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Research paper

Multi-nutrient stoichiometry of Chinese hickory (*Carya cathayensis*) saplings: plant organs vary in their response to nitrogen fertilization

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Nitrogen (N) enrichment from excessive fertilization in managed forests affects biogeochemical cycles on multiple scales, but our knowledge of how N availability shifts multi-nutrient stoichiometries (including macronutrients: N, phosphorus, potassium, calcium, magnesium and micronutrients: manganese, iron and zinc) within and among organs (root, stem and leaf) remains limited. To understand the difference among organs in terms of multi-nutrient stoichiometric homeostasis responding to N fertilization, a six-level N supply experiment was conducted through a hydroponic system to examine stem growth, multi-nutrient concentrations and stoichiometric ratios in roots, stems and leaves of 2-year-old Chinese hickory (*Carya cathayensis* Sarg.) saplings. Results showed that N supply significantly enhanced leaf length, width, basal diameter and sapling height. Increasing the rates of N also significantly altered multi-nutrient concentrations in roots, stems and leaves. Macronutrients generally respond more positively than micronutrients within organs. Among organs, leaves and stems generally responded more actively to N supply than roots. The stoichiometric ratios of nutrients within different organs changed significantly with N supply, but their direction and degree of change varied by organ. Specifically, increased N supply reduced the ratios of both macronutrients and micronutrients to N in plant organs, while increased N supply elevated the ratios of P to other nutrients. With N fertilization, ratios of micronutrients decreased in leaves and stems and increased in roots. In particular, leaf N and stem Mn stoichiometries responded strongly to N availability, indicating stimulated N uptake but a decreased risk of Mn²⁺ accumulation to excessive N. Overall, Chinese hickory saplings responded positively to increasing N availability in terms of stem growth, but the multi-nutrient stoichiometric homeostasis was distinctively organ-dependent. These results are expected to enhance our understanding of N-induced changes in homeostasis of multiple nutrients at the organ level and may offer new insights into how plants adapt to increasing N fertilization.

Keywords: Chinese hickory, multnutrient, nitrogen fertilization, organs, stoichiometric homeostasis.

Introduction

In order to meet the growing agricultural and forestry needs from explosive population growth, China has become the largest fertilizer consumer in the world (Erisman et al. 2008). Nitrogen (N) fertilizers are widely used in agroforestry practices as N is required in the fundamental building blocks of plants (chlorophyll, protein, enzymes, etc.) and plays a significant role in regulating plant metabolism and activities (Tian et al. 2016; Cleland and Harpole, 2010). Results from both short-term N

manipulative experiments and long-term investigations have established that N loading can stimulate plant growth and subsequently increase carbon sequestration rates and ecosystem productivity (Pregitzer et al. 2008, Thomas et al. 2010). But excessive N accumulation, which exceeds optimal N levels, diminishes positive N effects (Dong et al. 2022). Studies have demonstrated that excessive N fertilization has profound negative effects on terrestrial ecosystems, including soil acidification and nutrient imbalances (Han et al. 2015). At the species

level, the ability to maintain relatively consistent elemental nutrient composition and ratios (i.e., nutrient homeostasis) is fundamental to tree growth, but nutrient regimes could be altered by environmental changes (Mayor et al. 2014, Kou et al. 2017, Hogan et al. 2021). In managed agroforestry systems of subtropical regions, land managers generally obtain higher yields through massive N fertilization, often by exceeding the N requirement of trees (Zhang et al. 2016, 2017). Therefore, it is important to identify tree response to excessive N stress associated with increasing N fertilization (Ye et al. 2018).

Plants require at least 17 elemental nutrients to grow and support their complete life cycle; among them, macronutrients [N, phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg)] and micronutrients [here refers to manganese (Mn), iron (Fe) and zinc (Zn)] are both indispensable for plant growth and metabolic processes (Watanabe et al. 2007, Krämer 2010, Lin et al. 2014). These nutrients support not only growth and cellular functioning but also act as a safeguard against periods of reduced resource availability (Kaspari and Powers 2016). Previous studies have prioritized N and P, the most functionally and quantitatively important nutrients for plants. Base cations (K, Ca and Mg) and metal cations (Mn, Fe and Zn) have received less attention although they are physiologically essential in maintaining plant fitness and function (Elser et al. 2007, Vitousek et al. 2010, Ågren and Weih 2012). For instance, K plays a fundamental role in controlling water conductance and transpiration (Sardans and Peñuelas 2015) while Mg is indispensable for leaf photosynthesis (Laing et al. 2000). Nitrogen fertilization influences the nutrient stoichiometries of plants via microbe-mediated N transformations (mineralization, nitrification and denitrification) or cation exchanges, which mainly occur in leaves or roots. Increased N availability can improve plant N uptake; however, too much N input results in soil acidification, thus decreasing P availability by binding PO_4^{3-} to metal ions (Bünemann et al. 2011). Acidified soil also reduces K^+ , Ca^{2+} and Mg^{2+} availability in the soil but increases free Mn^{2+} and Fe^{3+} (Vadeboncoeur 2010, Tian and Niu 2015a, Cusack et al. 2016, Bourgeois et al. 2019). Therefore, these effects impact a wide range of plant physiological processes and morphological traits (Sant'Anna-Santos et al. 2006). Since the response of nutrients within biogeochemical cycles is dependent on their relationship with other nutrients, it is imperative to seek a holistic understanding of the direction and magnitude of multi-nutrient response to excessive N fertilization within an individual tree organ.

Nutrient stoichiometry not only serves as the conserved proportions of molecules that are necessary to support metabolic processes but also is a powerful approach to examine the balance of multi-nutrients in ecological processes (Yu et al. 2015). Many studies have used stoichiometric homeostasis to characterize shifts in the magnitude and direction of nutrient limitation and estimate alterations to forests from a changing

environment (Mooshammer et al. 2012, Pretzsch et al. 2015, Tian et al. 2016). But previous studies have been largely focused on N:P ratios because N or P can limit primary production (Elser et al. 2007), while the stoichiometric relationships of K, Ca, Mg, Mn, Fe and Zn have received minimal attention. Nitrogen fertilization may also alter the concentrations and stoichiometric ratios of these nutrients in tree organs due to differences in physio-chemical properties and biogeochemical pathways (Han et al. 2011). Although the stoichiometric homeostasis hypothesis established that plants need to maintain an optimal ratio or relatively stable ratios among these nutrients (Ågren 2008, Kaspari and Powers 2016), it remains unclear how the stoichiometric relations of these multiple nutrients shift with N availability at the organ level.

The optimal partitioning theory postulates that plants growing in different stages or environments allocate nutrients among organs based on resource demands (Abrahamson and Caswell 1982, Gargaglione et al. 2010). However, the balance could be altered by massive N input, leading to reduced transportation and absorption efficiency of nutrients (Niklas and Enquist 2002, Knorr et al. 2005). A recent hypothesis suggests that plants have the ability to maintain a relatively balanced element supply status (i.e., N, K, Na, Ca, Mg and Al) by increasing transpiration under high N inputs, but the results were synthesized from studies of leaf data only, which may distort the inferences of N enrichment on the regulation of nutrients more broadly (Lu et al. 2018). In general, leaves respond more actively to N additions than roots and stems. However, there are insufficient data to determine if roots and stems can also maintain nutrient homeostasis under excessive N fertilization, even though nutrient utilization and storage capacity are directly dependent on nutrient uptake by roots and species (Mayor et al. 2014, McCormack and Iversen 2019, Paradosio et al. 2020). The degree to which N-induced changes in multi-nutrient stoichiometries are reflected in different organs is still unclear in the early stage of plant growth. Consequently, manipulated experiments become an effective approach to understanding the responsive patterns of multi-nutrient to N fertilization within and among organs.

Chinese hickory (*Carya cathayensis* Sarg., hereafter, hickory) is distributed throughout eastern China and is greatly valued for its economical and highly nutritious nuts (Xu et al. 2016). However, due to the extensive use of fertilizers, a decline in the yield and nut quality has hindered the development of hickory as an economic crop in China (Liu et al. 2011). Therefore, understanding the nutrient uptake strategy of hickory saplings and their phenotypic and stoichiometric responses to N availability is economically important. In this study, two-year-old hickory saplings were subjected to a hydroponics experiment to test the effects of elevated N fertilization on sapling phenotypic plasticity, macro- and micronutrient concentrations and their stoichiometric relationships within and among

organs. We hypothesized that: (H1) regardless of plant organ, increasing N would elevate the concentration of macronutrients before reaching an 'optimal' N level while the concentrations of micronutrients would be reduced; (H2) leaves and stems would be more responsive to increasing N than roots; and (H3) stoichiometric homeostasis of macro- and micronutrients would differ among organs with N fertilization.

Materials and methods

Plant material and growth conditions

Two-year-old healthy hickory saplings with approximately uniform growth status (average height 80.0 cm; average basal diameter 6.5 mm) were obtained from the Tianze Hickory Company (Lin'an, Hangzhou). These saplings (bareroot stocks) were then transplanted into plastic pots (height: 30.5 cm; top diameter: 25 cm) that contained a full-strength fertilizer solution. The composition of the fertilizer solution was the same as in other published studies (Jin et al. 2011). It comprised of 1.25 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM $\text{Ca}(\text{H}_2\text{PO}_4)_2$, 1.0 mM K_2SO_4 , 0.1 mM MgSO_4 , 12.5 μM H_3BO_3 , 1.0 μM MnSO_4 , 1.0 μM ZnSO_4 , 0.25 μM CuSO_4 , 0.1 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ and 10 μM EDTA-Fe. We selected 150 of the homogeneous hickory saplings described above for our N fertilization experiment. Prior to transplanting, we gently cleaned the roots with pure water.

The saplings were grown in the artificial intelligence greenhouse of Zhejiang A&F University (Lin'an, Hangzhou). The greenhouse had natural sunlight during the day, and the temperature was set at 30 (day) and 25 °C (night), respectively, under natural photoperiod. The relative humidity within the greenhouse was about 60% ($\pm 5\%$). We allowed the hickories to adapt to transplanting and greenhouse conditions for a month before initiating the treatments. The N fertilization experiment was conducted as a randomized complete block factorial design with six treatments (25 replicates). The treatments included a control with no $\text{Ca}(\text{NO}_3)_2$ supply (N0), N1, N2, N3, N4 and N5 levels containing 0.25, 0.75, 1.25, 2.0 and 3.0 mmol of $\text{Ca}(\text{NO}_3)_2$, respectively. We used $\text{Ca}(\text{NO}_3)_2$ to increase N levels due to its high solubility and limited effect on the solution pH (Rich 2021). Since altering $\text{Ca}(\text{NO}_3)_2$ levels also affected the Ca concentration in the solution, CaCl_2 was added to the treatments along with N to ensure that Ca and electrical conductivity were consistent across treatments. The nutrient solution was changed every 7 days and $\text{Ca}(\text{OH})_2$ and HCl were used to maintain a pH of 5.7. The nutrient solution was aerated in the pots with small oxygen pumps for the duration of the study.

Sampling, measurement and chemical analysis

Following 80 days of treatment, six saplings per treatment were randomly selected to measure phenotypic and chemical response to N fertilization. From each sampling, we selected 10 fully expanded mature leaves from four directions to measure leaf length, width, stem basal diameter and stem height

with digital Vernier calipers, to compare phenotypic growth responses associated with N fertilization. Upon removing the saplings from the plastic pots, we washed them with deionized water to remove any adhering solution and dried them with absorbent paper. Using caution, we separated the saplings into roots (belowground organ), stems (aboveground organ separate from leaves) and leaves. All samples were labeled and chilled immediately. Samples were oven-dried (105 °C for 1 h and 65 °C for 48 h) to a constant weight and finely ground with a mixer mill (Retsch GmbH MM400, Haan, Germany). The total N concentration in plant organs (roots, stems and leaves) was determined with a C-N auto analyzer (Elementar VarioMAX, Germany) while total P, K, Ca, Mg, Mn, Fe and Zn were obtained by inductively coupled plasma-optical emission spectrometry (ICP-OES, PEO 5300 DV, Waltham, MA, USA) after digestion of the tissue samples in concentrated HNO_3 .

Statistical analysis

Data normality (Kolmogorov–Smirnov test) was evaluated before performing the following statistical analyses. If data were non-normal, they were log-transformed or square root-transformed to improve normality and reduce heteroscedasticity. The effects of N fertilization rate on individual phenotypic characteristics (leaf length, leaf width, stem basal diameter and stem height) were estimated by one-way analysis of variance (ANOVA), with N fertilization as the fixed effect. When statistically significant ($P < 0.05$), Tukey's HSD (Honestly Significant Difference) test was used to identify differences among the N fertilization treatments. Two-way ANOVAs were used to test the effects of N fertilization treatment, plant organ, and their interactions on the concentrations and stoichiometries of multi-nutrients. When significant effects were detected, one-way ANOVAs were used to test the effects of N fertilization rate and organ type on the individual concentrations and stoichiometries of multi-nutrients. Significant differences between means were compared using Tukey's HSD test. Statistical analyses above were performed using SPSS 20.0 (IBM SPSS Statistics, USA) with the statistical significance level set at $\alpha = 0.05$.

To quantify the magnitude of changes of a variable to N fertilization, we calculated a response ratio (RR) using the methods developed by Gill et al. (2021). The RR of multi-nutrient concentrations and stoichiometries within organs is equal to the natural log of the ratio of the mean value for a given variable under N fertilization treatment to that of the control treatment for each organ, as shown in Equation (1).

$$\text{RR} = \ln (x_{\text{average N}}/x_0) \quad (1)$$

where $x_{\text{average N}}$ and x_0 represent the mean value of the N fertilization treatment and the value of control treatment in each organ, respectively. $\text{RR} > 0$ indicates that N fertilization increased the parameter of interest, while $\text{RR} < 0$ indicates that

Table 1. Response of phenotypic characteristics to N fertilization in hickory saplings.

N level	Leaf length (cm)	Leaf width (cm)	Basal diameter (cm)	Height (cm)
N0	12.78 ± 0.63bc	4.12 ± 0.16a	9.07 ± 0.81c	100.2 ± 4.92c
N1	12.26 ± 0.69c	3.56 ± 0.35b	10.93 ± 0.78b	106.6 ± 9.29c
N2	13.02 ± 0.40abc	3.86 ± 0.21bc	10.98 ± 0.97b	109.6 ± 5.03bc
N3	14.38 ± 0.91a	4.28 ± 0.36a	11.90 ± 0.43b	117.4 ± 7.40b
N4	14.08 ± 1.75ab	4.32 ± 0.67a	13.28 ± 0.80a	129.8 ± 9.15a
N5	14.48 ± 1.37a	4.44 ± 0.86a	13.67 ± 1.23a	134.7 ± 10.47a

Note: Data are mean ± SD ($n = 6$). N0, N1, N2, N3, N4 and N5 indicate an N fertilization gradient of 0, 0.25, 0.75, 1.25, 2.0 and 3.0 mmol $\text{Ca}(\text{NO}_3)_2$, respectively. Different letters from within the same measured trait show significant differences between N fertilization levels ($P < 0.05$). Level of significance (two-tailed) by Tukey's HSD test.

Table 2. Effects of N fertilization rate, organ and their interactions on the multi-nutrient concentrations in hickory saplings (F -value).

Variables		Source of variation		
		N supply rate	Organ	N supply rate × organ
Macronutrients	N	168.677**	1128.326**	65.239**
	P	31.345**	743.649**	16.398**
	K	50.776**	1403.417**	9.714**
	Ca	71.847**	2433.712**	35.085**
	Mg	121.299**	4836.460**	45.183**
Micronutrients	Mn	106.060**	3935.645**	46.234**
	Fe	176.352**	6746.979**	32.472**
	Zn	65.443**	641.047**	31.364**

** $P < 0.01$.

N fertilization decreased the parameter. The greater absolute value of the RR indicates that N fertilization had a stronger impact on the parameter of interest (Gill et al. 2021). We computed the means and 95% confidence intervals (CI) of each parameter. Figures were made by Origin 9.1 (OriginLab, USA) and R 3.6.2 statistical platform using ggplot2.

Results

Response of phenotypic characteristics to N fertilization

Leaf length, basal diameter and height of hickory saplings all significantly increased with elevated N, while leaf width was smaller at lower N concentrations (N1 and N2), it increased in width at higher N concentrations (N3, N4 and N5) (Table 1, $P < 0.05$).

Multi-nutrient concentrations in response to N fertilization

Nitrogen fertilization level, plant organ and their interaction had significant impacts on the concentrations of micronutrients and macronutrients (Table 2), but the responses were organ-dependent (Figure 1). Generally, moderate N fertilization resulted in the accumulation of other macronutrients. At higher N fertilization rates, the accumulation of macronutrients decreased. However, the threshold for positive N fertilization response depended on plant organ.

In roots, elevated N significantly increased N concentration. Root P and K concentrations increased with N up to N4 but

decreased significantly at the highest N dosage (N5). Nitrogen fertilization rates had a negligible effect on Mg concentrations without a discernable response pattern. Root Ca concentration was highly variable across the N rates. At the lowest dosage (N1), root Ca concentration decreased to levels lower than those without N and remained lower for treatments N2, N3 and N4. At the highest N dosage, root Ca concentration was like those without NO_3^- supply, both having the highest Ca concentrations of all the treatments (Figure 1).

In stems, N concentrations were positively associated with N rates. Stem P, K and Ca concentrations displayed concomitant increases with increasing N supply; however, this positive effect was diminished at the highest dosage (N5). Stem Mg concentration was more sensitive to N rates, with initial increases following moderate N concentrations (N1 and N2) but decreasing positive effects thereafter (Figure 1).

In leaves, N concentrations were positively associated with N supply, but this positive effect was diminished at N5. Leaf P, K, Ca and Mg concentrations all had positive responses with the lowest N treatment but declined substantially when N concentrations exceeded N2 or N3. Leaf Mg concentration was the lowest level at the highest N rate (N5), which was similar to Mg concentrations without N fertilization. Generally, macronutrients in leaves were more responsive and sensitive to N supply than those in the roots or stems, especially for leaf N and Mg (Figure 1).

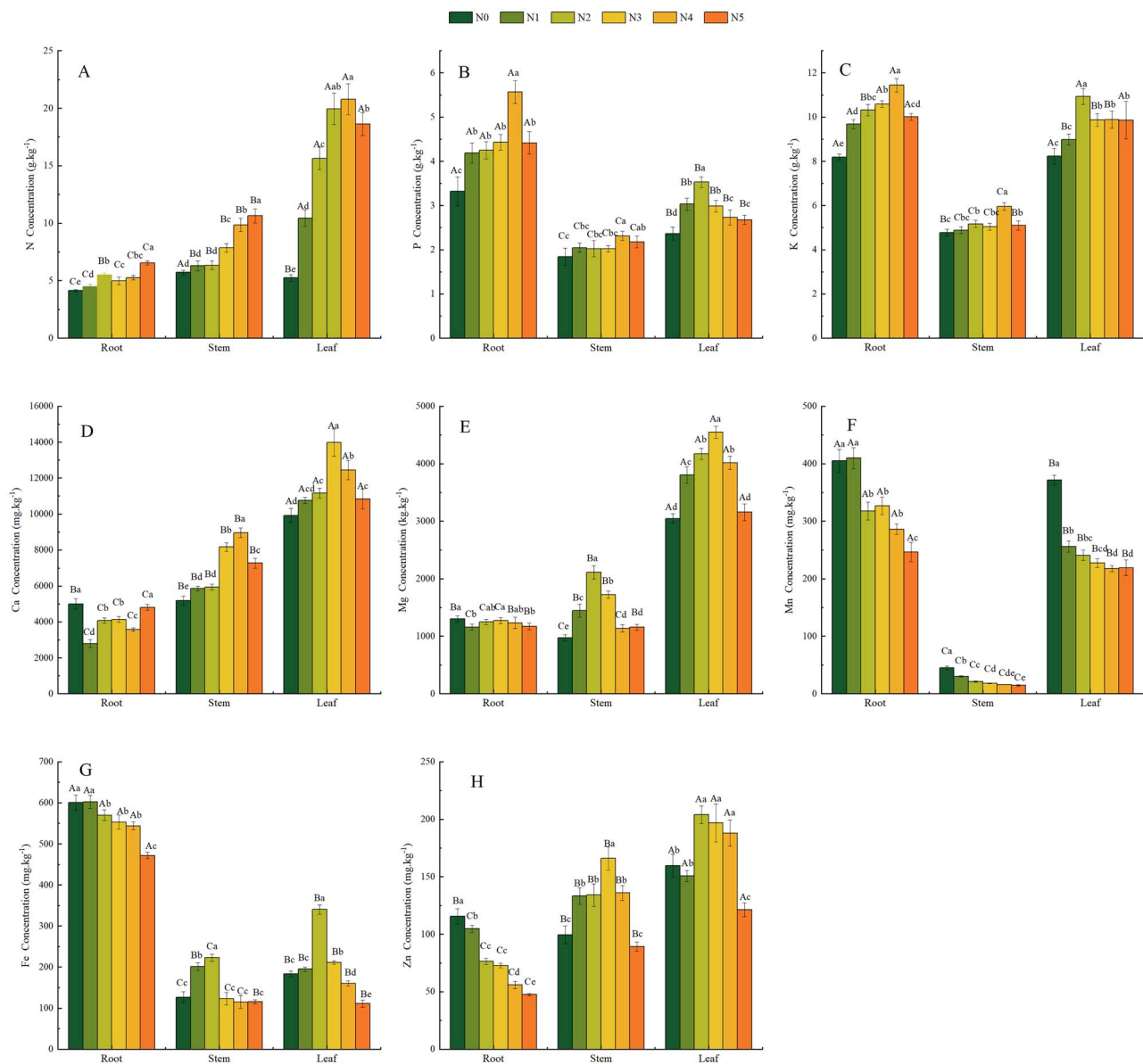


Figure 1. Multi-nutrient concentrations in response to N fertilization in hickory saplings. Data are mean $\pm$ SD ($n = 6$). N0, N1, N2, N3, N4 and N5 indicate an N supply gradient of 0, 0.25, 0.75, 1.25, 2.0 and 3.0 mmol $\text{Ca}(\text{NO}_3)_2$, respectively. Different uppercase letters from the same N fertilization level show significant differences between organs ($P < 0.05$). Different lowercase letters from the same organ show significant differences between N fertilization rates ($P < 0.05$). Level of significance (two-tailed) by Tukey's HSD test.

For micronutrients, significant negative effects of N supply were observed. Specifically, Mn concentrations responded negatively to increasing N fertilization among plant organs. Stems and leaves had generalizable trends across micronutrients. Iron and Zn were positively associated with moderate N fertilization, but these positive outcomes were diminished when treatments exceeded N2 and N3 (Figure 1).

Multi-nutrient stoichiometries in response to N fertilization

Nitrogen fertilization rate, plant organ and their interaction all had significant effects on the stoichiometries of the eight

nutrients (Table 3), but the responsive pattern varied by organ (Figures 2 and 3).

In roots, higher N fertilization (N4 or N5) significantly enhanced the RRs of P:K, P:Mg, K:Mg, Mn:Fe, Mn:Zn, Mn:N, Fe:Zn, P:Fe, P:Zn, K:Fe, K:Zn, Ca:Zn and Mg:Zn. However, higher N fertilization decreased the RRs for Mg:N, Fe:N and Zn:N. The following nutrients in roots responded to increasing N fertilization: P:N, K:N, Ca:N, P:Ca, K:Ca, Ca:Mg and Ca:Fe, but the response did not fit into a discernable pattern. The RRs for P:Mn, K:Mn, Ca:Mn, Mg:Mn and Mg:Fe were generally less responsive to increasing N fertilization than other nutrients RRs (Figures 2 and 3).

Table 3. Effects of N fertilization rate, organ and their interactions on the multi-nutrient stoichiometries in hickory saplings (*F*-value).

Variables		Source of variation		
		N rate	Organ	N rate × organ
Within macronutrients stoichiometries	P:N	33.963**	1816.394**	28.111**
	K:N	105.828**	2602.100**	54.548**
	Ca:N	122.309**	18.135**	51.308**
	Mg:N	125.082**	123.776**	61.443**
	P:K	2.220	149.543**	3.659**
	P:Ca	57.352**	2141.065**	62.517**
	P:Mg	26.254**	1416.111**	21.335**
	K:Ca	64.555**	2844.424**	77.461**
	K:Mg	29.578**	1959.031**	30.668**
Within micronutrients stoichiometries	Ca:Mg	163.898**	813.499**	96.957**
	Mn:Fe	78.154**	1412.558**	44.590**
	Mn:Zn	71.689**	2517.107**	103.864**
	Fe:Zn	88.510**	5677.108**	124.240**
	Mn:N	457.108**	4782.377**	131.485**
	Fe:N	85.685**	2786.899**	21.167**
	Zn:N	200.330**	28.396**	37.536**
Macronutrients:micronutrients stoichiometries	P:Mn	42.487**	930.415**	34.161**
	P:Fe	48.47**	173.078**	11.419**
	P:Zn	84.344**	1102.268**	66.766**
	K:Mn	141.565**	3024.735**	107.64**
	K:Fe	101.794**	616.730**	36.466**
	K:Zn	148.05**	1670.460**	90.765**
	Ca:Mn	319.443**	5467.081**	268.874**
	Ca:Fe	105.091**	932.664**	32.88**
	Ca:Zn	127.102**	50.768**	18.194**
	Mg:Mn	177.13**	3451.965**	126.136**
	Mg:Fe	35.16**	1075.094**	24.732**
	Mg:Zn	45.446**	433.509**	29.311**

***P* < 0.01.

In stems, increasing N fertilization significantly decreased the RRs of N:P, N:K, P:Ca, K:Ca, Mn:N, Mn:Fe and Mn:Zn. Although increasing N fertilization increased the RRs of P:Mn, K:Mn and Ca:Mn, there was no significant N fertilization effects detected for P:K. At lower levels of N fertilization, stem Mg:N, Fe:Zn, Fe:N, Zn:N, Mg:Mn, Mg:Fe and Mg:Zn increased (N1 and N2); however, at higher rates (N3, N4 and N5), they decreased. Conversely, stem P:Mg, K:Mg, Ca:Mg, P:Fe, P:Zn, K:Fe, K:Zn, Ca:Fe and Ca:Zn decreased with lower levels of N fertilization but increased with additional fertilization (Figures 2 and 3).

In leaves, increasing N fertilization significantly decreased P:N, K:N, Ca:N, Mg:N, P:K, Mn:N, Fe:N and Zn:N. Nitrogen fertilization was positively associated with leaf K:Mn. Leaf P:Ca, P:Mg, K:Ca, P:Fe, P:Zn, K:Zn, Mg:Fe and Mg:Zn respond in an 'up-down-up' pattern to increased N fertilization. However, leaf Fe:Zn, P:Mn, Ca:Mn, and Mg:Mn exhibited an 'up-down' pattern. Conversely, leaf Ca:Mg, Mn:Fe, Mn:Zn, K:Fe, K:Mg, Ca:Fe and Ca:Zn exhibited a 'down-up' pattern to increasing N fertilization (Figures 2 and 3).

Response ratios of multi-nutrient concentrations and stoichiometries to N fertilization

Nitrogen fertilization had varying degrees of impact on the concentrations of macronutrients and macronutrients within organs. In general, N supply increased the concentrations of all the nutrients ($RR > 0$) in leaves and stems except for Mn, but decreased the concentrations of Ca, Mg and micronutrients (Mn, Fe and Zn) in roots (Figure 4). In particular, N supply strongly increased leaf N concentration while it decreased Mn concentrations in both leaves and stems. Not surprisingly, among organs, fundamental macronutrients (N, P and K) increased with N fertilization, but concentrations of Mn decreased (Figure 4).

Nitrogen fertilization elicited strong responses in the stoichiometries of the eight nutrients within organs. Contrary to the responses of nutrient concentrations, N fertilization reduced the ratios of both macronutrients and micronutrients to N in plant organs, although root P:N and K:N displayed slight increases. The ratios of P to other nutrients (exclusive of N) were mostly elevated by the N supply (14 out of 18) among plant organs.

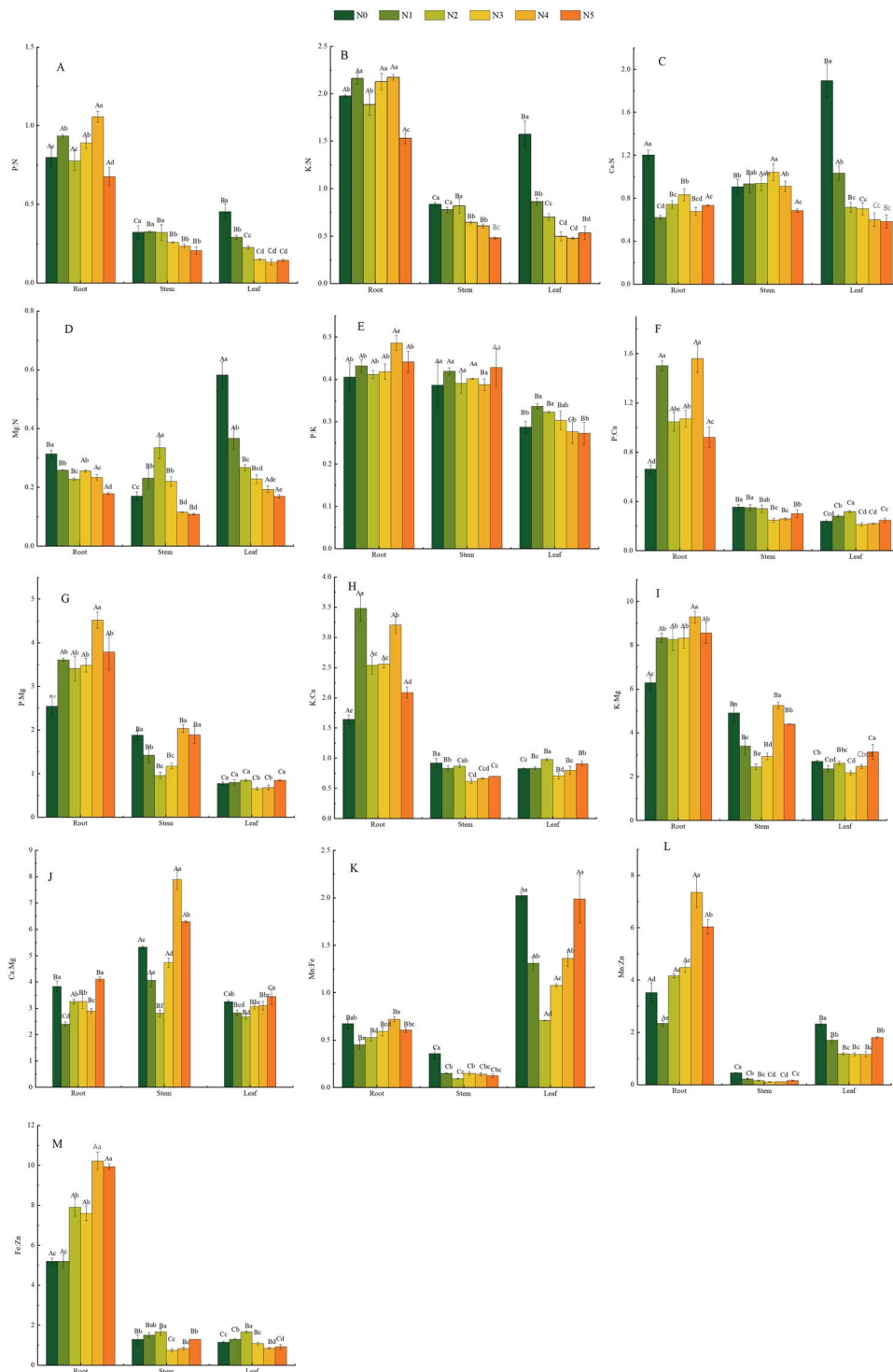


Figure 2. Multi-nutrient stoichiometries in response to N fertilization in hickory saplings. Data are mean $\pm$ SD ($n = 6$). N0, N1, N2, N3, N4 and N5 indicate an N fertilization gradient of 0, 0.25, 0.75, 1.25, 2.0 and 3.0 mmol $\text{Ca}(\text{NO}_3)_2$, respectively. Ratios are based on mass concentrations. Different uppercase letters from the same N fertilization rate show significant differences between organs ($P < 0.05$). Different letters from the same organ show significant differences between N fertilization rates ($P < 0.05$). Level of significance (two-tailed) by Tukey's HSD test.

Similarly, ratios of K to Ca, Mg and micronutrients were generally improved by N fertilization (10 out of 15). The ratios of Ca:Mg decreased with increasing N while the ratios of Ca and Mg to micronutrients increased among organs. Decreasing ratios between micronutrients were generally observed in leaves and

stems while they increased in roots (Figure 5). Across organs, the RRs following N fertilization of leaf N-related and stem Mn-related stoichiometries were significantly greater than that of other nutrients, indicating their reduced capacity to maintain homeostasis with increasing fertilization (Figure 5).

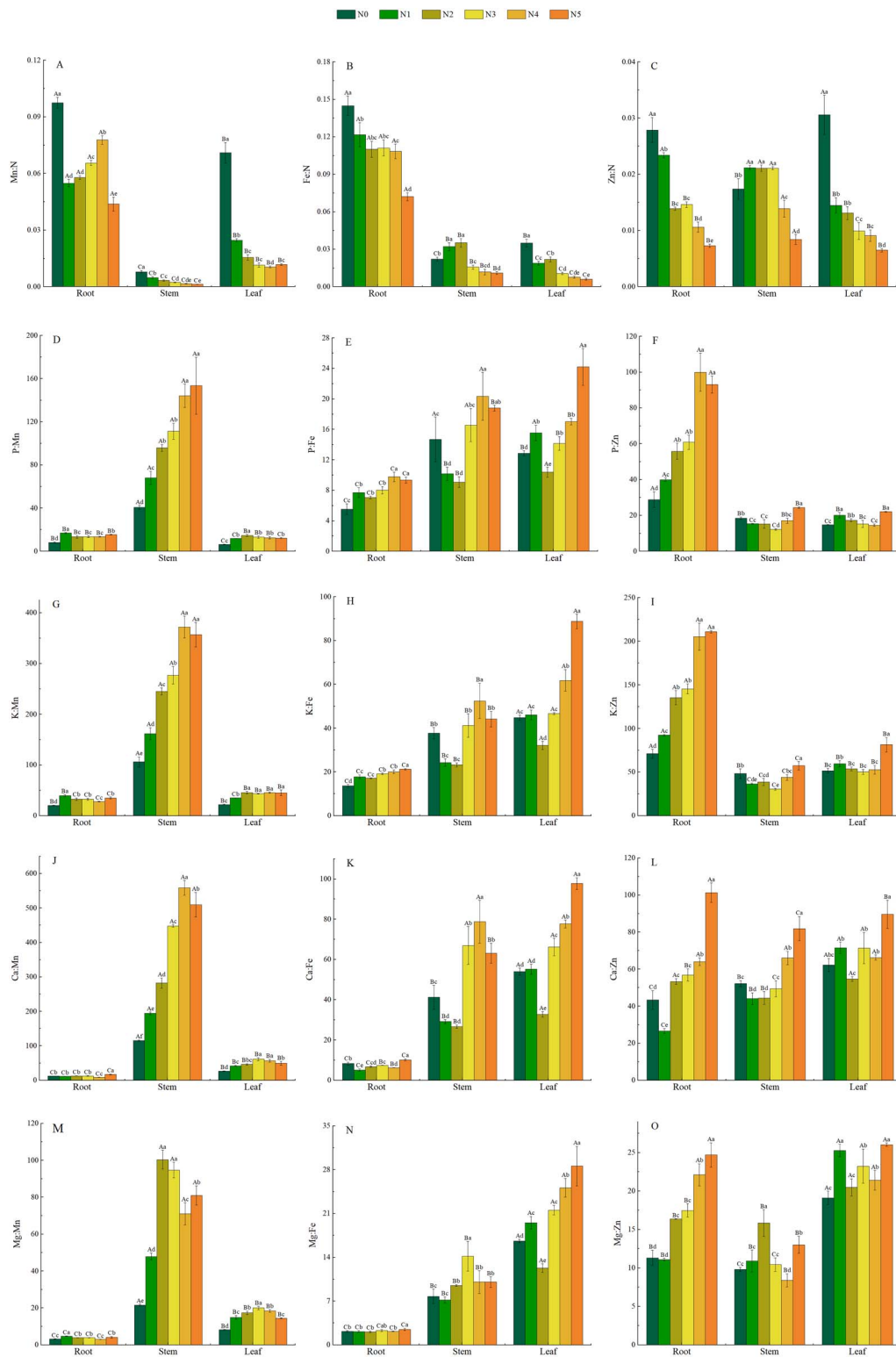


Figure 3. Multi-nutrient stoichiometries in response to N fertilization in hickory saplings. Data are mean $\pm$ SD ($n = 6$). N0, N1, N2, N3, N4 and N5 indicate an N fertilization gradient of 0, 0.25, 0.75, 1.25, 2.0 and 3.0 mmol $\text{Ca}(\text{NO}_3)_2$, respectively. Ratios are based on mass concentrations. Different uppercase letters from the same N fertilization rate show significant differences between organs ($P < 0.05$). Different letters from the same organ show significant differences between N fertilization rates ($P < 0.05$). Level of significance (two-tailed) by Tukey's HSD test.

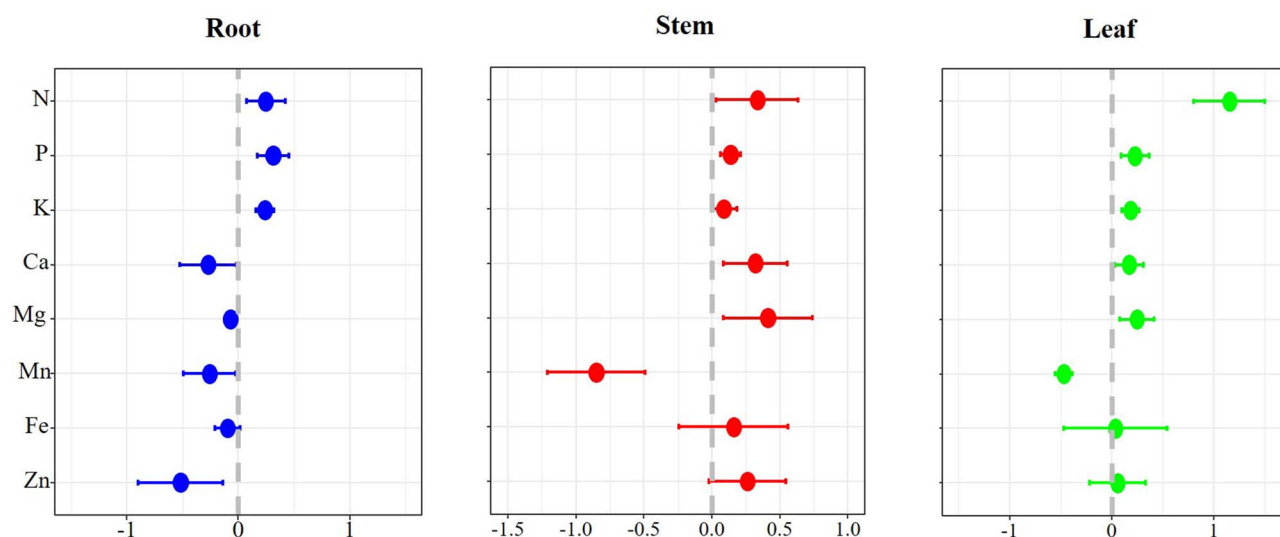


Figure 4. Response ratios of multi-nutrient concentrations to N fertilization in hickory saplings. Symbols indicate the means with their 95% bootstrapped CI. If the 95% CI does not overlap with the grey dashed line ($\log RR = 0$), it indicates significant changes in multi-nutrient concentrations caused by N fertilization.

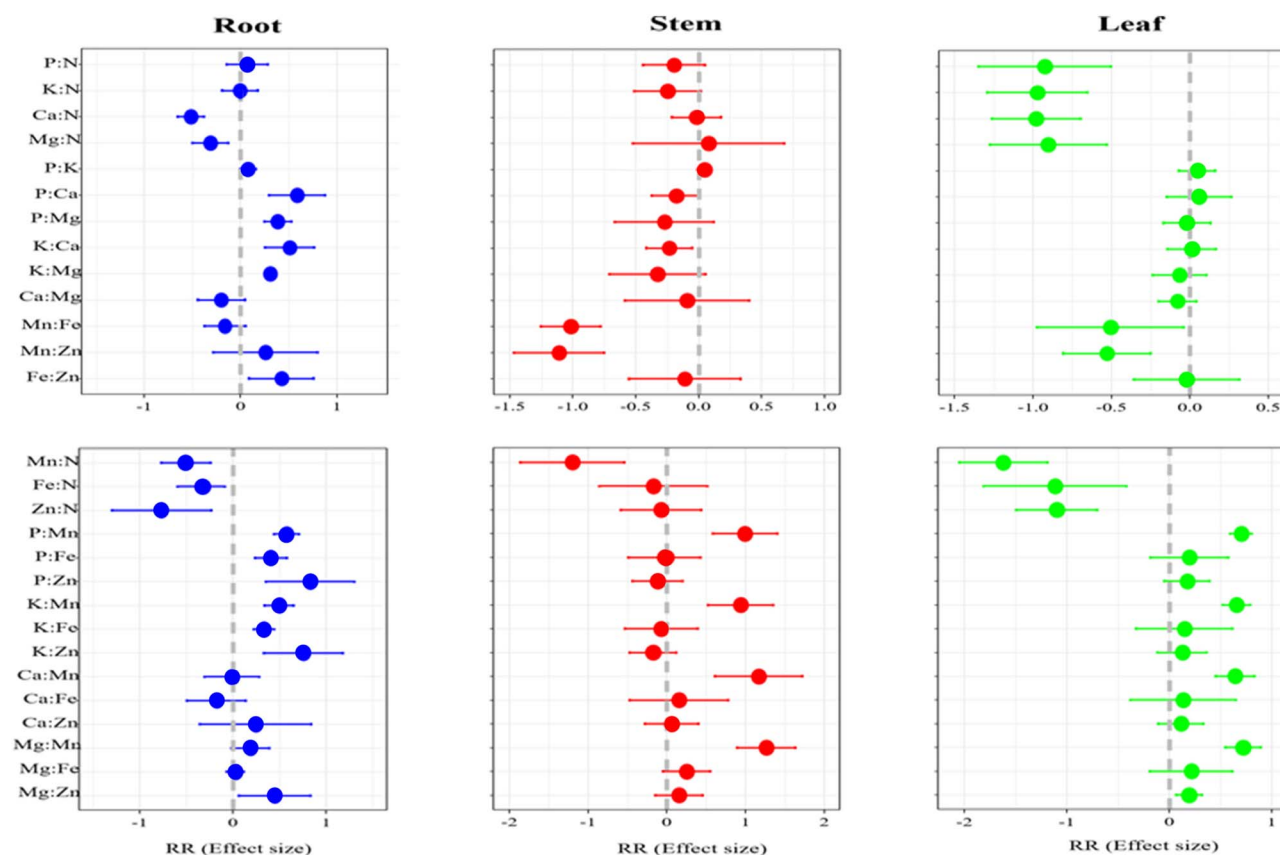


Figure 5. Response ratios of multi-nutrient stoichiometries to N fertilization in hickory saplings. Symbols indicate the means with their 95% bootstrapped CI. If the 95% CI does not overlap with the gray dashed line ($\log RR = 0$), it indicates significant changes in multi-nutrient stoichiometries caused by N fertilization.

Discussion

This study examined the response of hickory sapling growth and multi-nutrient stoichiometries to N fertilization through a

hydroponics experiment. We provided new experimental evidence that the stoichiometric relations of multi-nutrients are altered by N fertilization both within and among tree organs,

which is supported by previous empirical studies (Elser et al. 2010, Ågren and Weih 2012, Mo et al. 2015, Sardans et al. 2017). Greater phenotypic growth, such as increased leaf length, width and stem basal diameter and height, was observed with increasing N availability. However, leaf length and width appear sensitive to increased N supply with maximum gains when N rates ranged from 1.5 to 2.5 mmol. In contrast, the other growth parameters (stem basal diameter and height) increased with rising N availability. More importantly, we found divergent stoichiometric relationships among both macro- and micronutrients in different organs of hickory saplings, as explained below.

Variable response of plant organs to N fertilization

Results from this study show that N fertilization significantly alters the concentration of macro- and micronutrients in hickory saplings. However, these responses are organ-dependent. Among organs, leaves and stems increased nutrient concentrations greater than roots when measured by the RRs of multi-nutrient concentrations. This is consistent with our second hypothesis (H2) but contrasts with prior studies indicating that absorptive roots are more responsive to N than leaves when the number of nutrients and stoichiometric ratios are considered (Schreeg et al. 2014, Kou et al. 2018). This contrast with prior experiments is likely related to the differences in study design and species-specific response. The first difference may reflect the experimental application of N absorption and the study duration. For example, we are reporting results from a short-term hydroponics experiment compared with Kou et al. (2018), who conducted a 3-year soil N-manipulative experiment. Species may also be an important consideration for examining response to increasing N. Our study examined broadleaved saplings of Chinese hickory whereas Kou et al. (2018) conducted their study with primarily mature conifers (mainly *Pinus elliottii*). Plants may respond differentially to N availability in the short- and long-term. Short-term N input generally has less impact on base cations than long-term high N input (Perakis et al. 2013, Tian and Niu 2015b). However, this contrasts our short-term findings in which reduced concentrations of micronutrients were detected with N fertilization. Plant response to N fertilization may also differ between hydroponic systems and soil-based systems. Our hydroponic system excludes the interference of soil nutrient heterogeneity and depletion, which may increase the observed effects of N fertilization (Okemwa 2015). Additional work should be conducted to examine N response differences derived from experiments in natural soils versus those that are hydroponically controlled.

The stronger response of leaves may be related to their roles as specialists in photosynthesis and transpiration, in which many nutrients play significant and essential roles (Lu et al. 2012). For example, P participates in the formation of the photosynthetic membrane directly, and Mg is a constituent

element of chlorophyll, while K acts as an activator of certain enzymes (Schreeg et al. 2014, He et al. 2016). Prior studies in conifers have suggested that the response of leaves to N additions is species-dependent (Walter and Schurr 2005). We provide findings to suggest that the organs of broadleaved trees may also have variable responses to increasing N availability. Overall, our results demonstrate that hickory sapling leaves and stems are more responsive to the increasing N supply. In contrast, roots reduced base cations and metal cations with increasing N fertilization. However, we should also be aware that root nutrients could be accumulated when the vertical transport from roots to shoots is limited by external stress, thus resulting in an increase of N-induced changes in nutrient concentrations (Cernusak et al. 2010).

Differential responses of nutrient concentrations to N fertilization

The concentrations and allocations of nutrients in organs reflects the capacity of plants to respond to stress. Plants may become more vulnerable to external stressors with greater alterations in the concentration and allocation patterns of nutrients. (Hogan et al. 2021). In this study, the concentrations of macronutrients in roots, stems and leaves were significantly improved by increases in N before reaching the N 'optimal' point (i.e., N3 or N4). When N input increased beyond these optimal points, the concentrations of macronutrients began to decrease. This is somewhat consistent with N addition experiments that have been tested in temperate and tropical forests (Chen et al. 2016, McNulty et al. 2017), and confirms our first hypothesis (H1). Further, leaves generally have lower optimal N levels than roots or stems in terms of macronutrients concentrations (such as leaf N, P, K and Ca). Magnesium was the least resistant to high dosages of N in both stems and leaves, but root Mg concentrations remained stable even with excessive N.

Micronutrients responded differently to N fertilization compared with macronutrients. Increasing N supply reduced the concentrations of Mn, Fe, Zn in roots and Mn in both stems and leaves. These response patterns differ from the results of other studies (Kou et al. 2018), which reported that N addition in subtropical forests reduced the concentrations of root Zn but elevated root Mn, Fe and leaf Mn, Fe, Zn. Relatively speaking, micronutrients were more negatively associated with increasing N rates, as indicated by the reductions in Mn, Fe and Zn when N fertilization rate increased. Although not directly measured by our study, the varied response pattern of micronutrients in trees could be related to its growth stage because, as expected, adult trees and saplings generally have contrasting stoichiometric responses to environmental changes (Sun et al. 2016). Nevertheless, the mechanistic links of N supply and nutrient-specific responses remain controversial, especially for the belowground resource-acquiring absorptive roots and stem, and thus warrant further research (He and Dijkstra 2014).

Overall, among all the nutrients evaluated in this study, N and Mn were more responsive than other nutrients, but their patterns were contrasting, with N increasing and Mn decreasing with elevated N. Therefore, plants may develop corresponding functional traits to alleviate the stress from excessive N input (Chen et al. 2014).

Stoichiometric homeostasis of macro- and micronutrients to N fertilization

The results of the stoichiometric ratio changes are consistent with our third hypothesis (H3), in which N-related and Mn-related stoichiometric ratios displayed lower homeostasis (i.e., greater degree of change) across organs while P-related stoichiometric ratios were more stable (i.e., less degree of changes). We detected decreased responses of X:N ratios (i.e., ratios of P, K, Ca, Mg to N) in leaves, stems and roots except root P:N. Phosphorus-related ratios exhibited weaker but (6 out of 9) increasing response to N fertilization among organs. By contrast, we observed more decreasing responses on micronutrient dominant ratios, especially in stems. For example, Mn-dominant ratios were strongly reduced. These results suggested that the increase of N and P were greater than other macronutrients while reduction of Mn was higher than other micronutrients under increasing N input. This is somewhat incongruent with a synthetic result from Tian et al. (2016), who found positive N-dominant ratios but more negative P-dominant ratios under experimental N supply. This difference may be attributed to their results not considering organ-dependent N fertilization responses. The larger absorption of N and P might be attributable to plants at the sapling stage requiring more fundamental nutrients (N and P) for stem growth and differentiation (Bown et al. 2010). We also detected reductions in Mn concentrations and Mn-related stoichiometric ratios with increasing N supply. This finding may reflect that saplings are generally more sensitive to trace cation toxicity than adult trees, so they develop self-protective mechanisms to avoid accumulating excessive metal elements (Boquete et al. 2021). Furthermore, it is worth noting that roots were more sensitive than leaves and stems to the accumulation of metal cations. This is illustrated from this study by the decreased metal cation concentration in roots. This may be related to the submerged vertical position of roots in the nutrient solution, whereas stems and roots accumulate nutrients through translocation, which is significantly slower than direct root absorption (He and Dijkstra 2014). These combined results provide a more holistic understanding of multi-nutrient stoichiometric coupling in response to N fertilization. The results also offer possible insights for improving stoichiometric growth models at a broader scale. Moreover, although hickory saplings exhibited growth response to the additional N, further studies are needed to determine if adult hickories similarly respond to successive exogenous N. We expect that adult trees generally have contrasting stoichiometric

responses to environmental change compared with saplings, but the degree of these differences is not well understood.

Conclusion

In this study, hickory saplings exhibited high adaptability to increasing N fertilization in terms of stem growth, but the stoichiometric homeostasis of multi-nutrients varied significantly among organs. Concentrations of macronutrients generally responded with a hump-shaped pattern, while micronutrients displayed continuous reductions with increasing N fertilization. The stoichiometric ratios of multinutrients were also organ-dependent, in which N-related and Mn-related stoichiometric ratios showed lower homeostasis, while P-related stoichiometric ratios were relatively more stable among organs. Leaves and stems responded more actively (higher RR) than roots to increasing N. More importantly, we found that plants may develop related functional traits, such as increased fundamental nutrients to ensure stem growth but decreased metal cations to avoid the risk of metal toxicity through nutrient coupling. Therefore, the results from this study are expected to benefit our understanding of the balance of multiple nutrients in saplings and its impact on adaptive functional traits at the organ level with increasing N supply. What is more, implications from which nutrients or organs are more vulnerable to N input may also help forest managers reduce the negative effects of excessive N fertilization or by developing targeted fertilization strategies.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

The views and conclusions contained in this document are those of the authors and should not be interpreted as representing the opinions or policies of the funding agencies and supporting institutions. The authors declare that they have no conflict of interest.

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Authors' contributions

H.X. carried out the fieldwork and laboratory analysis, prepared figures and wrote the manuscript. X.L., Y.T. and L.S.P.K. revised

the manuscript, and S.J. contributed substantially to the study design and supervised the field and laboratory personnel.

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