strain, 1996; Liu, Wu, et al., 2016; Xu, Xie, Wu, Wang, & He, 2019). Previous studies found that leaf [N] increases with N deposition (Liang et al., 2020; Palmroth, Bach, Nordin, & Palmqvist, 2014; Zheng & Ma, 2018), leading to a risk of N-meditated nutrient imbalance including decreases in leaf [P] (De Long et al., 2016; Jung, Kwak, Gilliam, & Chang, 2018; Yan, Qu, Sun, et al., 2018). Furthermore, increases in N deposition also decrease leaf total structural carbohydrates ([TSC], Liu, Wu, et al., 2016; Shan et al., 2018) and [Lignin] (Shan et al., 2018), but increase leaf [Protein] (De Long et al., 2016). However, there is a lack of direct evidence to reveal whether the responses of leaf traits with a traditional N addition approach, UAN, differ from those of atmospheric N deposition involving the actual input into soil.

Understorey plant species, important components of forest ecosystems, can be affected by atmospheric N deposition and this may result in changes in species composition, forest structure and ecosystem functioning (Gilliam, 2007; Hedwall et al., 2019; Lu et al., 2010). The effects of N deposition on growth or defence of understorey species can be assessed by measuring leaf traits across plant species (Gilliam, Billmyer, et al., 2016; Gilliam, Welch, et al., 2016; Mao, Lu, Wang, Zhou, & Mo, 2017; Palmroth et al., 2014). However, many assessments of N deposition on leaf traits are based on experiments of understorey addition of N (UAN), that is, by application of N at or near the soil surface (Humbert et al., 2016; Mao, Lu, Mo, Gundersen, & Mo, 2018; Mao et al., 2017), and only a few assessments are based on experiments of canopy addition of N (CAN; Gilliam, Billmyer, et al., 2016; Gilliam, Welch, et al., 2016), to simulate actual atmospheric N deposition. UAN may overestimate the effects of N deposition on understorey vegetation, especially in N-limited ecosystems where plants strongly respond to N addition, because it bypasses canopy processes, for example, canopy N uptake (Houle, Marty, & Duchesne, 2015; Nair, Perks, Weatherall, Baggs, & Mencuccini, 2016), of atmospherically deposited N (Liu, Mao, et al., 2020; Wortman et al., 2012), leading to differences in the quality and quantity of N that reaches the forest understorey and forest soils from those of natural N deposition (Huang et al., 2019; Liu, Mao, et al., 2020).

In the present study, the effects of CAN and UAN on leaf traits were compared for the dominant understorey plant species, including four shrubs and two herbs, after 5 years of treatments. We tested the following three hypotheses: (a) both CAN and UAN alter leaf traits of the understorey species, but the effects of UAN will be greater than those of CAN due to naturally occurring canopy processes (Houle et al., 2015; Nair et al., 2016; Wortman et al., 2012); (b) changes in leaf CC caused by both CAN and UAN will result from increases in leaf [TNC] (Griffin et al., 1996) and organic nitrogen compounds (e.g. leaf [Protein], De Long et al., 2016; Lafitte & Loomis, 1988) in response to N addition, due to changes in these chemical compounds by increasing N availability; and (c) plant defence will decline via changing leaf traits like leaf [Lignin] and [TSC] under both CAN and UAN treatments, since increasing N availability reduces environmental stress imposed by N deficiency (De Long et al., 2016; Shan et al., 2018).

2 | MATERIALS AND METHODS

2.1 | Experimental design and sampling

The experiment was conducted in an evergreen broad-leaved forest at Shimentai (SMT) National Nature Reserve (24°22′–24°31′N, 113°05′–113°31′E), Guangdong Province, China (Figure 1). This reserve has a subtropical monsoon climate with alternating wet (April–September) and dry (November–March) seasons. The mean annual precipitation and temperature over the past 60 years are 2,364 mm and 20.8°C respectively (Zhang et al., 2015). The forest is dominated by *Castanea henryi*, *Castanopsis eyrei*, *Schima superba*, *Machilus chinensis* and *Engelhardtia roxburghiana* in the canopy layer; by *Blastus cochinchinensis*, *Psychotria rubra*, *Ardisia quinquegona* and *Macaranga sampsonii* in the shrub layer; and by *Alpinia chinensis* and *Cibotium barometz* in the herbaceous layer. The forest has a mean canopy height of 13.8 m and a mean tree DBH of 18.6 cm (Zhang et al., 2015). The atmospheric N deposition in rainfall near this reserve is ~34.0 kg N ha^{-1} year^{-1} (Zhang et al., 2015).

The experimental system and plots were described previously by Zhang et al. (2015) and detailed by some researchers (Jiang et al., 2018). In brief, both CAN and UAN involved three rates of N addition, that is, 0, 25 and 50 kg N ha^{-1} year^{-1}; these were in

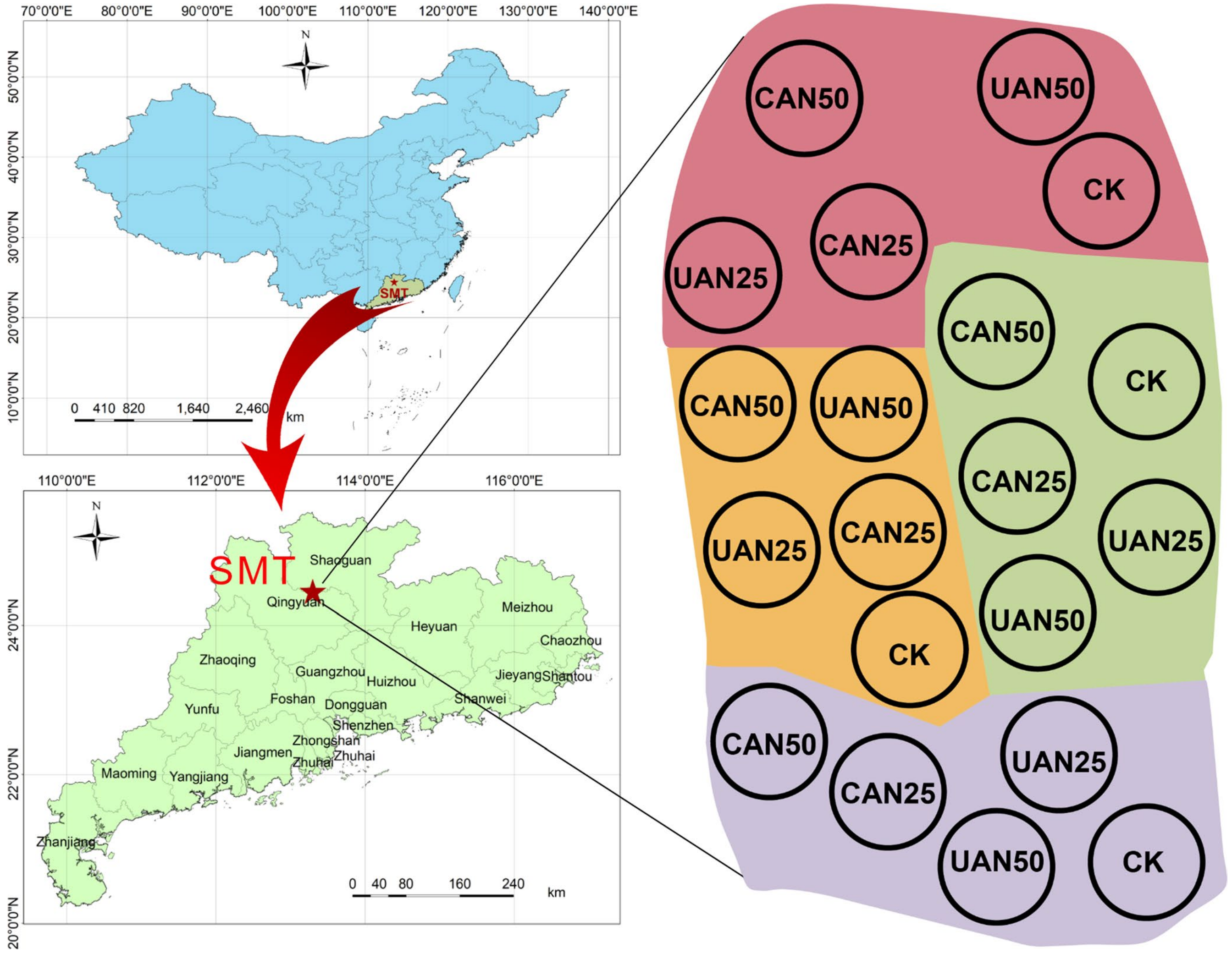

FIGURE 1 Location of the experimental site and plot map of the N treatments. CAN: canopy addition of N at 25 or 50 kg N ha^{-1} year^{-1}. UAN: understorey addition of N at 25 or 50 kg N ha^{-1} year^{-1}. CK: addition of 0 kg N ha^{-1} year^{-1} N. The four colours indicate four blocks, and each circle represents a plot

addition to background, natural N deposition. The three treatments were applied to circular plots that were 34 m in diameter. The CAN treatments were applied via a forest canopy spraying system (35 m high, 5–8 m above the canopy) built in the centre of each CAN plot, while the UAN treatments were applied by five sprinklers that were evenly distributed 1.5 m above the ground in each UAN plot. The targeted N solution was made on-site by weighing appropriate amount of ammonium nitrate (NH_4NO_3) and mixed with surface water drained from a nearby pond. The spraying was performed monthly from April to October (seven times per year) beginning in 2012 and continuing through 2017, when samples were collected for the current study. The treatments were applied at a rate equivalent to 3 mm of precipitation per application. The total solution applied was equal to 21 mm of precipitation per year which represents only ~1% of the total annual precipitation at this reserve; as a consequence, any confounding effect caused by water addition was negligible (Zhang et al., 2015, Table S1). Spray equipment was not present in control plots (those receiving 0 kg N ha^{-1} $year^{-1}$), and water was not sprayed onto those plots.

Each of the three treatments was represented by one randomly assigned plot in each of four blocks in the forest, that is, each treatment was represented by four replicate plots. The plots were separated by buffer zones of at least 20 m to minimize lateral contamination of N solution between plots, and polyvinylchloride (PVC) boards were inserted between adjacent plots.

Leaves of the above six dominant understorey species (four shrubs and two herbs, Table 1) in the evergreen broad-leaved forest were collected in October 2017. At least 10 mature, current year, fully expanded and healthy leaves from at least three mature individuals per species per plot were collected. To minimize the influence of plant age, the individuals of the same species selected for sampling were of similar diameter for shrubs or height for herbs in each plot. After sampling, the leaves were placed in ice bags and immediately transported to the laboratory.

2.2 | Leaf trait measurements and calculations

In the laboratory, the leaves were cleared and divided into two parts. One part was used to measure leaf area (Li–COR Inc.) and then dried to a constant weight (65°C for 72 hr) for calculation of SLA. The other part was freeze-dried and ground for chemical analyses. Leaf [Lipid] was determined using chloroform:methanol:water (2:2:1; v:v:v) according to Poorter et al. (1997). Leaf [SP] and concentrations of soluble sugars ([SS]) and insoluble sugars ([ISS]) were determined with Folin–Ciocalteu and anthrone reagents respectively (Blainski, Lopes, & de Mello, 2013). Leaf [TNC] was the sum of [SS] and [ISS]. Leaf [Lignin] and [TSC] were calculated based on [C] and [N] in the residue after 3% (v:v) HCl extraction (Poorter & Villar, 1997).

Leaf [C] and [N] were determined with an elemental analyser (Isoprime 100, Elementar Isoprime, UK). Leaf [P] was determined via molybdenum–antimony colorimetry after digestion by sulphuric acid. Leaf $[NO_3^-]$ was determined according to Cataldo, Haroon, Schrader, and Youngs (1975). Leaf ash concentration and ash alkalinity were determined acidimetrically after the leaf powder was combusted in a muffle furnace at 550°C for 6 hr (Poorter & Villar, 1997).

Leaf organic acid concentrations ([OA]) were calculated by multiplying the difference between ash alkalinity (meq/g) and $[NO_3^-]$ (meq/g) by 62.1 (the average molecular weight of one OA, Equation 1); [Mineral] was derived from subtracting ash alkalinity (meq/g) multiplied by 30 (the weight of carbonate per equivalent of charge) from the sum of Ash and $[NO_3^-]$ (mg/g; Equation 2); and [Protein] was calculated by multiplying the difference of [TN] and $[NO_3^-]$ by 6.25 (Equation 3, Poorter et al., 1997). Leaf CC (g glucose g^{-1}) was calculated according to Poorter et al. (2006; Equation 4):

$$[\mathrm{OA}] = \left(\mathrm{Ash\,alkalinity} - \left[\mathrm{NO_3^-}\right]\right) \times 62.1, \tag{1}$$

$$[\mathrm{Mineral}] = \mathrm{Ash} + \left[\mathrm{NO_3^-}\right] - \mathrm{Ash\,alkalinity} \times 30, \tag{2}$$

$$[\mathrm{Protein}] = \left(\left[\mathrm{TN}\right] - \left[\mathrm{NO_3^-}\right]\right) \times 6.25, \tag{3}$$

$$\mathrm{CC} = \left(-1.041 + 5.077 \times \mathrm{C_{om}}\right) \times (1 - \mathrm{M}) + \left(5.325 \times \mathrm{N_{org}}\right), \tag{4}$$

where C_{om}, M and N_{org} are concentrations of leaf C, minerals and organic N respectively.

2.3 | Statistical analyses

As noted above, the CK was considered equivalent to an N addition rate of 0 kg N ha^{-1} $year^{-1}$ in both CAN and UAN treatments

TABLE 1 The six understorey species in this study. Species names follow Flora of China available online at www.efloras.org

Species	Family	Types	Mean height (m)
Blastus cochinchinensis Lour.	Melastomataceae	Shrub	1.2
Psychotria asiatica Wall.	Rubiaceae	Shrub	0.8
Ardisia quinquegona Blume	Myrsinaceae	Shrub	1.0
Macaranga sampsonii Hance	Euphorbiaceae	Shrub	1.2
Alpinia chinensis Hayata	Zingiberaceae	Herbaceous	1.0
Cibotium barometz (L.) J. Sm.	Dicksoniaceae	Herbaceous	1.2

(CAN0 and UAN0 respectively). Differences in leaf traits between N-application approaches and among the N addition rates (0, 25 and 50 kg N ha^{-1} year^{-1}) were tested using general linear mixed effect models (GLMEMs), with species and blocks as the random effects (Crawley, 2007). Differences between the N-addition treatments and among the rates were determined using Tukey's HSD post hoc tests and conducted by lsmeans function in LSMEANS packages after processing GLMEMs.

The effect sizes of N addition approach and rate on leaf CC were determined via structural equation model (SEM) using Amos (IBM SPSS). Briefly, we built SEM by introducing those leaf traits that were significantly different between addition of N approaches and/or among addition of N rates based on GLMEMs results. After running the a priori model, we removed all non-significant paths ($p > 0.05$) based on SEM results, and ran this new model again. We assessed goodness of our model based on multiple goodness-of-fit criteria, including the ratio of chi-square to degrees of freedom (Chi-square/DF), comparative fit index (CFI) and root mean squared error of approximation (RMSEA), when Chi-square/DF ≤ 2 ($p > 0.05$), 0 ≤ RMSEA ≤ 0.05 and CFI ≥ 0.95 suggested the model had a good fit (Schermelleh-Engel, Moosbrugger, & Müller, 2003). All statistical analyses, except for SEM, were performed using the R software platform (R Development Core Team, 2019), with the NLME (Crawley, 2007), LSMEANS (Lenth, 2016) and MULTCOMP (Hothorn, Bretz, & Westfall, 2008) packages. Significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Effects of N addition on leaf CC

The effects of UAN on leaf CC were greater than those of CAN. In comparison with UAN0, UAN25 or UAN50 significantly decreased leaf CC (Figure 2); CAN, in contrast, did not significantly affect leaf CC. In a second analysis (within each application rate), leaf CC was significantly lower under UAN than under CAN treatment (Figure 2).

3.2 | Traits related to plant growth

As indicated in Table 2, the interaction between N addition approach and addition rate was not significant for any of the traits related to growth; leaf [C] and [P] were the only traits significantly affected by N addition approach; and N addition rate did not significantly affect any trait related to plant growth, except for leaf [C]. In comparison with the control, UAN, but not CAN, significantly decreased leaf [C] (Figure 3a); no other trait related to growth was significantly affected by UAN or CAN at 25 or 50 kg N ha^{-1} year^{-1} (Figure 3a,c–f). In addition, leaf [C] and [P] were significantly lower under UAN than under CAN25 or CAN50 (Figure 3b,d).

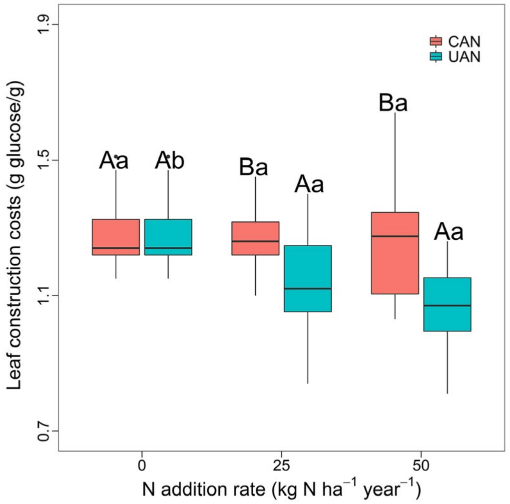

FIGURE 2 Leaf construction costs (CC) of the dominant understorey species in the evergreen broad-leaved forest as affected by N addition approach (UAN vs. CAN) and N addition rates. Different lower case and capital letters indicate significant differences among N addition rates within each N addition approach and differences between N addition approaches within each N addition rate, respectively, based on linear mixed effect models (post hoc Tukey test, $p < 0.05$). Boxes in each boxplot show the first and third quartiles and the median; the upper and lower whiskers indicate the largest and smallest values away from 1.5 × IQR (inter-quartile range) of the third quartiles and first quartiles respectively; black points in each figure are values that fell outside the whiskers

TABLE 2 Effects (as indicated by *F* values) of N addition approach (CAN vs. UAN) and N addition rate (0, 25 and 50 kg N ha^{-1} year^{-1}) on SLA and leaf concentrations of carbon ([C]), nitrogen ([N]), phosphorus ([P]), nitrate ([NO_3^-]) and chemical compounds of the dominant understorey species in an evergreen broad-leaved forest. The effects were tested using general linear mixed effect models, with species and blocks as random effects (trait ~ approach × rate, random = ~1|block/species). *, ** and *** indicate $p < 0.05$, <0.01 and <0.001 respectively

		Independent variables		
Functions	Dependent variables (leaf traits)[a]	N addition approach	N addition rate	Approach × Rate
Growth	SLA	0.17	1.40	0.88
	[C]	10.43**	4.95**	3.03
	[N]	3.99	1.75	1.00
	[P]	11.89***	0.54	3.04
	[Mineral]	2.63	1.48	2.64
	[NO_3^-]	2.36	1.32	0.59
Defence	[Lignin]	5.87*	17.30***	2.03
	[Lipid]	2.56	11.87***	1.00
	[OA]	13.58***	12.79***	3.50*
	[SP]	1.28	2.52	0.39
	[TNC]	40.27***	9.22***	21.25***
	[TSC]	8.13**	19.65***	3.04

[a] Brackets indicate concentrations.

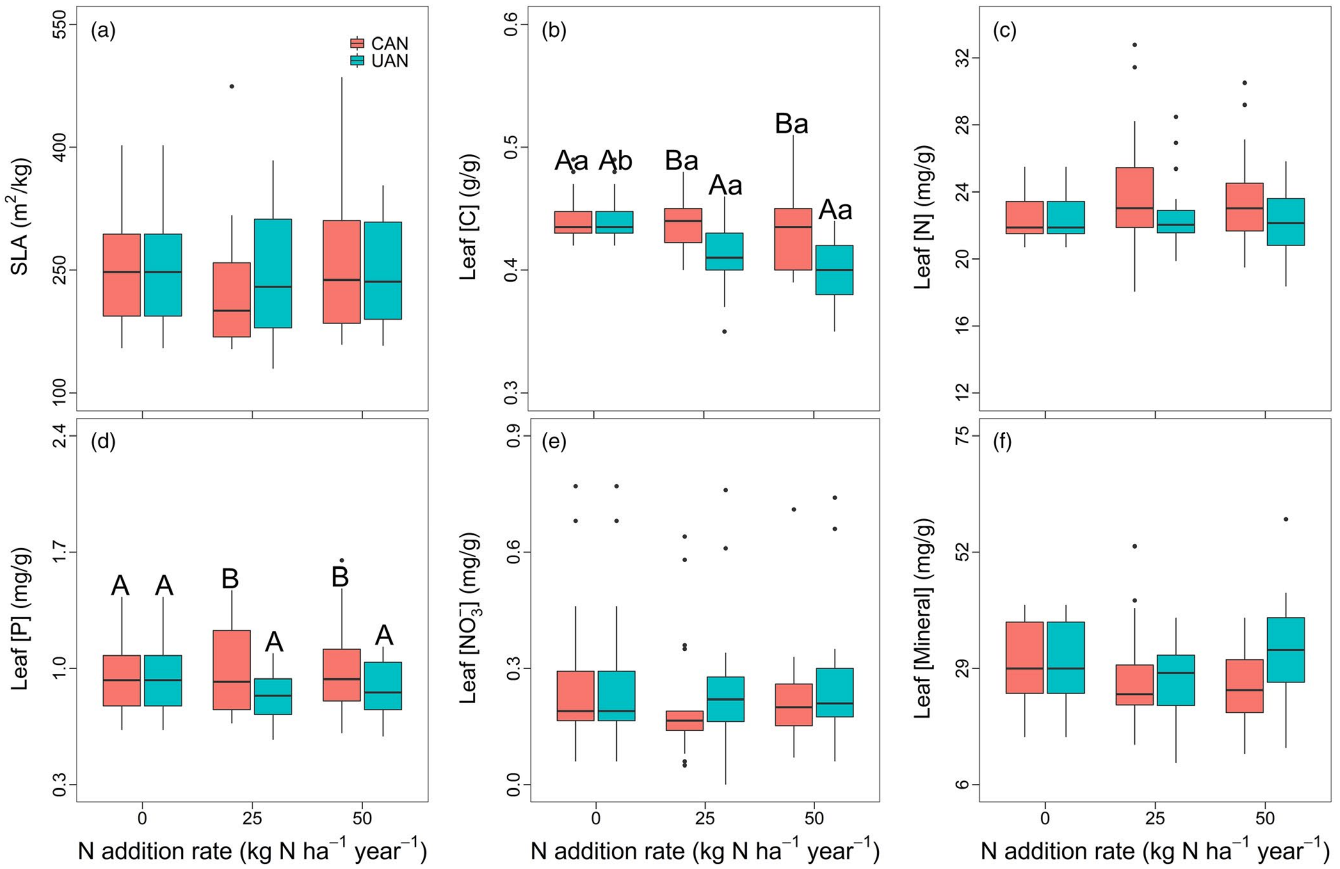

FIGURE 3 Leaf traits associated with plant growth, including SLA and concentrations of leaf carbon ([C]), nitrogen ([N]), phosphorus ([P]), nitrate ([NO$_3^-$]) and minerals ([Mineral]) of the understorey species as affected by N addition approach (UAN vs. CAN) and N addition rate. Different lower case and capital letters indicate significant differences among N addition rates within each N addition approach and differences between N addition approaches within each N addition rate, respectively, based on linear mixed effect models (post hoc Tukey test, $p < 0.05$). The absence of lower case or capital letters indicates that the effect of addition rate or approach was not significant. Boxes in each boxplot show the first and third quartiles and median; the upper and lower whiskers indicate the largest and smallest values away from 1.5 × IQR (inter-quartile range) of the third quartiles and first quartiles respectively; black points in each figure are values that fell outside the whiskers

3.3 | Traits related to defence

As indicated in Table 2, the interaction between N addition approach and addition rate was significant for leaf [OA] and [TNC], but not for the other defence-related traits; N addition approach significantly affected leaf [Lignin], [OA], [TSC] and [TNC], but not leaf [Lipid] or [SP]; and N addition rate significantly affected all defence-related traits, except [SP]. The following comparisons are relative to the control (0 kg N ha^{-1} year^{-1}): leaf [Lignin] was significantly reduced by CAN25, UAN25 and UAN50 (Figure 4a); leaf [Lipid] was significantly increased by CAN25, CAN50 and UAN50 (Figure 4b); leaf [OA] was not significantly affected by UAN25 or UAN50, but was significantly increased by CAN25 and CAN50 (Figure 4c); leaf [TNC] was not significantly affected by CAN25 or CAN50, but was significantly reduced by UAN25 (Figure 4d); leaf [TSC] was significantly increased by CAN50 and by UAN25 and UAN50 (Figure 4e). In a second analysis (within each application rate), the effect of CAN50 on [Lignin] was greater than that of UAN50 (Figure 4a); the effects of CAN25 and CAN50 on [OA] were greater than those of UAN25 and UAN50 (Figure 4c); the effects of CAN25 and CAN50 on [TNC] were greater than those of UAN25 and UAN50 (Figure 4d); and the effect of UAN50 on [TSC] was greater than that of CAN50 (Figure 4e).

3.4 | Principle of N addition influencing leaf CC

N addition approach and rate had negative effects on leaf CC directly and indirectly via influencing leaf traits related to defence and growth (Figure 5a,b). Specifically, N addition approach indirectly influenced leaf CC via negative effects on leaf [C], [P] and further on [Lignin], [OA]; however, N addition rate indirectly increased and decreased leaf CC via declining leaf [C], [Lignin] and enhancing leaf [OA] respectively (Figure 5a,b). In addition, both growth and defence had positive effects on leaf CC, while the effects of growth were greater than those of defence (Figure 5b).

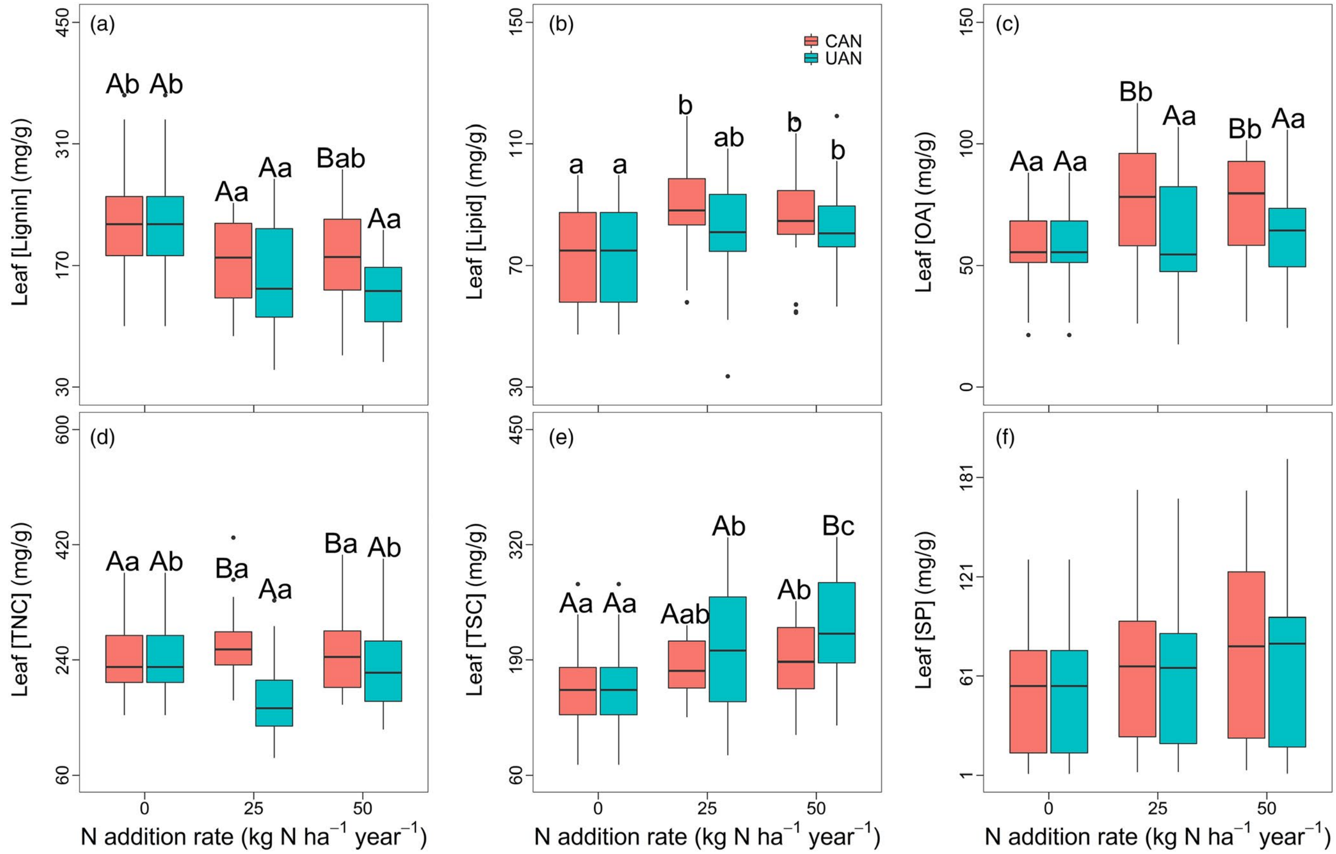

FIGURE 4 Leaf traits associated with plant defence, including leaf concentrations of lignin ([Lignin]), lipids ([Lipid]), organic acids ([OA]), and total non-structural carbohydrates ([TNC]), total structural carbohydrates ([TSC]), soluble phenolics ([SP]), of the dominant understorey species in an evergreen broad-leaved forest as affected by N addition approach (UAN vs. CAN) and N addition rate. Different lower case and capital letters indicate significant differences among N addition rates within each N addition approach and differences between N addition approaches within each N addition rate, respectively, based on linear mixed effect models (post hoc Tukey test, $p < 0.05$). The absence of lower case or capital letters indicates that the effect of addition rate or approach was not significant. Boxes in each boxplot show the first and third quartiles and median; the upper and lower whiskers indicate the largest and smallest values away from 1.5 × IQR (inter-quartile range) of the third and first quartiles respectively; black points in each figure are values that fell outside the whiskers

4 | DISCUSSION

Leaf CC indicates not only a plant's response to environmental change (Cavatte et al., 2012; Poorter et al., 2006), but also plant C budgets and investment strategies (Liu, Wu, et al., 2016). Our results show, for the first time, that the understorey species responded differently to N addition approaches or N addition rates in C budgets or investment, which implies that understorey N addition may lead to a biased assessment on plant responses to atmospheric N deposition (Nair et al., 2016; Zhang et al., 2015).

Nitrogen and P are required for plant growth, and SLA is a proxy for a plant's growth potential (Lambers & Poorter, 1992). Nitrogen addition increases [N] in leaves and SLA, and thus the plant growth rate (Lambers & Poorter, 1992; Liang et al., 2020; Palmroth et al., 2014; Zheng & Ma, 2018). Nitrogen addition, however, may reduce [P] in leaves of understorey species, because N addition may enhance growth more than P uptake, and thus dilutes leaf [P] (Jung et al., 2018; Yan, Qu, Song, et al., 2018). In the present study, neither SLA nor leaf [N] of the understorey species was significantly affected by UAN nor CAN, and only leaf [P] differed between UAN and CAN at 25 and 50 kg N ha^{-1} year^{-1} after 5 years of treatments (Table 2; Figure 3a,c,d). The results were inconsistent with some UAN experiments (Liang et al., 2020; Mao et al., 2018; Palmroth et al., 2014). Leaf [P] mainly depends on the supply of soil P (Cleveland et al., 2011), but the responses of leaf [P] and [N] as well as that of SLA to both canopy and understorey N addition (Figure 3) indicate that N addition approach affected leaf [P] (Figure 3d), which is likely due to more N added to the soil surface under UAN than under CAN, leading to soil acidification (Lu, Mao, Gilliam, Luo, & Mo, 2014). The different effects of UAN versus CAN on the understorey plants were probably explained by the canopy closure of the forest (Zhang et al., 2015), canopy retention of N (Wortman et al., 2012), leading to a greater N input into soil and to lower availability of soil P due to soil acidification, under UAN than CAN treatments (Lu et al., 2014), the abundant N availability (Fang et al., 2011) and the limiting P supply (Cleveland et al., 2011; Hou

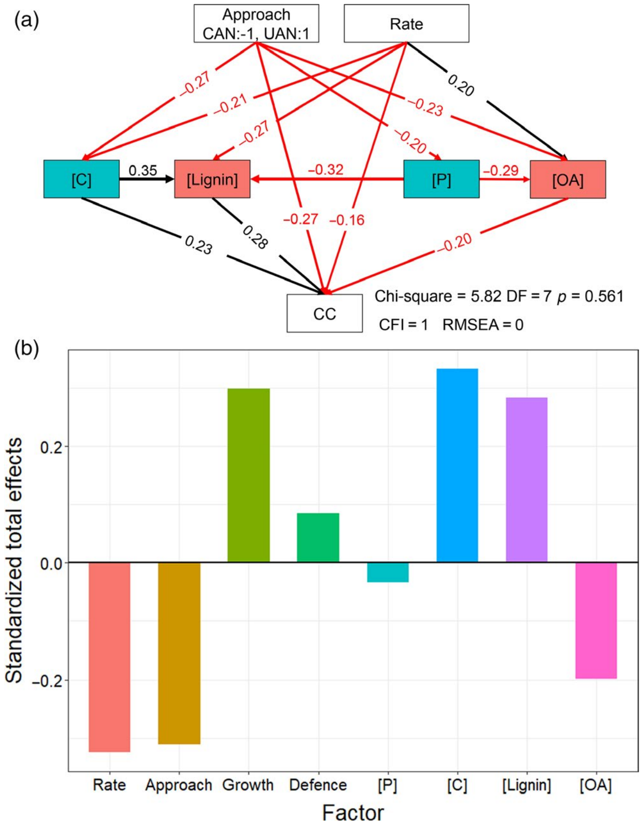

FIGURE 5 Effects of N addition approach and rate on leaf carbon ([C]), phosphorus ([P]), lignin ([Lignin]), organic acids ([OA]) and construction costs (CC) as indicated by a structural equation modelling (SEM) (a). And standardized total effects (direct plus indirect effects) derived from the SEM, Growth and Defence refer to the total effects of traits related to plant growth and defence (Table 1), respectively, on leaf CC (b). Goodness-of-fit statistics for SEM is shown in the lower right corner (DF, degrees of freedom; CFI: comparative fit index; RMSEA, root mean squared error of approximation). Numbers adjacent to arrows are standardized path coefficients. Line width corresponds to parameter size. The red and black lines indicate significantly negative and positive effects ($p < 0.05$) respectively

et al., 2020) in subtropical forest soils. This finding partly supports our first hypothesis, which stated that leaf traits of understorey plants would be affected more by UAN than by CAN.

In this study, the significant increase of leaf [OA] under CAN (Figure 4c) was likely partly due to the generation of hydroxyl ions associated with the reduction and assimilation of leaf NO_3^- (Argyriadis, Dijkshoorn, & Lampe, 1976; Dijkshoorn, Lathwell, & De Wit, 1968). We also found that leaf [Lignin] was decreased by both CAN and UAN (Figure 4a), consistent with previous reports (Koricheva, Larsson, Haukioja, & Keinanen, 1998; Shan et al., 2018). However, the factors influencing the change in leaf [Lignin] differed from those studies (De Long et al., 2016; Koricheva et al., 1998; Shan et al., 2018). For instance, a decrease in leaf [Lignin] was associated with an increase in leaf [N] (or decreases in C/N ratio; Bryant, Chapin, & Klein, 1983; Koricheva et al., 1998; Shan et al., 2018). In the present study, although leaf C/N ratio was decreased by UAN (Figure S1), UAN did not affect leaf [N], but decreased leaf [C] and increased [TSC] (Figures 3b,c, and 4e). We assume that the decrease in leaf [Lignin] of understorey plants in this study may be associated with greater allocation of C to leaf [TSC], leading to lower cost of defence (Figure 4a,e). The differences associated with decreasing leaf [Lignin] between previous and ours findings may be due to difference in ecosystems (wetlands; Shan et al., 2018; boreal forest, Bryant et al., 1983) or functional types (woody plants; Koricheva et al., 1998; trees, De Long et al., 2016) from that of our study.

Increased N availability would promote plant growth, while excess N would have opposite effect due to soil acidification, nutrient imbalance and competition with fast-growing species (Dirnboeck et al., 2018; Lu et al., 2014; Mao et al., 2018; Tian et al., 2017). As mentioned earlier, because of the biological and physical processes in the canopy (Houle et al., 2015; Wortman et al., 2012), N inputs into the forest floor are less with CAN than with UAN, suggesting that UAN is more likely than CAN to inhibit plant growth (Mao et al., 2018). The differentially changed leaf [Protein] of dominant canopy plant species, *Schima superba*, between CAN and UAN treatments confirms the substantial influence of canopy N uptake on atmospherically deposited N (Liu, Zhang, Huang, Wang, & Cai, 2020). We, therefore, suggest that responses of leaf traits of understorey species in subtropical forests to atmospherically deposited N might be different from the responses to the experimental addition of N via UAN. The significant differences in leaf [TNC] and [TSC] between CAN and UAN at higher N addition rates (Figure 4) also suggest that UAN may overestimate the effects of increases in N deposition in subtropical evergreen broad-leaved forests in the short term. The results partly support our first and third hypothesis that both CAN and UAN will change leaf [Lignin] and [TSC], but do not support the statement that N addition will decrease plant defence, since we observed increases in leaf [TSC] and decreases in leaf [Lignin] under both treatments (Figure 4a,e).

Differences in leaf CC can be explained by the correlations between leaf CC and some chemical compounds (Cavatte et al., 2012; Griffin et al., 1996; Poorter & De Jong, 1999). In the present study, the N addition approach and addition rate significantly impacted leaf CC via altering leaf [C], [P], [Lignin] and [OA] (Figure 5), UAN significantly declined leaf CC of understorey species (Figure 2). High N addition rate increased leaf [OA] and decreased leaf [Lignin] and [C]; those effects were more negative for UAN than for CAN (Figure 5a). The results do not support our second hypothesis and differ from those of Griffin et al. (1996), Griffin, Thomas, and Strain (1993) and Lafitte and Loomis (1988), who found that differences in leaf CC were associated with differences in leaf [TNC] or [Protein]. Although increased soil N availability might increase leaf [Protein], and thus increase leaf CC (Lafitte & Loomis, 1988), this would be different in our experimental forest, because of the low availability of soil P ($N/P = 25.7 \pm 4.8 > 20$, $p < 0.001$). The high rate of N deposition in our experimental field (Zhang et al., 2015) would result in relatively stable leaf [Protein] of understorey plants (Figure S2). Exposed to N addition, understorey plants preferentially increased low-cost compounds (e.g. OA) to balance the reduction of nitrate, associated with reduced investment in lignin (Endara & Coley, 2011; Shan et al., 2018). UAN treatment, however, had significantly negative impacts on leaf [C] and [Lignin] of understorey plants compared with CAN treatment, which most likely result from an excessive N input (Mao et al., 2018).

5 | CONCLUSIONS

Two approaches simulating increasing N deposition, UAN and CAN, had different effects on leaf traits including leaf CC of understorey plants in subtropical evergreen broad-leaved forests. For traits related to growth, N addition approach and rate affected leaf [C], but only N addition approach affected leaf [P]. For leaf traits, except for [SP], related to plant defence were significantly affected by N addition approach and rate. The decline in leaf CC under UAN is ascribed to the declines in leaf [Lignin]. Further study will be needed to evaluate the ecological processes and functioning of forest ecosystems responding to atmospheric N deposition via both CAN and UAN experiments over a longer time frame.

ACKNOWLEDGEMENTS

This study was jointly supported by the Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (No. GML2019ZD0408) and the National Natural Science Foundation of China (No. 41471443, 41771522, 31570401). The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Y.K. designed the study; L.Z., S.T., W.R. and X.L. performed the experiments; S.T., L.Z. and Y.K. analysed the data; S.T., L.Z. and Y.K. drafted the manuscript; H.L., E.H. and S.F. revised the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/1365-2745.13496.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.0gb5mkkzj (Tang et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Tang S, Zhang L, Lambers H, et al. Addition of nitrogen to canopy versus understorey has different effects on leaf traits of understorey plants in a subtropical evergreen broad-leaved forest. *J Ecol*. 2021;109:692–702. https://doi.org/10.1111/1365-2745.13496