Patterns of physical, chemical, and metabolic characteristics of sugar maple leaves with depth in the crown and in response to nitrogen and phosphorus addition

Characteristics of sugar maple leaves

Young et al.

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Abstract

Few previous studies have described the patterns of leaf characteristics in response to nutrient availability and depth in the crown. Sugar maple has been studied for both sensitivity to light, as a shade-tolerant species, and sensitivity to soil nutrient availability, as a species in decline due to acid rain. To explore leaf characteristics from the top to bottom of the canopy, we collected leaves along a vertical gradient within mature sugar maple crowns in a full-factorial nitrogen (N) by phosphorus (P) addition experiment in three forest stands in central New Hampshire, USA. Thirty-two of the 44 leaf characteristics had significant relationships with depth in the crown, with the effect of depth in the crown strongest for leaf area, photosynthetic pigments and polyamines. Nitrogen addition had a strong impact on the concentration of foliar N, chlorophyll, carotenoids, alanine and glutamate. For several other elements and amino acids, N addition changed patterns with depth in the crown. Phosphorus addition increased foliar P and boron (B); it also caused a steeper increase of P and B with depth in the crown. Since most of these leaf characteristics play a direct or indirect role in photosynthesis, metabolic regulation or cell division, studies that ignore the vertical gradient may not accurately represent whole-canopy performance.

Key words: amino acids; chlorophyll; leaf traits; light environment; nutrients; polyamines

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Introduction

Leaves in forest canopies experience heterogeneous light environments that influence physical, chemical and metabolic leaf characteristics (Evans and Poorter 2001, Niinemets et al. 2015). Leaves at the tops of crowns shade leaves lower in crowns, reducing the light available for photosynthesis (Hirose and Bazzaz 1998, Le Roux et al. 2001). In response to gradients in light, temperature and humidity, leaves at the tops of crowns are small and thick, while leaves at the bottom are large and thin (Reich et al. 1997, Coble and Cavaleri 2017). Nutrient availability also affects leaf characteristics (Cramer et al. 2000), but whether these effects differ with crown position is unknown.

Most tree foliage studies have focused on collecting and comparing sunlit leaves from the tops of crowns, even though the majority of leaves in the crown are partially shaded (Stark et al. 2012, Keenan and Niinemets 2016). Studies that examine within-crown variation in leaf characteristics provide information on the plasticity of leaf traits (Baldocchi and Harley 1995, Bonan et al. 2012, Raabe et al. 2015). Of these traits, the variation in leaf area, leaf mass and leaf nitrogen (N) reflects a trade-off between light interception and resource investment per leaf (Peltoniemi et al. 2012). Shade-tolerant species, in particular, have high plasticity in leaf area (Poorter et al. 2009). Ignoring the seasonal and vertical gradient of N in a late-successional, shade-tolerant species, sugar maple (*Acer saccharum* Marsh.) led to a ~60% underestimate of whole crown photosynthesis across a growing season (Coble et al. 2016).

Although soil nutrients and leaf characteristics have been used extensively to indicate tree health (Wargo et al. 2002Long et al. 2002; St Clair et al. 2005), the extent to which soil nutrients affect the patterns of leaf characteristics with depth in the crown has never been assessed. The distribution of photosynthetic pigments in leaves is related to light interception (Hollinger 1996), but photosynthetic pigment concentrations are also a consequence of soil nutrient availability (Hikosaka and Terashima 1995). Soil resource availability also impacts cellular metabolism (Minocha et al. 2010, Minocha et al. 2015, 2019). Evaluating leaf metabolites alongside leaf chemistry and physical leaf characteristics will advance our understanding of these processes and improve the estimates of whole-canopy photosynthesis (Díaz and Cabido 2001, Smith et al. 2019).

In this recently deglaciated region with young parent materials, N could be expected to be relatively more limiting than phosphorus (P) (Walker and Syers 1976). A meta-analysis of studies in the region reported greater forest growth responses to N than to P (Vadeboncoeur 2010), but no single study had tested growth responses to both N and P. To test the relative importance of N and P limitation, we implemented a study of Multiple Element Limitation in Northern Hardwood Ecosystems (MELNHE), adding low levels of N and P in a full factorial design beginning in 2011. These treatments elevated the availability of N and P in soils (Fisk et al. 2014, Fisk 2022). Foliar chemistry reflected the addition of each of the nutrients and predominantly indicated P limitation (Gonzales and Yanai 2019, Hong et al. 2022, Gonzales et al. in press). After 4 years of treatment, the relative<<Query: Since coloured text is not used in tables, please indicate the positive and negative effects another way, such as with bold type. Ans: **rdyanai@syr.edu:** We used red and black, following an accounting convention for positive and negative numbers. Bold is used for emphasis, and we don't want to emphasize one direction over another. Can we have italics for what was red befo

re (positive effects in italics), and an underline for what was black (negative effects in underline)?>> diameter growth of the average tree was greater under P addition (Goswami et al. 2018) but the large trees grew more in response to N addition (Hong et al. 2022).

Changes in N and P availability may alter foliar chemistry and metabolites, serving as early warning signals of changes in growth (Minocha et al. 2014, Singh et al. 2018). For example, elevated concentrations of a few amino acids, particularly arginine, proline, alanine and γ-aminobutyric acid), indicate elevated N availability within cells because these amino acids can salvage, store and redistribute excess N (Ericsson 1995, Näsholm et al. 1997, Minocha et al. 2015, 2019, Chen et al. 2017). Amino acids are also known for their role in protein synthesis, growth and development, nutrition and stress responses (Hildebrandt et al. 2015). However, more recently, the metabolism of amino acids has also been linked to energy release during carbon starvation, carbon (C) and N budgets, lipid metabolism, photorespiration, hormone and secondary metabolite production (e.g., polyamines, alkaloids and phytoalexins) and stress modulation (Yang et al. 2020).

Polyamines are aliphatic amines that mediate C and N metabolism within cells (Handa et al. 2018) and play a critical role in coordinating responses to stress or promoting growth (Minocha et al. 2014, Singh et al. 2018). Polyamines confer stress tolerance by lowering ammonium toxicity and scavenging free radicals (Minocha et al. 2014). The concentration of polyamines also facilitates carbon dioxide transport to Rubisco, suggesting an indirect involvement in C assimilation (Yasumoto et al. 2018).

The goal of this research was to describe the relationships of physical, chemical and metabolic leaf characteristics as a function of depth within tree crowns and in response to N and P addition. We measured the leaf characteristics of mature sugar maples in the Bartlett Experimental Forest in the White Mountains of central New Hampshire, USA, in the seventh year of the MELNHE nutrient treatments. We hypothesized that leaf characteristics would differ substantially with depth in tree crowns, and we tested for the effects of N and P addition on these relationships.

Materials and methods

Field site and sample collection

Trees in this study were located in three mature forest stands in the Bartlett Experimental Forest (44.06, −71.28). These stands regenerated following harvest in ~1890 and are dominated by American beech (*Fagus grandifolia* Ehrh.), sugar maple and yellow birch (*Betula alleghaniensis* Michx.; Goswami et al. 2018). Soils are well drained Spodosols formed in granitic glacial drift

(Vadeboncoeur et al. 2012, Vadeboncoeur et al. 2014). These forests have a history of acid deposition, including substantial N deposition (Galloway et al. 2003). The regional climate is humid continental with average annual precipitation of 130 cm and an average annual temperature of 4.4 °C (Bailey 2003).

Twelve mature sugar maple trees were studied, one in each of four treatment plots in three stands (Table 1). Since 2011, annual additions of N (30 kg ha^{-1} NH_4NO_3) and P (10 kg ha^{-1} NaH_2PO_4) and a combined treatment of N + P at the same concentrations were applied to experimental treatment plots (Fisk et al. 2014). Control plots did not receive N or P. The selected trees were in dominant or codominant canopy positions, free of disease or decay and located at least 10 m from the edge of nutrient addition plots.

Table 1 Characteristics of the three forest stands and 12 trees used in this study. These mature stands in the Bartlett Experimental Forest, NH, are part of a study of Multiple Element Limitation in Northern Hardwood Ecosystems (MELNHE). Treatments are control (C) and the additions of nitrogen (N), phosphorus (P) and combined nitrogen and phosphorus (N + P). Tree diameter was measured at breast height (1.37 m).

					Tree diameter (cm)				**Tree height (m)**				**Crown depth (m)**			
Stand	**Year clearcut**	**Elevation (m)**	**Aspect**	**Slope (%)**	**C**	**N**	**P**	**N + P**	**C**	**N**	**P**	**N + P**	**C**	**N**	**P**	**N + P**
C7	1890	440	ENE	5–10	59.3	42.6	64.5	49.3	24	20	24	20	14	8	8	6
C8	1883	330	NE	5–35	50.7	40.8	49.5	52.9	25	23	24	25	10	6	10	10
C9	1890	440	NE	10–35	55.7	44	59.8	53.8	25	24	23	22	10	12	10	12

Branches were collected on 31 July and 1 August, 2017 using a pole pruner and minimally invasive rope access techniques (Jespon 2000, Anderson et al. 2015). Foliar samples were collected in a vertical transect within 1 m of the climbing rope that went over a branch close to the top of the tree and followed the main stem to the base of the tree. Leaves free of herbivory and physical damage were collected by cutting branches every 2 m from the top to the bottom of each tree crown. From the pool of leaves collected from a branch, 2–3 leaves were used for metabolites, 10 leaves were used for physical measurements, and the rest were ground for elemental analysis. The trees in this study ranged in height from 20 to 25 m and in crown depth from 6 to 14 m, resulting in 3–7 branches collected per tree for a total of 60 samples. The trees in this study ranged in height from 20 to 25 m and in crown depth from 6 to 14 m, resulting in 3–7 branches collected per tree for a total of 60 samples.

Sample processing

Physical leaf characteristics

Ten leaves from each branch were prepared in a standard leaf press for 2 days and then imaged as a high-resolution TIFF at a constant distance, including a ruler for scale, with an OLYMPUS TG4 camera (Center Valley, PA, USA). ImageJ software (https://imagej.nih.gov/ij/) was used to convert the image of leaves on a white background to a binary black to assess the number of leaf pixels and thus quantify leaf surface area (Chianucci et al. 2019). After imaging, these same 10 leaves were oven-dried at 60 °C to constant mass. Leaf surface area and leaf dry mass are reported as the average of these leaves for each branch. Specific leaf area (SLA) is calculated as the average leaf surface area divided by the average leaf mass.

Total leaf element concentrations

Additional leaves from each branch were pooled, dried and ground using a Wiley mill through a 40-mesh screen. Leaf C and N concentrations were quantified using a CN analyzer (FlashEA 1112 analyzer, Thermo Fisher Scientific, Inc Waltham, MA, USA). National Institute of Standards and Technology standard reference material 1515, 1545 and 1575 were run as standards. Total P, calcium (Ca), magnesium (Mg), manganese (Mn), aluminium (Al), boron (B) and zinc (Zn) were measured by microwave digestion (MARS6 Microwave digestion system CEM Corporation, Matthews, NC, USA) using ~ 0.25 g of oven-dried leaf tissue in 10 ml of 15.8 N nitric acid, brought to 50 ml with DI water and then quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 5300 DV, Perkin-Elmer, Inc. Waltham, MA, USA). One duplicate sample, one blank and two replicates of standard reference material were processed with each group of 25–36 samples. During ICP-OES, a calibration blank and in-house quality control were run after every five samples. We recalibrated the machine if drift exceeded 5% of the in-house quality control. Tissue standard and recovery values were within 4% of the certified values for Ca (average recovery 100%), 10% for N (average recovery 94%), 17% for Mg (average 88%), 7% for Mn (average recovery 76%), 28% for Al (average recovery 76%), 11% for P (average recovery 93%), 14% for B (average recovery 101%) and 12% for Zn (average recovery 93%) (Young et al. 2020).

Polyamines, amino acids, chlorophyll, carotenoids and soluble protein

We used a 6-mm hole punch, avoiding leaf veins, to collect leaf material from two to three leaves from each branch. These were stored in a cooler with ice in the field, refrigerated overnight and then transported in a cooler to the laboratory. Subsamples were used for the following analyses.

Each 0.2-g subsample was placed in a 2-ml microfuge tube with 1 ml of cold 5% perchloric acid to extract polyamines and amino acids, stored at –20 °C, then thawed and frozen three times to break cellular membranes and centrifuged at 14,000 × *g* for 10 min. The supernatants were used to determine the concentrations of polyamines (putrescine, spermidine and spermine) and amino acids (Minocha et al. 2000). Polyamines and amino acids were dansylated (Minocha and Long 2004) with modifications described here. Samples were incubated at 60 °C for 30 min, cooled for 3 min and then microcentrifuged at 14,000 × *g* for 30 s. Dansylation was terminated by the addition of 45 µl of glacial acetic acid. Sample tubes were kept open for 3 min under a flow hood to allow CO_2 to escape. Filtered HPLC-grade methanol was added to bring the final volume to 2 ml. A Perkin Elmer (Waltham, MA, USA) series 200 HPLC system fitted with a fluorescence detector was used to separate and quantify polyamines and amino acids in standards and samples. Data were processed using Perkin Elmer TotalChrom software (version 6.2.1).

To determine the concentrations of chlorophyll a, chlorophyll b and carotenoid pigments, 5–10 mg of thawed leaf tissue was incubated in 1 ml of 95% ethanol in the dark at 65 °C for 16 h and then centrifuged at 13,000 × *g*. Chlorophyll a, chlorophyll b and carotenoids were analyzed using a Hitachi U2010 spectrophotometer (Tokyo, Japan) by scanning absorbances in the range of 350–710 nm and then quantified using equations found in Lichtenthaler (1987) and Minocha et al. (2009).

Soluble protein was extracted from 50 mg of thawed leaf tissue in 500 µl of extraction buffer (Jones et al. 1989) by three cycles of freezing and thawing and quantified (Bradford 1976) using a Bio-Rad (Hercules, CA, USA) protein dye reagent based on absorbance recorded at 595 nm using a Hitachi U2010 spectrophotometer and UV solutions software (v. 2.0).

Data analysis

A total of 44 leaf characteristics were examined in this study. The response variables related to physical leaf characteristics were leaf area, leaf mass and SLA. Photosynthetic pigments were the concentrations of chlorophyll (chlorophyll A and B were combined for analysis) and carotenoids. The metabolic characteristics examined were the concentrations of polyamines (putrescine, spermidine and spermine) and amino acids (glutamic acid, arginine, alanine, γ-aminobutyric acid and valine). The response variables related to leaf chemistry were the total concentrations of leaf N, P, Ca, Mg, Mn, Al, B, Zn and iron (Fe).

Several decisions were made for the statistical analysis of leaf characteristics. In this within-crown study, we scaled the depth in the crown from 0 to 1 (0 being the top and 1 being the bottom) because models that used the scaled depth in the crown had lower AIC values compared with models that used depth in the crown in units of meters. We chose to examine foliar concentrations per unit mass, as mass-normalized units are the most commonly reported in the literature (Wright et al. 2004) and because previous work indicates strong vertical gradients of sugar maple leaf thickness and density (Coble and Cavaleri 2014). We recognize that variation in SLA across the global flora is associated with area-proportional variation due to leaf structure and leaf lifespan (John et al. 2017, Osnas et al. 2018), but we focus on mass-normalized traits here.

The experimental units for the analyses were the 12 trees, which were used as a random effect in a randomized block design linear mixed-effect model (nlme package in R; Pinheiro et al. 2016). The blocks were the three replicate stands (Bates et al. 2015). The model included the main effects of N addition, P addition and depth in the crown and all their interactions. The coefficients for the main effects and interaction terms were compared to assess the direction of the effects for each leaf characteristic. For Figure 2, we normalized the effect sizes across response variables by dividing the model coefficient by the mean effect size for each response variable to allow the comparisons of variables with different units.

We used R version 3.5.1 (R Core Team 2018): the R package ggplot2 (Wickham 2016) for data visualization.

Statistical results for leaf characteristics not reported here, namely, C; sulfur (S); potassium (K); strontium (Sr); the ratio of N:P, lysine; proline; aspartic acid; isoleucine; leucine; chlorophyll a; chlorophyll b; the ratio of chlorophyll a:b; and the concentrations of soluble ions Al, Ca, K, Mn, P, Mg and Zn are presented in Supplemental information 1 available as Supplementary data at *Tree Physiology* Online. Raw data are available in a data package (Young et al. 2020).

Results

Leaf characteristics as a function of depth in the crown

Many leaf characteristics differed with depth in the crown based on the leaves that were sampled along the main stem of mature sugar maple trees in this closed-canopy forest. As expected, the mass of individual leaves decreased with depth in the crown ($P = 0.01$, Figure 1) while the area of individual leaves increased steeply with depth in the crown ($P < 0.001$, Figure 1). This resulted in a strong increase in SLA with depth in the crown, with thick and small leaves at the top of crowns and thin leaves with higher surface area at the bottom of tree crowns.

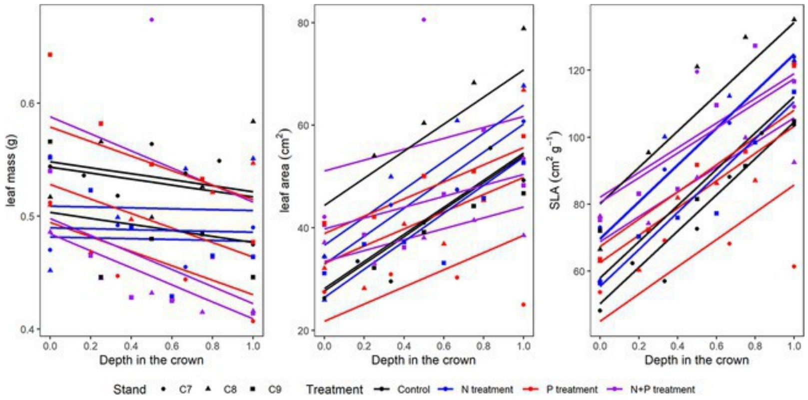

Figure 1 Leaf area, leaf mass and SLA as a function of depth in the crown in control, N, P and N + P treatment plots for mature sugar maple trees at the Bartlett Experimental Forest, NH. Data points represent the characteristics of leaf samples collected through the crowns of 12 trees. Depth in the crown was scaled from the top of the crown (0) to the bottom (1). The slopes of the lines represent the model estimates for the nutrient treatments; the intercepts are fit for each of the trees. These three characteristics have $P < 0.05$ for the main effect of depth (Table 2).

Of the 44 leaf characteristics included in this study, 32 had significant ($P \leq 0.05$) positive or negative relationships with depth in the crown (Table 2, Appendix Table S1 available as Supplementary data at *Tree Physiology* Online). In general, depth in the crown had the strongest effect on physical characteristics and on the concentrations of polyamines putrescine, spermidine and spermine, which all decreased with depth, significantly so for putrescine and spermidine (Figure 2). Photosynthetic pigments and most amino acids increased considerably with depth in the crown, as did a majority of the chemical characteristics. Each of these variables will be described in detail below.

Table 2 *P*-values of the main effects (depth in the crown, N addition and P addition) and all their interactions in linear mixed-effect models of the 23 leaf characteristics reported in the paper. Additional characteristics are reported in Appendix Table S1 available as Supplementary data at *Tree Physiology* Online.

Leaf characteristic		**Depth**[1]	**N addition**[1]	**P addition**[1]	**Depth*N**[1]	**Depth*P**[1]	**N*P**[1]	**Depth*N*P**[1]
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	Mass	0.01	0.44	0.68	0.81	0.09	0.85	0.64
	Area	<0.001	0.59	0.61	0.69	0.02	0.49	0.60
	SLA	<0.001	0.23	0.61	0.88	0.03	0.37	0.80
Elements								
	N	<0.001	0.01	0.53	0.91	0.07	0.35	0.71
	P	0.01	0.32	< 0.001	0.62	0.02	0.12	0.48
	Ca	<0.001	0.91	0.84	0.54	0.62	0.97	0.38
	Mg	<0.001	0.09	0.96	0.46	0.17	0.49	0.28
	Mn	0.00	0.01	0.26	0.35	0.15	0.79	0.34
	Al	0.78	0.01	0.55	0.38	0.05	0.42	0.70

Leaf characteristic		Depth[1]	N addition[1]	P addition[1]	Depth*N[1]	Depth*P[1]	N*P[1]	Depth*N*P[1]
	Fe	0.34	0.01	0.75	0.84	0.03	0.94	0.67
	B	<0.001	0.21	0.001	0.91	0.003	0.09	0.52
	Zn	<0.001	0.65	0.86	0.06	0.21	0.74	0.15
Amino acids and soluble protein								
	Glutamic acid	<0.001	0.06	0.32	0.03	0.76	0.57	0.75
	Arginine	0.01	0.28	0.37	0.003	0.24	0.51	0.21
	Alanine	0.09	0.01	0.62	0.003	0.48	0.80	0.94
	GABA	0.003	0.20	0.66	0.01	0.30	0.51	0.10
	Valine	0.01	0.09	0.31	0.004	0.22	0.62	0.33
	Protein	<0.001	0.88	0.32	0.52	0.61	0.60	0.16
Polyamines								
	Putrescine	<0.001	0.82	0.84	0.21	0.81	0.50	0.10
	Spermidine	0.05	0.33	0.51	0.83	0.34	0.79	0.46
	Spermine	0.52	0.75	0.40	0.004	0.42	0.28	0.97

Leaf characteristic		Depth[1]	N addition[1]	P addition[1]	Depth*N[1]	Depth*P[1]	N*P[1]	Depth*N*P[1]
Photosynthetic pigments								
	total_chl	<0.001	0.02	0.37	0.69	0.31	0.73	0.55
	carot	<0.001	0.01	0.47	0.32	0.46	0.40	0.45

[1] Positive effects are underlinedshown in black text; negative effects are italicized.shown in red. A positive effect of N or P addition is a greater value under N or P addition; for depth in the crown, a positive effect indicates greater values at the bottom of the crown. A positive interaction indicates that the trend with depth in the crown is amplified by nutrient addition; a negative interaction indicates that the trend is dampened by nutrient addition.

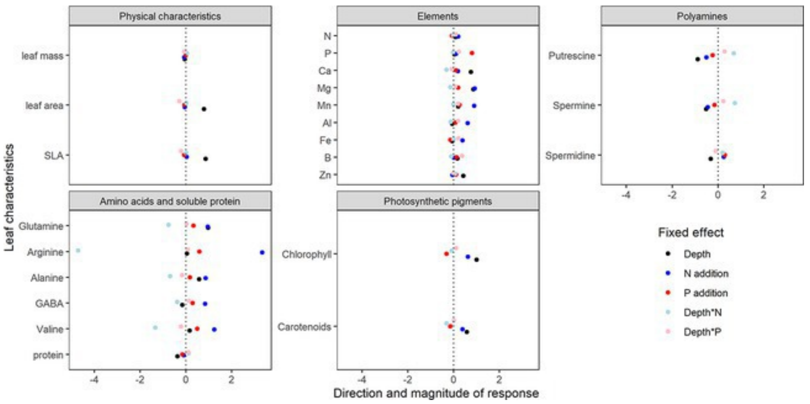

Figure 2 The direction and magnitude of the main effects and the interaction of depth in the crown and N or P addition are shown for each leaf characteristic. There were no significant three-way interactions of N, P and depth. The magnitude of the effect is represented by the model coefficient divided by the mean coefficient for each leaf characteristic, to allow the comparison of variables with different units.

Leaf characteristics respond to N and P addition

Nitrogen addition

The addition of N caused a strong increase in many chemical and metabolic leaf characteristics. Trees that received added N had 23% higher foliar N ($P < 0.01$, Figure 3). Nitrogen addition increased the concentrations of other elements in the leaves, resulting in 34% higher Fe ($P = 0.01$), 49% higher Mn ($P = 0.1$) and 34% higher Al ($P = 0.01$). Trees that received N had a steeper increase in Zn with depth in the crown ($P = 0.05$ for the interaction of depth and N addition).

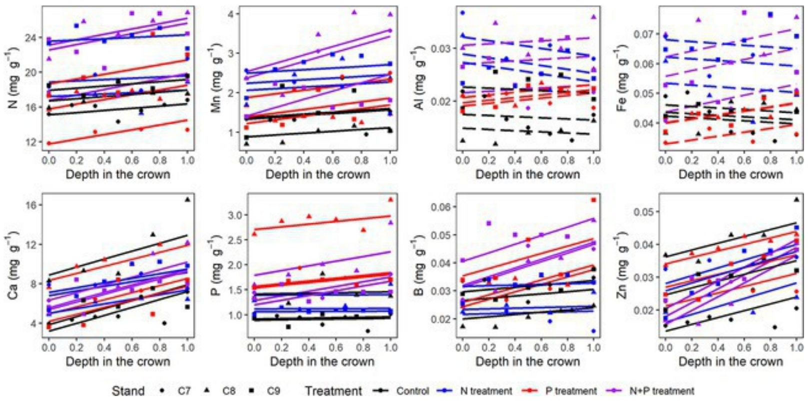

Figure 3 The concentrations of foliar N, Mn, Al, Fe, Ca, P, B and Zn in mature sugar maple leaves as a function of depth in the crown in control, N, P and N + P treatment plots for mature sugar maple trees at the Bartlett Experimental Forest, NH. Data points represent the concentrations of leaf samples collected through the crowns of 12 trees. Depth in the crown was scaled from the top of the crown (0) to the bottom (1). Slopes of the lines represent the model estimates for the nutrient treatments; the intercepts are fit for each of the trees. Solid lines indicate $P < 0.05$ for the main effect of depth or the interaction of depth and N or P addition (Table 2).

Most amino acids had higher concentrations at the top of the canopy and N addition commonly increased the steepness of the decline with depth in the crown (Figure 4). The amino acid alanine had a 37% increase with N addition ($P = 0.01$, the main effect of N addition) and a more rapid decrease with depth in the crown with N addition ($P < 0.01$ for the interaction of depth by N addition). This interaction of depth and N addition interaction was also seen in glutamic acid ($P < 0.01$), arginine ($P < 0.01$), γ-aminobutyric acid ($P < 0.01$) and valine ($P < 0.01$).

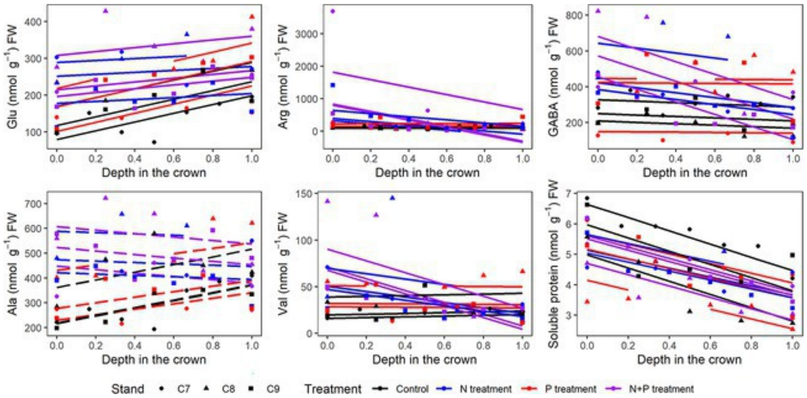

Figure 4 The concentration of glutamic acid (Glu), arginine (Arg), γ-aminobutyric acid (GABA), alanine (Ala), valine (Val) and soluble protein as a function of depth in the crown in control, N, P and N + P treatment plots for mature sugar maple trees at the Bartlett Experimental Forest, NH. Data points represent the concentrations of leaf samples collected through the crowns of 12 trees. Depth in the crown was scaled from the top of the crown (0) to the bottom (1). The slopes of the lines represent the model estimates for the nutrient treatments; the intercepts are fit for each of the trees. Solid lines indicate $P < 0.05$ for the main effect of depth or the interaction of depth and N or P addition. Dashed lines indicate leaf characteristics that were $P > 0.05$ for the main effect of depth or interaction of depth and N or P addition. Broken lines indicate missing data.

Surprisingly, none of the polyamines responded consistently to N addition ($P \geq 0.33$ for the main effect of N) (Figure 5). The foliar concentrations of polyamines decreased steeply with depth in the crown for putrescine ($P < 0.01$, main effect of depth) and for spermidine ($P = 0.03$, the main effect of depth). For spermine, concentrations decreased with depth in the crown in the absence of N addition but increased with depth in the crown under N addition ($P = 0.01$ for the interaction of depth and N addition, Figure 5).

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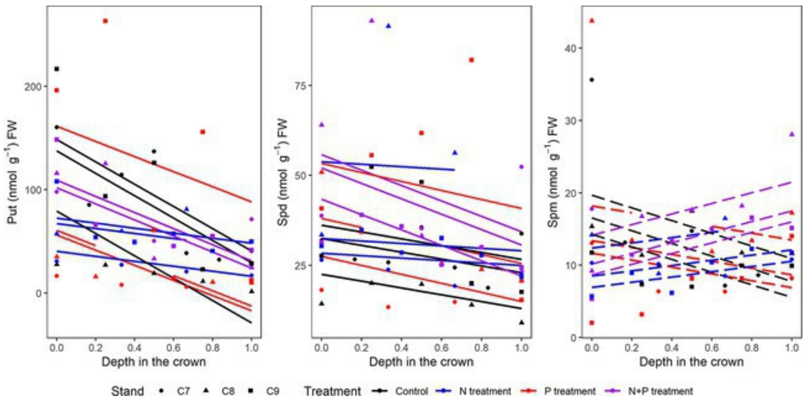

Figure 5 The concentration of putrescine (Put), spermidine (Spd) and spermine (Spm) as a function of depth in the crown in control, N, P and N + P treatment plots for mature sugar maple trees at the Bartlett Experimental Forest, NH. Data points represent the concentrations of leaf samples collected through the crowns of 12 trees. Depth in the crown was scaled from the top of the crown (0) to the bottom (1). The slopes of the lines represent the model estimates for the nutrient treatments; the intercepts are fit for each of the trees. Solid lines indicate $P < 0.05$ for the main effect of depth or the interaction of depth and N or P addition. Dashed lines indicate leaf characteristics that were $P > 0.05$ for the main effect of depth or interaction of depth and N or P addition. Broken lines indicate missing data.

The concentrations of chlorophyll and carotenoids increased steeply with depth in the crown ($P = 0.02$ and $P = 0.01$, respectively, for the main effect of N addition, Table 2). The concentrations of chlorophyll were 47% higher for trees that received N ($P = 0.02$ for the main effect of N addition, Figure 5) and carotenoids were 33% higher for trees that received N ($P = 0.01$ for the main effect of N addition, Figure 5). The pattern in photosynthetic pigment concentrations per unit mass of leaf reflects the choice of units; top-crown leaves had the highest photosynthetic pigment concentrations per unit leaf area, but when expressed per unit mass, top-crown leaves have the lowest photosynthetic pigment concentrations.

Phosphorus addition

The addition of P affected relatively few of the leaf characteristics in this study (Table 2). When P was added, the concentration of foliar P increased by 48% ($P < 0.01$, the main effect of P addition, Figure 3) and foliar B increased by 36% ($P < 0.01$, the main effect of P addition, Figure 3).

There were several significant interactions of P addition with depth in the crown, meaning that the addition of P caused a change in the vertical distribution of leaf characteristics. Trees that received P had steeper increases with depth in the crown for concentrations of P ($P = 0.01$), B ($P < 0.01$), Al ($P = 0.05$) and Fe ($P = 0.03$, Figure 3). The relationship of increasing leaf area and SLA with depth was less steep for trees that received P addition (Figure 1); trees receiving P did not increase leaf area with depth in the crown as steeply as those that did not ($P = 0.02$ for the interaction of depth and P addition). There was a concomitant interactive effect on SLA, in which trees that received P had a less steep increase in SLA with depth in the crown ($P = 0.03$ for the interaction of depth and P addition).

No significant interactions were observed between N and P or among depth, N and P for any leaf characteristic.

Discussion

The patterns of leaf characteristics with both depth in the crown and nutrient availability have rarely been reported in the literature. In this study of 12 mature sugar maple trees sampled vertically through the crown in an N by P factorial nutrient manipulation study, many leaf characteristics responded to depth in the crown, several responded to nutrient addition, and in a few cases, the relationship of depth in the crown changed as a consequence of N and P addition (Figure 2).

Light environment was more important to physical leaf characteristics, polyamines and photosynthetic pigments than whether N or P was added. In contrast, nutrient addition was more influential than depth in the crown for foliar elements and amino acids (Figure 2).

Within-crown plasticity in leaf characteristics

The most noticeable difference in leaf characteristics was the substantial increase in leaf area with depth in the crown, which, combined with a slight decrease in leaf mass, resulted in the high SLA values at the bottom of the crown (Figure 1). This variation in SLA has been well studied in sugar maple (Coble and Cavaleri 2014, 2017, Coble et al. 2016) and is an important strategy for shade-tolerant deciduous trees (Sack et al. 2006, Liu et al. 2016). The increase in SLA with depth in the crown is consistent with leaves at the top of the crown being thicker than leaves at the bottom of the crown, which are thinner and have higher surface area (Aranda et al. 2004, Coble and Cavaleri 2017, Osnas et al. 2018).

The increase in amino acids (glutamic acid, arginine, γ-aminobutyric acid and valine) with depth in the crown was accompanied by a decline in soluble proteins. It has been suggested that the accumulation of proline, alanine, arginine and γ-aminobutyric acid often indicates a disequilibrium in C and N (Chen et al. 2017). At Harvard Forest Experimental Forest, MA (Minocha et al. 2015) and Bear Brook Watershed, ME (Minocha et al. 2019), foliage accumulated these amino acids under N addition, indicating the storage of this excess N in non-toxic organic usable forms. These adjustments in the N metabolism might help re-establish C and N equilibrium by using C from photosynthesis and glycolysis at a later stage (Chen et al. 2017).

Putrescine is a diamine known for its role in photosynthesis, cell division and response to biotic and abiotic stress (Yasumoto et al. 2018, Seo et al. 2019, Alcázar et al. 2020). The trend we observed of putrescine decreasing with depth in the crown is consistent with its known functions since stress from multiple environmental factors is often higher at the top of the crown and so is the photosynthetic rate.

Chlorophyll and carotenoids were more strongly controlled by depth in the crown than by N addition (Figure 6), suggesting that the leaves in tree crowns acclimate to their light environment and proportionately distribute chlorophyll and N to maximize light-harvesting capacity (Kura-Hotta et al. 1987, Lei et al. 1996).

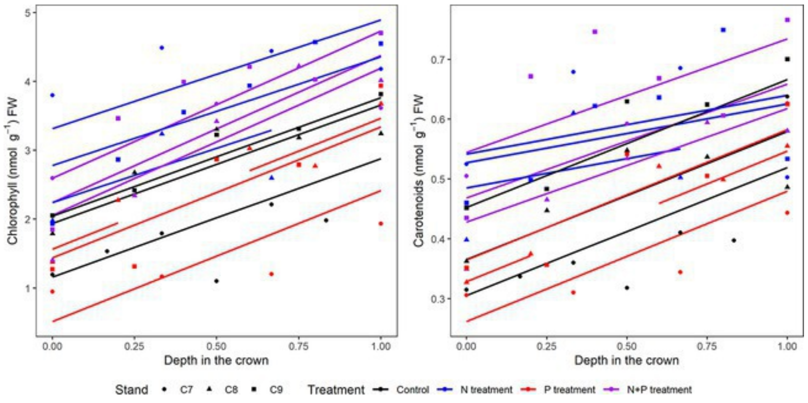

Figure 6 The concentrations of chlorophyll and carotenoids as a function of depth in the crown in control, N, P and N + P treatment plots for mature sugar maple trees at the Bartlett Experimental Forest, NH. Data points represent the concentrations of leaf samples collected through the crowns of 12 trees. Depth in the crown was scaled from the top of the crown (0) to the bottom (1). The slopes of the lines represent the model estimates for the nutrient

treatments; the intercepts are fit for each of the trees. Solid lines indicate $P < 0.05$ for the main effect of depth or the interaction of depth and N or P addition. Dashed lines indicate leaf characteristics that were $P > 0.05$ for the main effect of depth or interaction of depth and N or P addition. Broken lines indicate missing data.

Effects of N addition

Trees that received N had higher foliar concentrations of photosynthetic pigments, several elements and many amino acids than trees that did not receive N. Nitrogen addition increased the foliar concentrations of glutamic acid, especially at the top of the crown. Glutamic acid is the first organic metabolite synthesized from ammonia (Lea and Miflin 2018) and is a precursor for dozens of metabolites including polyamines and several amino acids that are involved directly or indirectly in the photosynthetic process (Young and Ajami 2000). Since these metabolites are interconnected and many of them link major C and N metabolic pathways, a change in the concentration of one metabolite often leads to shifts in the concentrations of other metabolites (Mohapatra et al. 2010). The accumulation of amino acids at the top of the crown with N addition (especially the ones that are known as salvage or storage amino acids) is evidence of a change in upper canopy N metabolism. Rubisco and other stromal proteins are needed at this level because of the higher photosynthetic capacity in the upper part of the crown (Johnson et al. 2010).

When trees experience excessive N associated with N saturation, elevated concentrations of several N metabolites are often accompanied by a high concentration of putrescine (Minocha et al. 2000, Bauer et al. 2004). The stress indicator putrescine was not elevated under N addition in this study. The concentrations of spermidine were not elevated with N addition, but its concentrations are known to be more tightly controlled under stress and do not fluctuate as widely as those of putrescine (Minocha et al. 2014). Along with putrescine and spermidine, spermine decreased with depth in trees not receiving N, but when N was added, the concentrations of spermine increased with depth in the crown, which may reveal a positive effect of N addition. Putrescine, spermidine and spermine all play a role in cell division; thus, the effects of nutrient addition on polyamines may relate to growth effects with N addition.

On the other hand, the addition of N raised the concentrations of foliar toxins Al and Mn for the trees in this study, suggesting that the addition of N may not have been entirely positive. Putrescine concentration was associated with high Al and Mn in unlimed compared with limed sugar maple stands on the Allegheny Plateau (Wargo et al. 2002). Although we observed higher Al and Mn in trees receiving N, this was not accompanied by an increase in putrescine, suggesting that this change was within the tolerance range of sugar maple. We observed more leaf characteristics to be sensitive to N addition than P addition, possibly because many of the leaf characteristics we measured contain N, whereas P in plants is primarily in nucleic acids and membrane phospholipids, which we did not measure.

Leaf response to P addition varied with depth in the crown

Phosphorus is critical to plant growth and plays an essential role in photosynthesis (Hammond and White 2008) and cellular growth (Carstensen et al. 2018). Low P availability can lead to decreased ATP synthase activity, causing reduced ATP production in the chloroplast stroma and decreased CO_2 fixation. This, in turn, reduces the flow of protons from the thylakoid lumen to the chloroplast stroma causing lumen acidification and inhibition of linear electron flow, thus negatively affecting the quantum yield of Photosystem II. Low P availability may also impair protein synthesis due to the requirement for P in nucleic acids and ribosomes.

As expected, trees that received P had higher foliar P. Trees that received P also had higher foliar B, perhaps due to their shared transport mechanism (Brown and Shelp 1997, Mühlbachová et al. 2017). Boron plays an essential role in the structural and functional integrity of the cell wall and membranes, ion fluxes across the membranes, N metabolism, carbohydrate transport and phenol metabolism and transport (Shireen et al. 2018). An unexpected effect of P addition was a decrease in the rate at which leaf area increased with crown depth.

The concentrations of foliar Zn have been linked to sugar maple health, with unhealthy sugar maples having Zn concentrations $<29\ \mu g\ g^{-1}$ (Horsley et al. 2002, St Clair et al. 2005). In this study, many trees had foliar Zn $<29\ \mu g\ g^{-1}$ at the top of the crown. Trees that received P addition had a steeper increase of Zn concentrations with depth in the crown, which is a treatment response that we would have missed if we had observed leaves only high in the canopy.

Leaf sampling strategies

By prioritizing the sampling of sunlit leaves, previous studies have excluded a considerable fraction of the foliage on the earth, which is shaded (Niinemets et al. 2015, Keenan and Niinemets 2016). The emphasis on sunlit leaves is understandable because the leaves at the top of the crown typically have the highest photosynthetic activity, and their characteristics can be observed with remotely sensed earth observation techniques. Collecting samples across the vertical gradient of tree crowns allowed us to explore physical, chemical and metabolic leaf characteristics of mature sugar maple trees. We found that the effect of nutrient addition on several amino acids and photosynthetic pigments had an interaction with depth in the crown, such that sampling leaves from only one canopy position could give misleading results. Research regarding physical, photosynthetic, chemical and metabolic leaf characteristics throughout the crown of these trees increases understanding of resource partitioning within tree crowns.

Acknowledgments

Chris Costello, Dan Hong, Alexandrea Rice, Claudia Victoroff and Trey Turnblacer assisted with field collections. The Bartlett Experimental Forest is operated and maintained by the USDA Forest Service, Madison, WI.

Supplementary data

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

Data availability statement

The data are available in the Environmental Data Initiative repository: A.R. Young, R.D. Yanai, R. Minocha, S. Long and J.E. Drake. 2020. https://doi.org/10.6073/pasta/3da4e463ebb199309a453e4377d7b1f1 (accessed 28 April 20232-04-26, date last accessed).

Conflict of interest

None declared.

Funding

The USDA Forest Service, Northern Research Station, provided support for the metabolic analyses conducted in Durham, NH. Steve Stehman improved our approach to data analysis. This paper is a product of the Multiple Element Limitation in Northern Hardwood Ecosystems project, which is funded by the National Institute of Food and Agriculture (2019-67019-29464) and the National Science Foundation (DEB 0949324) and forms part of the Hubbard Brook LTER (Long Term Ecological Research) Network, also funded by NSFNational Science Foundation (DEB 1633026).

Authors’ contributions

A.R.Y. and R.D.Y. planned and designed the research. A.R.Y. climbed the trees and collected the leaves. A.R.Y. analyzed leaf physical and chemical characteristics; R.M. and S.L. analyzed leaf metabolic characteristics. A.R.Y., R.D.Y. and J.E.D. analyzed the data. All the authors engaged in interpreting the results and learned a lot from one another. A.R.Y. and R.D.Y. led the writing of the manuscript with input from R.M.

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