



Responses of stomatal density and carbon isotope composition of sugar maple and yellow birch foliage to N, P and CaSiO₃ fertilization

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Stomatal density, stomatal length and carbon isotope composition can all provide insights into environmental controls on photosynthesis and transpiration. Stomatal measurements can be time-consuming; it is therefore wise to consider efficient sampling schemes. Knowing the variance partitioning at different measurement levels (i.e., among stands, plots, trees, leaves and within leaves) can aid in making informed decisions around where to focus sampling effort. In this study, we explored the effects of nitrogen (N), phosphorus (P) and calcium silicate (CaSiO₃) addition on stomatal density, length and carbon isotope composition ($\delta^{13}\text{C}$) of sugar maple (*Acer saccharum* Marsh.) and yellow birch (*Betula alleghaniensis* Britton). We observed a positive but small (8%) increase in stomatal density with P addition and an increase in $\delta^{13}\text{C}$ with N and CaSiO₃ addition in sugar maple, but we did not observe effects of nutrient addition on these characteristics in yellow birch. Variability was highest within leaves and among trees for stomatal density and highest among stomata for stomatal length. To reduce variability and increase chances of detecting treatment differences in stomatal density and length, future protocols should consider pretreatment and repeated measurements of trees over time or measure more trees per plot, increase the number of leaf impressions or standardize their locations, measure more stomata per image and ensure consistent light availability.

Keywords: MELNHE, nutrients, stomata, stomatal length, variance partitioning.

Introduction

Stomata are crucial for photosynthesis, respiration and transpiration. The density and size of stomata together influence the capacity for photosynthesis and transpiration (Sack et al. 2003, Wang et al. 2015). Along with regulation of stomatal conductance, these characteristics can influence water-use efficiency (Franks et al. 2015). Changes in stomatal density and length could scale up to influence water-use efficiency (Mastrotheodoros et al. 2017) and net primary production (Wang et al. 2015) at the ecosystem scale. Therefore, in the context of increased CO₂ and increasingly variable precipitation expected with global climate change (Campbell et al. 2009), it is important to understand influences on stomatal characteristics to understand how individual plants, species and ecosystems will respond.

Stomatal characteristics have a recognized genetic basis (Shimada et al. 2011), varying strongly with phylogeny (Liu et al. 2018), and are influenced by environmental factors such as light availability (Gay and Hurd 1975), atmospheric CO₂ concentrations (Woodward and Kelly 1995), soil moisture (Sun et al. 2014) and temperature (Beerling and Chaloner 1993, Hill et al. 2015). Stomatal density has also been found to vary with tree height, though the direction of this effect is inconsistent (Woodruff et al. 2010, Kenzo et al. 2012), perhaps due to confounding effects of canopy position. This plasticity is just one component of plants' overall water-use strategy (Deans et al. 2020, Xu et al. 2021); other biological influences on plant water use, in addition to stomatal density and length, include regulation of stomatal conductance

(Lawson and Violet-Chabrand 2019), leaf area and sapwood area (Wullschlegel et al. 1998), wood anatomy (Meinzer et al. 2013) and rooting architecture (Weigelt et al. 2021).

While effects of some environmental factors on stomatal characteristics are well known, studies on the effects of soil nutrient availability have yielded inconsistent results. In response to nitrogen (N) addition, stomatal density has increased in hybrid poplar (*Populus × euramericana* (Dode) Guiner var Dorskamp) and salmonberry (*Rubus spectabilis* Pursh; Siegwolf et al. 2001, van den Top et al. 2018) but decreased in *Arabidopsis thaliana* (L.) Heynh. (Cai et al. 2017). In response to the addition of phosphorus (P), stomatal density has increased in cowpea (*Vigna sinensis* L.) and chickpeas (*Cicer arietinum* L.; Sekiya and Yano 2008, Chtouki et al. 2022), or remained unchanged in *A. thaliana* and in six woody savanna species in Brazil (Cai et al. 2017, Costa et al. 2021). Effects of P addition on stomatal density were found to be positive in chickpea leaves when soil moisture was adequate (Chtouki et al. 2022) and positive in cowpea leaves when atmospheric CO₂ concentrations were high (Sekiya and Yano 2008). In response to calcium carbonate addition, no change in stomatal density in mature European beech trees (*Fagus sylvatica* L.) was detected (Forey et al. 2015). Other studies have observed a decrease in stomatal conductance with silicon fertilization (Gao et al. 2006, Vandeguer et al. 2021). Little research has been conducted on the effects of nutrient addition on stomatal characteristics in mature trees, and no research has been conducted on stomatal characteristics in a fully factorial N × P addition experiment.

Stomatal density and length can influence photosynthetic capacity and transpiration, but the actual rate of either process also depends on stomatal conductance. One way to observe the cumulative outcome of stomatal conductance over the lifetime of a leaf is to measure the stable carbon (C) isotope ratio ($\delta^{13}\text{C}$), which is related to the discrimination between ^{12}C and ^{13}C as leaves convert atmospheric carbon dioxide to photosynthate (Farquhar et al. 1982). $\delta^{13}\text{C}$ can thus provide insight on how nutrient addition might influence physiological function. $\delta^{13}\text{C}$ relates to intrinsic water-use efficiency (iWUE): the ratio of photosynthesis (A) to stomatal conductance (g_s), or of micromoles of CO_2 consumed in photosynthesis to moles of water lost due to transpiration (Pérez-Harguindeguy et al. 2013). The iWUE has been shown to increase with added N and P when adding N or P increases photosynthetic capacity (Raven et al. 2004), so we would expect $\delta^{13}\text{C}$ to also increase with N or P addition, particularly if N or P limits tree growth. Increases in transpiration with calcium (Ca) or silicate (SiO_3) addition (Green et al. 2013) could result in decreased iWUE and thereby a decrease in $\delta^{13}\text{C}$, though iWUE could increase if photosynthetic capacity were to increase more than transpiration or if total leaf area were to increase, both of which are possible given increases in forest production following calcium silicate (CaSiO_3) addition (Battles et al. 2014, Fahey et al. 2022). Other environmental factors that can influence $\delta^{13}\text{C}$ include climate (Rao et al. 2017), tree height and light availability, though comparisons of $\delta^{13}\text{C}$ in shaded vs open-canopy trees suggest that light availability and canopy position are most influential than tree height in influencing these characteristics (Vadeboncoeur et al. 2020).

Stomatal characteristics are easy and inexpensive to measure, but measuring a sufficient number to detect treatment effects in a large study can be time consuming. Decisions must be made at a variety of levels; for example, researchers must decide how many trees to sample (per species, plot, treatment, etc.), how many leaves of each tree to sample, how many impressions to make, the area of the leaf blade to study, and how many stomata for which to measure length. It is wise to consider the sampling intensity at each level to enhance the ability to detect treatment effects. Variance partitioning analysis, in which a linear mixed-effects model is run on data with no fixed effects and with nested random effects (Messier et al. 2010), is often used in trait studies to quantify variability at different taxonomic or otherwise hierarchical levels (Albert et al. 2010, Messier et al. 2010, Burton et al. 2017, Hecking et al. 2022). By showing at which level(s) variance is highest, researchers can optimize sampling intensity across multiple levels of organization.

Here, we measure the effects of N, P and CaSiO_3 addition on stomatal density, stomatal length and $\delta^{13}\text{C}$ and determine how variance is partitioned across sites, stands, plots, trees and leaves in our experimental design. Our research took place in a study of Multiple Element Limitation in Northern Hardwood Ecosystems (MELNHE), a long-term nutrient addition experiment in New Hampshire, USA that investigates the effects of long-term, low-dose applications of N (ammonium nitrate), P (monosodium phosphate) and CaSiO_3 (wollastonite). We studied two of the most abundant tree species—yellow birch (*Betula alleghaniensis* Britton) and sugar maple (*Acer saccharum* Marsh)—for this analysis. We expected to see an increase in stomatal density with nutrient addition and an increase in $\delta^{13}\text{C}$ with N and P addition and a decrease in $\delta^{13}\text{C}$ with CaSiO_3 addition, consistent with observed trends in iWUE

with nutrient addition (Raven et al. 2004, Green et al. 2013). In addition to characterizing the effects of nutrient addition on stomatal characteristics, we aimed to consider how our variance partitioning analyses could improve future sampling protocols.

Materials and methods

Site description

The 11 even-aged forest stands used in this study were located in three sites in the White Mountains of New Hampshire: Jeffers Brook (44°02'N, 71°53'W), Hubbard Brook Experimental Forest (43°56'N, 71°44'W) and Bartlett Experimental Forest (44°03'N, 71°17'W). Mean precipitation at Hubbard Brook Experimental Forest is ~1400 mm per year (Campbell et al. 2010), and the mean monthly air temperature is −9 °C in January and 18 °C in July (USDA Forest Service 2022). The climate at Bartlett Experimental Forest and Jeffers Brook is similar to that of Hubbard Brook (PRISM Climate Group 2022). Six of these stands were located at Bartlett (C1, C2, C4, C6, C8 and C9), whereas three were located at Hubbard Brook (HBCa, HBM, HBO) and two at Jeffers Brook (JBM, JBO; Table 1). Six stands were mid-successional (clear-cut between 1970 and 1990), and the remaining five were mature, harvested between 1883 and 1915 (Table 1). Forests were dominated by typical northern hardwood species, namely, American beech (*Fagus grandifolia* Ehrh.), sugar maple, yellow birch, red maple (*Acer rubrum* L.), white birch (*Betula papyrifera* Marsh.) and pin cherry (*Prunus pensylvanica* L.f.). Mature forest stands were dominated by American beech, sugar maple and yellow birch, whereas mid-successional stands had greater proportions of red maple, white birch and pin cherry. Soils were acidic, sandy-loam Spodosols developed in glacial drift deposited 14,000 years ago (Vadeboncoeur et al. 2014).

Each stand had at least four plots, which were 50 × 50 m, consisting of a 30 × 30-m measurement area with a 10-m buffer (but HBM and JBM plots were 30 × 30 m, consisting of a 20 × 20-m inner plot with a 5-m buffer). Four plots in each stand were treated annually with N (as NH_4NO_3 , 30 kg ha^{−1} year^{−1}), P (as NaH_2PO_4 , 10 kg ha^{−1} year^{−1}), both N and P, or neither. These doses are relatively modest, but have resulted in increased nutrient concentrations in foliage (Hong et al. 2022, Gonzales et al. 2023). Stands C1, C6, C8, HBM, JBM and JBO have a fifth plot where calcium CaSiO_3 (wollastonite) was added once at 1150 kg ha^{−1}. This application rate matches that applied in Watershed 1 at Hubbard Brook (Battles et al. 2014). The HBCa stand at Hubbard Brook had only a control and CaSiO_3 plot. Treatments began in 2011 in all stands except for HBM, in which CaSiO_3 was added in 2015.

Foliar sampling and laboratory analysis

Sunlit foliage was sampled from two locations in canopies of dominant and codominant sugar maple and yellow birch trees (>10 cm diameter at breast height, Table 1) using a shotgun. Sugar maple and yellow birch were selected due to their abundance in the study sites. Foliage was sampled in late July and early August in 2021 at Hubbard Brook and Jeffers Brook and in 2022 at Bartlett; foliage from 85 sugar maple trees and 137 yellow birch trees were sampled in total (Table 1). Most trees were sampled from the measurement

Table 1. Characteristics of stands used in a study of nutrient addition effects on stomatal density in yellow birch (YB) and sugar maple (SM). Sites are Bartlett Experimental Forest (Bartlett), Hubbard Brook Experimental Forest (Hubbard Brook) and Jeffers Brook. The number of trees sampled (*n*) is listed for each species in each stand. Note that SM was not present in four mid-successional stands at Bartlett.

Site	Stand	Age	Year cut	Plots	Species (<i>n</i>)
Bartlett	C1	Mid-successional	1990	Control, N, P, NP, CaSiO ₃	YB (12)
	C2	Mid-successional	1988	Control, N, P, NP	YB (10)
	C4	Mid-successional	1978	Control, N, P, NP	YB (12)
	C6	Mid-successional	1975	Control, N, P, NP, CaSiO ₃	YB (15)
	C8	Mature	1883	Control, N, P, NP, CaSiO ₃	SM (15), YB (15)
	C9	Mature	~1890	Control, N, P, NP	SM (12), YB (12)
Hubbard Brook	HBM	Mid-successional	1970	Control, N, P, NP, CaSiO ₃	SM (14), YB (14)
	HBCa	Mature	~1910	Control, CaSiO ₃	SM (5), YB (6)
	HBO	Mature	~1900	Control, N, P, NP	SM (9), YB (12)
Jeffers Brook	JBM	Mid-successional	~1975	Control, N, P, NP, CaSiO ₃	SM (14), YB (15)
	JBO	Mature	~1900	Control, N, P, NP, CaSiO ₃	SM (15), YB (15)

area, though occasionally trees from the buffer were sampled if there were not enough suitable trees in the inner plot.

Three leaves from each tree were selected for stomatal density and length measurements. These leaves were air-dried and pressed in a plant press for at least 72 h (van den Top et al. 2018). To help account for the effect of variability in light availability due to differences in canopy position (Keenan and Niinemets 2016, Young et al. 2023), additional foliage with complete petioles and little to no evidence of insect damage were used to calculate specific leaf area (SLA), which is the ratio of leaf area to dry mass. Area was calculated in ImageJ (<https://imagej.net/ij/index.html>) using 300 d.p.i. computer scans and mass was measured on the same leaves dried at 60 °C. This additional foliage was subsequently ground using a Wiley mill with a 40-mesh sieve or a mortar and pestle if the dry mass was <1.0 g. Stable carbon isotope ratios were measured on ground, homogenized foliage subsamples (3.5–4.5 mg) using an Isoprime isotope-ratio mass spectrometer coupled with a Pyrocube combustion analyzer; there was one ground foliage sample per tree. Values are reported in delta notation (Coplen 2011). Thirteen of 222 samples were run in triplicate for quality control purposes. $\delta^{13}\text{C}$ values for NIST 1515 (apple leaf) tissue standards were within 0.25‰ of the certified value. Triplicates were within 0.25‰, averaging 0.11‰. Carbon isotope data were available for 81 of 85 sugar maple trees and 112 of 137 yellow birch trees due to equipment malfunction. Fortunately, these samples include trees from each species in each treatment across both stand ages in all three sites.

Stomatal density and length measurements

Stomata were visualized using nail polish impressions (Figure 1; Sonti et al. 2021). Clear nail polish was applied in three ~1 × 1-cm squares on each leaf: one near the base of the leaf, one in the middle of the leaf and one at the top of the leaf, adjacent to the midvein. Impressions were mounted on clear slides using transparent tape and viewed using light microscopy at ×400 magnification. One photograph was taken of each impression. Care was taken to photograph an area on the impression in which stomata were as clear as possible and veins were minimally present; this biased the count relative to the whole leaf but minimized variability due to vein presence. Stomata were counted in each image using ImageJ software and converted to units of stomata

per square millimeter. In total, 846 sugar maple images and 1354 yellow birch images were analyzed; for five yellow birch trees, we did not make images for one of the three leaves due to pubescence on the underside of leaves, and for an additional two yellow birch trees and one sugar maple tree, poor impressions led to unusable images (i.e., impressions where in no place could we get a clear image in which we could clearly recognize stomata). Length of three stomata was measured in each image for 15 yellow birch trees in JBM and for 35 sugar maple trees across HBM, HBO and JBM (405 stomata for yellow birch, 945 stomata for sugar maple). Stomata density and length measurements (Zukswert et al. 2023b) and microscope photos (Zukswert et al. 2023a) are published through the Environmental Data Initiative.

Data analysis

Linear mixed-effects modeling was used to quantify the effect of nutrient addition on stomatal density and $\delta^{13}\text{C}$. Analyses were performed in R (R Core Team 2022) with the ‘lme4’ and ‘lmerTest’ packages (Bates et al. 2015, Kuznetsova et al. 2017) using Type III sums of squares (SS) and Satterthwaite degrees of freedom (DF). We ran two separate models to analyze the effects of N and P on stomatal density (N × P analysis): one for sugar maple and one for yellow birch. We ran two additional models to analyze effects of N and P on $\delta^{13}\text{C}$ in sugar maple and yellow birch. Data for these four models came from stands that had a control, N, P and NP plot. Categorical fixed effects for each model included N addition, P addition, the interaction of N and P, stand age (successional and mature) and site. The N and P variables represent a factorial ANOVA. Because sites were sampled in two different years, ‘site’ effects are confounded with sampling year and primarily serve a blocking function. Random effects for stomatal density models included stand, plot within stand, tree within plot and leaf within tree. Random effects for $\delta^{13}\text{C}$ models included site, stand within site and plot within stand. Stomatal density was either logarithm or cube-root transformed to meet assumptions of normality and homoscedasticity of the residuals (Cox 2011, Gotelli and Ellison 2013). $\delta^{13}\text{C}$ was not transformed. Fixed-effect and random-effect adjusted R^2 approximations were calculated using the ‘rsq’ package (Zhang 2022). Main effects (i.e., mean differences between factor levels: P vs no P, CaSiO₃ vs no CaSiO₃) were calculated using the ‘diffsmeans’ function in the ‘lmerTest’ package.

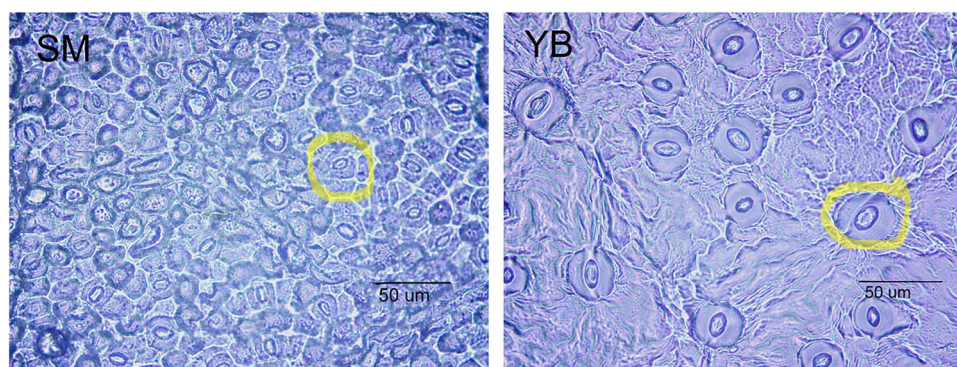


Figure 1. Examples of images used to measure stomatal density in sugar maple (SM, left) and yellow birch (YB, right). These leaves are from trees located in stand C8 plot 1, which is a P-addition plot at Bartlett Experimental Forest. Images were taken of clear nail polish films using a light microscope at $\times 400$ magnification. One stoma is circled in each image. All images used in this study are published through the Environmental Data Initiative (Zukswert et al. 2023a).

Stand-level random effects were zero for the sugar maple stomatal density analysis and $\delta^{13}\text{C}$ analysis for the $\text{N} \times \text{P}$ analysis, and plot-level random effects were zero for the yellow birch $\delta^{13}\text{C}$ analyses, resulting in singularities. For sugar maple, this meant that stand age and site were tested on the plot level as opposed to stand level, and for yellow birch, this meant that N, P and $\text{N} \times \text{P}$ were tested on the tree level as opposed to plot level, which increases the danger of accepting a significant effect that is not truly significant. Fortunately, for most of these tests, the effects were not significant. However, the positive effect of stand age on $\delta^{13}\text{C}$ in the sugar maple $\text{N} \times \text{P}$ analysis should be interpreted with caution.

Four linear mixed-effects models were constructed to quantify the effects of CaSiO_3 addition on stomatal density and $\delta^{13}\text{C}$ (CaSiO_3 analysis) for sugar maple and yellow birch. Data for these models came from stands that had control and CaSiO_3 plots. Categorical fixed effects included CaSiO_3 addition, stand age class and site. Random effects for stomatal density models included stand, plot within stand, tree within plot and leaf within tree. Random effects for $\delta^{13}\text{C}$ models included stand and plot within stand. Stomatal density was logarithm transformed to meet assumptions of normality for yellow birch; $\delta^{13}\text{C}$ was not transformed. Fixed-effect and random-effect adjusted R^2 approximations were calculated using the ‘rsq.lmm()’ function in the ‘rsq’ package (Zhang 2022). Singularity errors resulted for sugar maple stomatal density (stand-level effects were zero), yellow birch stomatal density (stand-level effects were zero) and yellow birch $\delta^{13}\text{C}$ (plot-level effects were zero), but in no cases did these instances of pseudoreplication produce statistically significant results.

To further evaluate sources of variability in stomatal density, we explored the relationship between SLA and stomatal density using a Spearman rho rank correlation test, calculated using the ‘Hmisc’ package (Harrell and Dupont 2023). To further aid in interpreting results, we also ran a linear mixed-effects model for SLA in yellow birch trees, with age as a fixed-effect factor and stand and plot within stand as random effects.

Nested random-effects models were used to perform a variance partitioning analysis to correspond with each of the four stomatal density models described above. Four nested random-effects models were also constructed for the four possible comparisons of stomatal length (sugar maple in

$\text{N} \times \text{P}$ plots, sugar maple in CaSiO_3 plots, yellow birch in $\text{N} \times \text{P}$ plots and yellow birch in CaSiO_3 plots). Random effects for stomatal density models were site, stand within site, plot within stand, tree within plot, leaf within tree and image within leaf, whereas stomatal length models also included a level for stoma within image. No fixed effects were included. Variance at each level was extracted from the model output and visualized graphically in R as the proportion of the total variance (R Core Team 2022). We assume in this analysis that any nutrient-addition treatment effect on variance would be present at the plot level and recognize that variance due to plot and treatment is indistinguishable given that there is only one plot of each treatment type in each stand. This analysis assumes that nutrient addition does not influence variance at other levels of measurement.

Upon performing a variance partitioning analysis on stomatal length and noting the extremely high variance within images and low variance at the plot level, we abandoned further stomatal length measurements and decided not to analyze these data statistically. These results are presented graphically.

Results

For sugar maple, stomatal density was 35 ± 19 stomata per mm^2 (8%) higher, on average, with P addition (P-addition effect for ln-transformed stomatal density $F = 4.10$, $P = 0.06$; Table 2), though stomatal density was not higher with P addition than the control in two of the six forest stands (Figure 2). Stomatal density was not consistently affected by N addition (N-addition effect $F = 0.02$, $P = 0.88$; Table 2), and the $\text{N} \times \text{P}$ interaction was not significant ($F = 0.01$, $P = 0.91$; Table 2). Overall, fixed effects in the sugar maple stomatal density $\text{N} \times \text{P}$ model explained 3.3% of variance and random effects explained 45.4% (Tables S1 and S2 available as Supplementary data at *Tree Physiology* Online). $\delta^{13}\text{C}$ in sugar maple was $0.51 \pm 0.23\text{‰}$ higher with N addition ($F = 4.05$, $P = 0.06$; Table 3, Figure 3), but not affected by P addition ($F = 0.08$, $P = 0.78$; Table 3) nor the $\text{N} \times \text{P}$ interaction ($F = 0.14$, $P = 0.71$, Table 3). Stomatal density was not influenced by CaSiO_3 addition ($F = 0.02$, $P = 0.88$; Table 2), but $\delta^{13}\text{C}$ was $0.76 \pm 0.38\text{‰}$ higher with CaSiO_3 addition ($F = 4.95$, $P = 0.08$; Table 3). $\delta^{13}\text{C}$ and stomatal density were not significantly correlated in sugar maple

Table 2. Effects of nutrient addition and stand age on stomatal density in sugar maple (SM) and yellow birch (YB) leaves based on four linear mixed-effects models. Each model is listed in *italics*. Analyses examined effects of N and P addition (SM $n = 588$ images, YB $n = 1000$ images) and effects of CaSiO_3 addition (SM $n = 258$ images, YB $n = 354$) in mid-successional and mature stands. Coefficients with standard error, SS, and numerator (Num.) and denominator (Den.) DF are displayed. Stomatal density was log-transformed for SM in the $\text{N} \times \text{P}$ analysis and YB in the Ca analysis, and cube-root transformed for YB in the $\text{N} \times \text{P}$ analysis, to meet normality assumptions for residuals (Shapiro–Wilk test $P \geq 0.05$). Random effects included stand, plot within stand, tree within plot and leaf within tree.

Fixed effect	SS	Num. DF	Den. DF	F	P
<i>SM: N × P (ln)</i>					
N	0.001	1	16	0.023	0.882
P	0.102	1	16	4.100	0.060
Age	0.013	1	16	0.538	0.474
Site	0.006	2	16	0.118	0.889
N × P	<0.001	1	16	0.014	0.908
<i>SM: CaSiO₃</i>					
CaSiO ₃	142.7	1	24	0.024	0.877
Age	376.2	1	24	0.065	0.802
Site	3558.4	2	24	0.306	0.740
<i>YB: N × P (cube root)</i>					
N	0.079	1	27	0.807	0.377
P	0.041	1	27	0.417	0.524
Age	0.112	1	6	1.135	0.329
Site	0.137	2	6	0.696	0.537
N × P	0.031	1	27	0.311	0.582
<i>YB: CaSiO₃ (ln)</i>					
CaSiO ₃	0.018	1	9	0.947	0.357
Age	0.058	1	9	3.121	0.113
Site	0.030	2	9	0.793	0.483

(Spearman $\rho = -0.04$, $P = 0.29$). $\delta^{13}\text{C}$ was higher in mature stands than younger forests for sugar maple trees, but this effect was only significant in the $\text{N} \times \text{P}$ analysis ($F = 9.57$, $P < 0.01$).

For yellow birch, though striking differences in stomatal density among nutrient treatments seem evident within several stands (Figure 2), stomatal density was not consistently affected by N or P addition across all the stands (N-addition $F = 0.81$, $P = 0.38$; P-addition $F = 0.42$, $P = 0.52$; Table 2). No patterns in $\delta^{13}\text{C}$ were evident with either N or P addition (N-addition $F = 1.18$, $P = 0.28$; P-addition $F = 0.84$, $P = 0.36$; Table 3). The interaction between N and P was not significant for either stomatal density or $\delta^{13}\text{C}$ ($P \geq 0.58$; Tables 2 and 3). Neither stomatal density nor length differed consistently due to CaSiO_3 addition in yellow birch (Table 2, Figures 2 and 4).

No consistent differences in stomatal length were observed among N or P addition treatments for either species (Figure 4). $\delta^{13}\text{C}$ was weakly positively correlated with stomatal density in yellow birch (Spearman $\rho = 0.05$, $P = 0.09$). In yellow birch, stomatal density was higher in thicker leaves with lower SLA values, more so for yellow birch (Spearman $\rho = -0.34$, $P < 0.01$) than for sugar maple (Spearman $\rho = -0.08$, $P = 0.03$; Figure 5). SLA in yellow birch was lower in mature stands than in mid-successional stands ($F = 8.367$, $P = 0.02$; Figure 6, Table S3 available as Supplementary data at *Tree Physiology Online*).

Most variance in stomatal density was found among images (i.e., within leaves) and among trees for both sugar maple and yellow birch in both the $\text{N} \times \text{P}$ analysis and the CaSiO_3 analysis (Figure 7). More than 50% of the variance was among images for sugar maple, with the next largest source of variance being among trees. For yellow birch, variance among

trees was highest, but nearly equivalent to variance among images in both analyses (Figure 7).

By far, most of the variance in stomatal length was measured among stomata within the same image: over 60% (Figure 7). In yellow birch, the next largest source of variance was among trees, whereas in sugar maple, variance was next highest among images in the CaSiO_3 analysis and among stands for the $\text{N} \times \text{P}$ analysis (Figure 7).

Discussion

Increased stomatal density in sugar maple with P addition (Table 2) is consistent with two previous studies of P addition and stomatal density, namely, a laboratory experiment involving cowpea (*Vigna sinensis* L.; Sekiya and Yano 2008) and a long-term (20-year) $\text{N} \times \text{P}$ fertilization field study of several woody plant species in a Brazilian savanna, though these increases in stomatal density were not statistically significant in the latter study (Costa et al. 2021). Our study is the first report of a possible effect of P availability on stomatal density in a temperate forest species. Other studies on the effects of P availability on stomatal density in herbaceous plants have not detected an effect of P addition, just as we did not observe an effect of P addition on yellow birch stomatal density. For example, Cai et al. (2017), who also compared effects of both N and P, did not find significant effects of P addition on stomatal density in *Arabidopsis thaliana* (L.) Heynh. The studies that do report a significant effect of P addition on stomatal density report significant interactions with soil moisture and atmospheric CO_2 , suggesting a greater effect of P on stomatal density in sufficiently wet conditions (Sekiya and Yano 2008, Chtouki et al. 2022) and with increasing atmospheric CO_2 (Sekiya and Yano 2008).

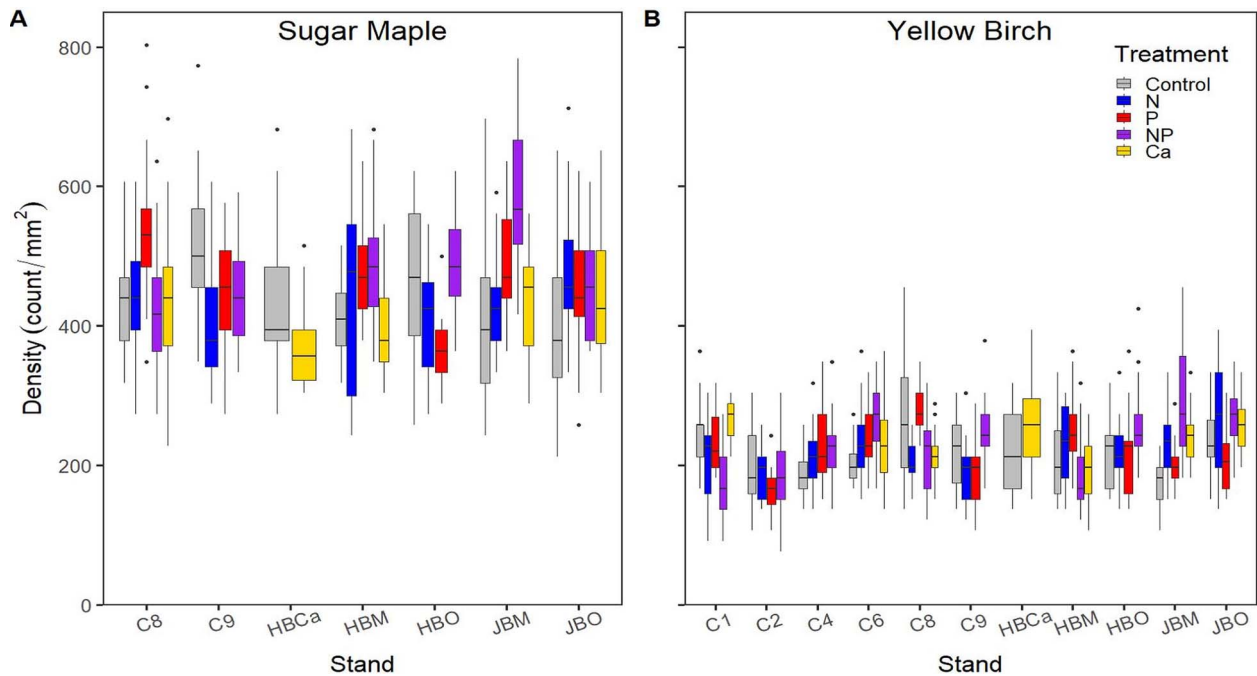


Figure 2. Stomatal density (count per mm²) of sugar maple (SM) leaves (A) and yellow birch (YB) leaves (B). Note that YB occurs in all 11 study stands but SM only occurs in seven.

Table 3. Effects of nutrient addition and stand age on $\delta^{13}\text{C}$ in sugar maple (SM) and yellow birch (YB) leaves based on four linear mixed-effects models. Each model is listed in *italics*. Analyses examined effects of N and P addition (SM $n = 67$ trees, YB $n = 92$ trees) and effects of CaSiO_3 addition (SM $n = 35$ trees, YB $n = 41$ trees) in mid-successional and mature stands. SS, and Num. and Den. DF are displayed. Random effects included stand and plot within stand.

Fixed effect	SS	Num. DF	Den. DF	<i>F</i>	<i>P</i>
<i>SM: N × P</i>					
N	1.707	1	18	4.052	0.059
P	0.034	1	19	0.081	0.779
Age	4.030	1	18	9.568	0.006
Site	1.167	2	18	1.385	0.276
N × P	0.061	1	18	0.144	0.709
<i>SM: CaSiO₃</i>					
CaSiO ₃	1.991	1	5	4.952	0.078
Age	1.467	1	3	3.650	0.164
Site	0.060	2	3	0.075	0.929
<i>YB: N × P</i>					
N	0.952	1	82	1.180	0.281
P	0.676	1	82	0.838	0.363
Age	0.772	1	5	0.957	0.373
Site	1.004	2	5	0.622	0.573
N × P	0.022	1	82	0.027	0.869
<i>YB: CaSiO₃</i>					
CaSiO ₃	1.514	1	36	2.660	0.112
Age	1.385	1	4	2.432	0.197
Site	0.007	2	4	0.007	0.994

Stomatal density and length can influence maximum stomatal conductance, though actual conductance is also influenced by pore area within stomata and the rate at which stomata open and close (Fanourakis et al. 2015). An increase in stomatal density with P addition can thereby increase the maximum stomatal conductance, which has implications for both photosynthesis and transpiration. Stomatal density and length are often negatively correlated (Bertolino et al. 2019), and small stomata are believed to open and close more quickly. As a result, leaf surfaces with higher densities of smaller

stomata can increase maximum conductance (and thereby increase photosynthetic capacity) in adequate environmental conditions while providing more control and faster rates of stomatal opening and closure in inadequate conditions, such as during drought or periods of high atmospheric water demand (Drake et al. 2013). We did not see a difference in stomatal length with P addition in our study, though we measured this variable on only a subset of trees, and variability in length among stomata within the same image was remarkably high (Figure 7).

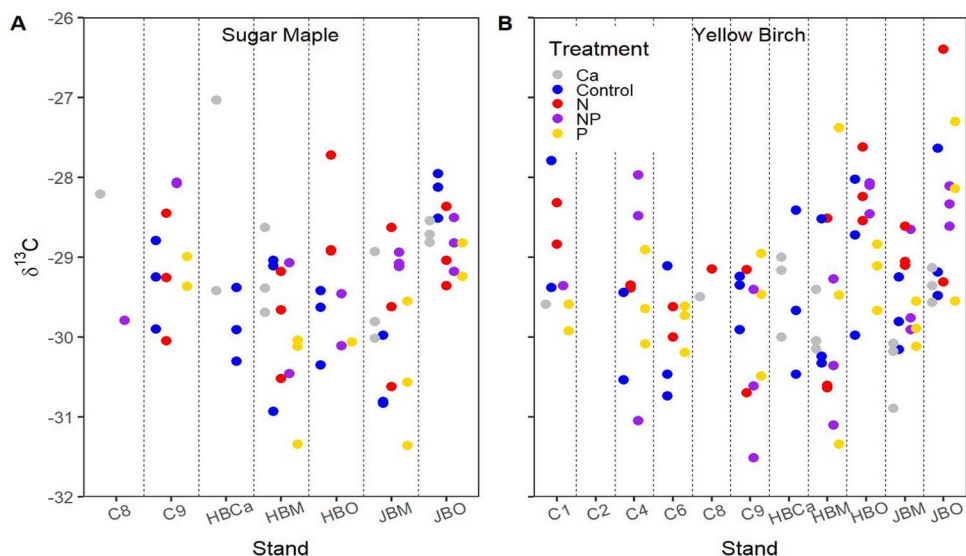


Figure 3. $\delta^{13}\text{C}$ in sugar maple leaves (A) and yellow birch leaves (B).

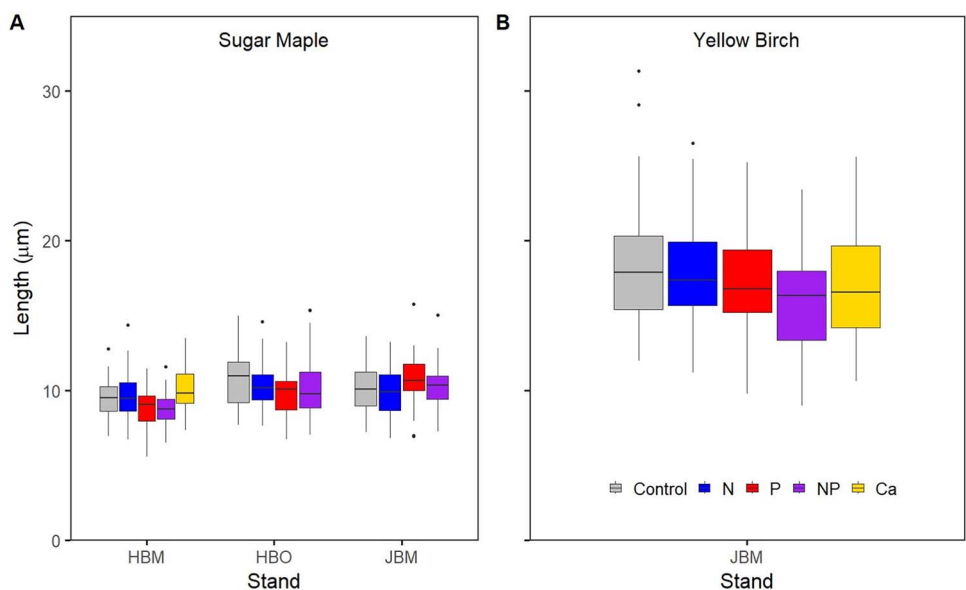


Figure 4. Stomatal length (in micrometers) in leaves of sugar maple leaves (A) and yellow birch leaves (B).

While P addition was associated with increased stomatal density in sugar maple, it was not associated with differences in $\delta^{13}\text{C}$. $\delta^{13}\text{C}$ increased with both N and CaSiO_3 addition in sugar maple. Under constant light and atmospheric conditions, an increase in $\delta^{13}\text{C}$ indicates lower internal CO_2 concentrations and therefore reduced stomatal conductance relative to photosynthesis, i.e., increased iWUE (Dawson et al. 2002); thus, iWUE increased with N and CaSiO_3 addition in these trees. Increases in iWUE with N addition are often attributed to an increase in photosynthesis, due to increases in RuBisCO and chlorophyll (Raven et al. 2004). Indeed, N addition, but not P addition, has been shown to increase chlorophyll concentrations in sugar maple trees in the MELNHE study (Young et al. 2023). While CaSiO_3 addition has previously been associated with increased transpiration, silicon in general has been observed to decrease stomatal conductance by reducing the flexibility of stomatal guard cell walls, which can increase iWUE (Gao et al. 2006, Nascimento-Silva et al. 2023).

The observation of greater $\delta^{13}\text{C}$ in sugar maple in mature stands is not surprising, as $\delta^{13}\text{C}$ and thereby iWUE are known to increase with tree height (Vadeboncoeur et al. 2020). Variability among trees in stand age effects on $\delta^{13}\text{C}$, and thereby iWUE, has been attributed to both tree- and stand-scale differences in tree height, as well as to canopy structure differences that affect sub-canopy vapor pressure deficit difference, and light availability, with iWUE increasing as these factors increase (Farquhar et al. 1989, Duquesnay et al. 1998, Vadeboncoeur et al. 2020). Stomatal characteristics sampled at the top of the canopy did not differ with stand age in our study, which is consistent with past findings of no relationship with stand age and inconsistent relationships with tree height (Woodruff et al. 2010, Kenzo et al. 2012, Li et al. 2021).

Our variance partitioning analysis addressed the variability at all the spatial scales involved in our sampling design and suggested that stomatal density measurements were most variable among trees and within leaves (Figure 7). The consistency in stomatal density within individual trees may reflect a

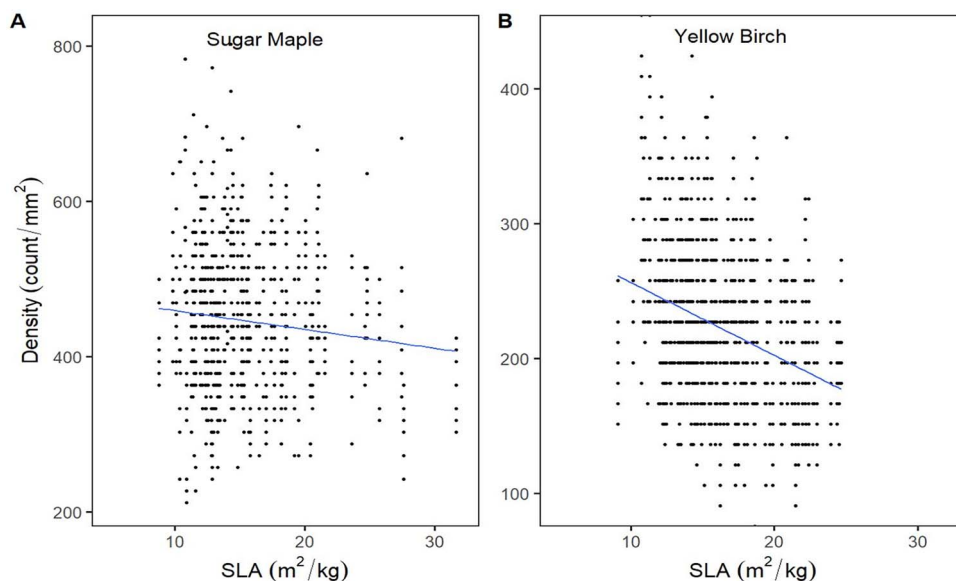


Figure 5. Relationship between SLA ($\text{m}^2 \text{kg}^{-1}$) and stomatal density in sugar maple (A) and yellow birch (B) trees across stands included in both N $\times$ P and CaSiO₃ analyses. The solid line represents a linear regression.

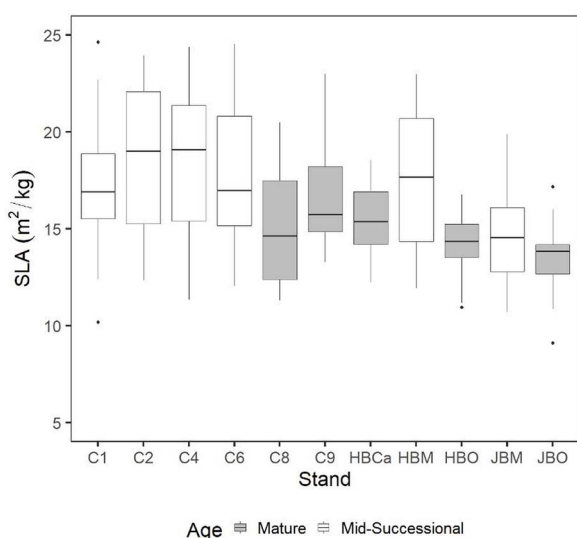


Figure 6. Relationship between SLA ($\text{m}^2 \text{kg}^{-1}$) and stand age in yellow birch trees. Mid-successional stands (open) were harvested 32–52 years ago, and mature stands (filled) were harvested 112–139 years ago.

genetic component of stomatal density (Shimada et al. 2011, Zhang et al. 2012) or differences in environmental conditions among trees. To improve the detectability of treatment effects on stomatal density, it may therefore be helpful to measure stomatal density over time in the same trees. Measuring edaphic indicators, such as predawn water potential at a dry time of year, or monitoring soil variables, such as soil moisture, at the tree level may also help future studies explain variability among trees or plots due to environmental conditions among trees and improve the detectability of a treatment effect.

The second highest level of variance in stomatal density occurred within leaves (i.e., among images), which could be attributed both to natural variability and to measurement error. The variability of stomatal density across leaf surfaces can vary naturally, and stomatal density measured near the

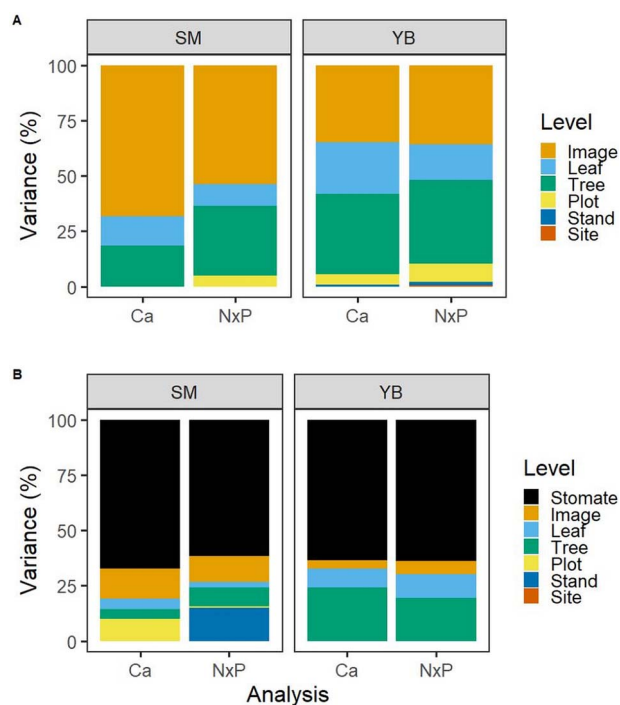


Figure 7. Percent of variance attributed to each level of measurement for stomatal density (A) and stomatal length (B) in sugar maple (SM) and yellow birch (YB) following N, P or CaSiO₃ addition.

edge of the blade has been shown to be lower than it is for the rest of the leaf in European beech (Forey et al. 2015). While we attempted to standardize the placement of the three impressions, we acknowledge that the impressions near the top and bottom of the leaf in particular could at times be influenced by proximity to the edge of the leaf. Future protocols could minimize this variability by taking more impressions, mostly in the middle of the leaf, or measuring more than three leaves and taking only one impression per leaf in a central location. Variability was relatively higher within leaves for sugar maple

than for yellow birch, which could be due in part to observer error. The sugar maple stomata tended to be very small and generally left less distinct impressions that were more difficult to recognize in comparison to yellow birch (Figure 1). The sugar maple leaves tended to have more small veins, which were difficult to exclude from images.

Variance in stomatal length was highest by far among stomata within the same images, ranging from 62 to 67% of variance. Only three stomata per image were measured in this protocol, as has been done by others (e.g., Sonti et al. 2021, though they also took three images on different spots within each impression, instead of one). Measuring more than three stomata per image, and thereby increasing the sampling intensity at this measurement level, would best reduce the uncertainty associated with stomatal length. Increasing the number of stomata per image, however, would substantially increase time and effort. Making all stomatal density and length measurements for one tree (e.g., three leaves) took 75 min on average, with 35 min devoted to stomatal length measurements. It is therefore important to account for the tradeoff between increasing sampling effort and resource constraints.

One way to substantially decrease time and effort for stomatal density measurements is to automate the counting process. Several programs using neural networks exist, such as Stomatal Counter (Fetter et al. 2019). We elected to count stomata manually to eliminate error due to automation, which we anticipated could be high especially for sugar maple or images of poor quality. In future studies, using a neural network system could save time and thereby potentially support a greater sample size.

Light availability could cause additional variation in stomatal density and length, as stomatal density, like many other leaf traits (Pérez-Harguindeguy et al. 2013, Coble and Cavaleri 2015), has been shown to change with light availability, especially early in leaf development (Gay and Hurd 1975). While we strove to sample only fully sunlit leaves in our study, it is possible that some of our leaves were not as fully sunlit as others, which may be more common than previously thought of 'sunlit leaves' (Keenan and Niinemets 2016). In yellow birch in particular, SLA was negatively correlated with stomatal density, and SLA was lower in the older stands (Figure 6). This could be due to differences in the size of these trees, as SLA has been shown to decrease with increasing tree height, likely because an increased SLA no longer increases growth rate in older trees, which tend to be taller (Liu et al. 2010, Falster et al. 2018). It could alternatively mean that these leaves were more fully sunlit than the leaves in the mid-successional stands in the N × P analysis, where the dense forest canopies impair visibility during shotgun sampling. Alternative methods such as tree climbing (Young et al. 2023) and unmanned aerial vehicles offer more control in selecting fully sunlit leaves for analysis (Schweiger et al. 2020), which could reduce variability due to light availability and thereby improve the detectability of treatment effects on stomatal characteristics.

Conclusions

Our study suggests that soil P availability might influence stomatal density in certain tree species, such as sugar maple. These increases in stomatal density could then have cascading effects on photosynthetic capacity and transpiration,

increasing the capacity for these trees to take in atmospheric CO₂ but also increasing their capacity to transpire. More research is needed, perhaps in more controlled conditions and with attention to variance at different measurement levels, to confirm these hypotheses. While N and CaSiO₃ addition did not seem to influence stomatal characteristics in either species, N and CaSiO₃ addition did positively correlate with $\delta^{13}\text{C}$ in sugar maple, suggesting a possible influence of N on photosynthetic capacity. Future studies should consider measuring stomatal characteristics in more trees or in the same tree over time, ideally considering pretreatment conditions where applicable, standardizing impression location or measuring more impressions per leaf, measuring more stomata per impression and controlling for canopy position.

Supplementary data

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

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Conflict of interest

None declared.

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Authors' contributions

J.M.Z., M.A.V. and R.D.Y. designed the study. Data were collected by J.M.Z. and M.A.V., and data were analyzed by J.M.Z. J.M.Z. wrote the manuscript with input from R.D.Y. and M.A.V.

Data availability

Stomatal density and length measurements and the corresponding microscope images are published in the Environmental Data Initiative. Zukswert JM, Weimer S, McGarry A, Fessenden A, Carter A, Yanai RD (2023) Multiple Element Limitation in Northeast Hardwood Ecosystems (MELNHE)—stomatal density and length 2021–2022 ver 1. Environmental Data Initiative. [10.6073/pasta/34850e37fda3b971214788c327ea21f2](https://doi.org/10.6073/pasta/34850e37fda3b971214788c327ea21f2). Zukswert JM, Weimer S, McGarry A, Fessenden A, Carter A, Yanai RD (2023) Multiple Element Limitation in Northeast Hardwood Ecosystems (MELNHE)—Raw images for the analysis of stomatal density and length 2021–2022 ver 1. Environmental Data Initiative. [10.6073/pasta/968fe01e9b507cbc9a4db9ee4ed671f1](https://doi.org/10.6073/pasta/968fe01e9b507cbc9a4db9ee4ed671f1).

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