



## Research paper

# Calcium and aluminum impacts on sugar maple physiology in a northern hardwood forest

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Received July 31, 2013; accepted October 8, 2013; handling Editor Heinz Rennenberg

Forests of northeastern North America have been exposed to anthropogenic acidic inputs for decades, resulting in altered cation relations and disruptions to associated physiological processes in multiple tree species, including sugar maple (*Acer saccharum* Marsh.). In the current study, the impacts of calcium (Ca) and aluminum (Al) additions on mature sugar maple physiology were evaluated at the Hubbard Brook Experimental Forest (Thornton, NH, USA) to assess remediation (Ca addition) or exacerbation (Al addition) of current acidified conditions. Fine root cation concentrations and membrane integrity, carbon (C) allocation, foliar cation concentrations and antioxidant activity, foliar response to a spring freezing event and reproductive ability (flowering, seed quantity, filled seed and seed germination) were evaluated for dominant sugar maple trees in a replicated plot study. Root damage and foliar antioxidant activity were highest in Al-treated trees, while growth-associated C, foliar re-flush following a spring frost and reproductive ability were highest in Ca-treated trees. In general, we found that trees on Ca-treated plots preferentially used C resources for growth and reproductive processes, whereas Al-treated trees devoted C to defense-based processes. Similarities between Al-treated and control trees were observed for foliar cation concentrations, C partitioning and seed production, suggesting that sugar maples growing in native forests may be more stressed than previously perceived. Our experiment suggests that disruption of the balance of Ca and Al in sugar maples by acid deposition continues to be an important driver of tree health.

**Keywords:** acid deposition, antioxidant activity, carbon partitioning, cations, frost injury, Hubbard Brook, membrane integrity, reproductive capacity.

## Introduction

Northeastern forests have been exposed to acidic inputs of anthropogenic origin for decades, which has resulted in the acidification of many forest soils (Likens et al. 1996, DeHayes et al. 1999, Driscoll et al. 2001). In addition to lowering pH levels in soils, acidic inputs leach important base cations like calcium (Ca) from soils (Schaberg et al. 2001, 2010). Because Ca is biologically essential, the leaching of this element has far-reaching consequences for forest health and productivity.

Calcium is necessary for both structural integrity (e.g., membrane stabilization and wood formation; Davies and Monk-Talbot 1990, Fromm 2010), and metabolic regulation/stress response within plant cells (e.g., coordination of carbohydrate metabolism and carbon (C) partitioning (Snedden and Fromm 2001), antioxidant activity (Halman et al. 2008) and flowering (Tretyn et al. 1994)). Sensitivity to reductions in Ca is often species specific, and some sensitive species have experienced declines in their populations on a regional basis coincident with recent anthropogenic Ca depletion (Schaberg et al. 2001). Perhaps

the most noteworthy example of this is red spruce (*Picea rubens* Sarg.) decline in northeastern North America. Anthropogenic Ca-depletion has been shown to reduce the cold tolerance of red spruce, and Ca-fertilization has been shown to bolster the cold tolerance and reduce the severity of foliar winter injury (Hawley et al. 2006, Halman et al. 2008, Schaberg et al. 2011). Given the extent to which acid deposition has impacted northeastern forests, it is not surprising that other Ca-sensitive species have also experienced health declines in recent years (Schaberg et al. 2001, 2006, Halman et al. 2011).

Sugar maple (*Acer saccharum* Marsh.) decline is described as the dieback of fine branches, loss of crown vigor and subsequent mortality of trees (Kolb and McCormick 1993). Examples of sugar maple decline extend from Missouri east to New Hampshire and north into Quebec (Duchesne et al. 2002). Similar to red spruce decline, numerous studies have linked sugar maple decline to soil base cation depletion (Kolb and McCormick 1993, Wilmot et al. 1995, Mohamed et al. 1997, Horsley et al. 2000, Kogelmann and Sharpe 2006, Schaberg et al. 2006, Huggett et al. 2007). In particular, soil Ca depletion has been shown to influence many processes in sugar maple, including radial growth, crown vigor, wound closure, antioxidant activity and reproductive success (St Clair et al. 2005, Juice et al. 2006, Schaberg et al. 2006, Huggett et al. 2007). The health of sugar maples may be jeopardized by these influences, contributing to maple decline.

Soil base cation depletion by acid deposition typically is accompanied by increases in the availability of aluminum (Al) as its solubility increases at low pH (Cronan and Schofield 1979). Increases in Al bioavailability are problematic because (i) Al competes with Ca for plant uptake and (ii) Al can be directly phytotoxic to many species (Marschner 2002), including sugar maple (Thornton et al. 1986). In particular, Al-exposure has been found to increase cellular membrane damage and oxidative stress (and associated antioxidant activity), and to impair tree C metabolism and growth (Rengel 1992, Kochian 1995, Ezaki et al. 2000, Schaberg et al. 2006). While considerable research has evaluated the influences of soil Ca depletion on sugar maple health, less is known about the impacts of elevated Al availability (Cronan and Grigal 1995). Aluminum can reduce the growth and increase the mortality of sugar maples at various life stages. For example, increased soil-Al availability has been shown to increase the mortality of sugar maple seedlings (Kobe et al. 2002, Bigelow and Canham 2010). In southern New England, Bigelow and Canham (2010) found that sugar maple seedling survival was greatest on sites with higher available Ca and was lowest on sites with higher available Al. For mature trees, the impacts of elevated Al concentrations on sugar maple include reductions in growth and crown vigor, and a higher incidence of decline (Mohamed et al. 1997, Schaberg et al. 2006).

Although there is growing literature on the general effects of both Ca and Al on sugar maple health, the influence of these

cations on C partitioning, defense and reproductive capacity has not been examined for mature trees. In order to more completely assess the impact of Ca and Al on sugar maple physiological ecology, we studied multi-tissue tree response (i.e., root to crown-based measures of physiological function), and implications for subsequent generations (e.g., flowering, seed yield and viability) for dominant sugar maple trees at a long-term soil Ca and Al addition study at the Hubbard Brook Experimental Forest (HBEF; Thornton, NH, USA). This study site is unique because control plots indicate tree performance on native soils (Ca depleted; Likens et al. 1996), Ca-addition plots demonstrate tree performance on soils with pre-pollution Ca levels, and Al-addition plots provide an example of tree function on soils with Ca and Al levels that reflect future conditions associated with continued acid loading. We hypothesized that soil Ca and Al additions would induce alterations in a wide spectrum of physiological characteristics in a range of tissues, resulting in shifts in stress response, C use and reproductive capacity. We anticipated that, in general, sugar maples on control plots would be intermediate in cation nutrition, C relations (e.g., growth and carbohydrate storage), and stress response (e.g., root membrane destabilization and altered antioxidant enzyme activity) compared with trees on Ca- and Al-addition plots. If tree performance on control plots matched that on Ca-addition plots, this would suggest that ambient Ca availability appeared adequate. In contrast, if tree performance on control plots better matched that on Al-addition plots, this would suggest that ambient Al availability was already altering tree health and productivity.

## Materials and methods

### Study site

The HBEF has been exposed to acidic inputs and subsequent soil Ca depletion for decades (Likens and Bormann 1974, Likens et al. 1998). To better evaluate the interaction of Ca depletion and Al mobilization on a northern hardwood forest, the Nutrient Perturbation (NuPert) study was initiated in 1995 west of the biogeochemical reference watershed (W6) at HBEF (N 43.95411°, W 71.74779°). The study area is on a south-facing slope, with an elevational range of 700–760 m, and most soils are classified as either Aquic Haplorthods or Aquic Haplumbredts (Berger et al. 2001). Twelve sugar maple-dominated plots were randomly assigned one of three treatments: Ca addition, Al addition or control (no addition), to yield four replicates of each treatment in the study. In addition to sugar maple, American beech (*Fagus grandifolia* Ehrh.) and yellow birch (*Betula alleghaniensis* Britt.) are co-occurring tree species in these plots, while hobblebush (*Viburnum lantanooides* Michx.) and striped maple (*Acer pensylvanicum* L.) dominate the understory. Calcium treatments began with annual CaCl<sub>2</sub> applications in fall or spring during leafless periods (Table 1). The use of CaCl<sub>2</sub> was halted in 1999 in favor of a one-time

Table 1. Dates and treatment applications to the long-term NuPert experiment at the HBEF, Thornton, NH, USA.

Date of application	Treatment (g m <sup>-2</sup> )		
	Calcium	Control	Aluminum
October 1995	2	–	0.9
May 1996	3	–	0.9
November 1996	2	–	0.9
May 1997	3	–	0.9
October 1999	38	–	1.8
May 2001	0	–	0.9
April 2002	0	–	0.9
May 2004	0	–	0.9
November 2005	0	–	0.9
May 2008	0	–	0.9
May 2011	0	–	0.9

application of wollastonite (CaSiO<sub>3</sub>, a slow-release form of Ca; [Peters et al. 2004](#)). The total addition of calcium to the plots was 38 g m<sup>-2</sup>, an amount similar to the estimated depletion of soil Ca during the 20th century ([Likens et al. 1996](#)); hence, this treatment represented remediation of lost Ca rather than a heavy fertilization. Addition of AlCl<sub>3</sub> occurred in annual applications in fall or spring during 1995–1999 followed by less frequent additions during 2001–2011 (Table 1). Unless otherwise noted, all tree-based measures (described below) were from the same five dominant sugar maples per plot collected during 2006–2009 ( $n = 60$  trees).

#### Root collection and electrolyte leakage testing

To test for differences in growing season cation concentrations and membrane integrity (a measure of stability/injury) in root cells, we excavated fine roots (<2 mm diameter) from dominant sugar maples in early July 2008. Primary roots were excavated from the base of each tree until fine roots could be identified and sampled. Fine roots were excised, wrapped in moist towels and stored in plastic bags for transport back to the laboratory for relative electrolyte leakage (REL) tests and quantification of cation concentrations. Relative electrolyte leakage from plant cells has been used as a measure of cellular membrane damage caused by a variety of stresses including changes in mineral nutrition ([David et al. 1994](#), [Branquinho et al. 1997](#)), acid mist treatment ([DeHayes et al. 1999](#), [Schaberg et al. 2001](#)) and freezing injury ([Schaberg et al. 2008](#), [Comerford et al. 2013](#)). Roots were cleaned and prepared for REL analysis using previously established methods ([Schaberg et al. 2008](#)). Roots were then cut into 5-mm segments, placed in polystyrene trays and soaked for 20 h in 3.5 ml of a 0.01% (v/v) Triton-X solution. Initial conductivity was measured using a multi-electrode conductivity meter (Wavefront Technologies, Ann Arbor, MI, USA), after which samples were dried for 72 h at 65 °C to induce mortality. Another 3.5 ml of Triton-X solution was then added to each sample, and roots were soaked for 24 h before measuring

final conductivities. For each sample, REL was calculated by dividing the initial conductivity by the final conductivity and then multiplying the quotient by 100 to represent the percent of electrolyte leakage relative to complete mortality.

#### Root cation concentrations

A subset of fine roots that did not undergo REL analysis was used to quantify cation concentrations. Roots were cleaned thoroughly as per standard methods ([Schaberg et al. 2008](#)), and dried at 65 °C for 2 weeks. Samples were ground to pass through a 2-mm mesh, and digested by heating with nitric acid and hydrogen peroxide using a block digester (adapted from [Jones and Case 1990](#)). Samples were analyzed for cation concentrations by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Perkin-Elmer Optima DV 3000, Perkin-Elmer Corp., Norwalk, CT, USA). Peach leaves from the National Institute of Standards and Technology (SRM 1547), sample duplicates and blanks were analyzed for procedural verification. Assayed tissue standards were within 5% of certified values.

#### Sugar concentrations and C partitioning

To assess C relations within woody tissues, branches were collected from the upper third of tree crowns using shotguns in early August 2009. Branches of ~1 cm diameter were collected, bagged and placed on dry ice for transport to the laboratory. Samples were prepared for carbohydrate analysis by removing bark, phloem, cambium and pith, and processed using methods described by [Wong et al. \(2003\)](#). After sample preparation, soluble carbohydrates were extracted with 80% ethanol in an 80 °C water bath and centrifuged (HN-SII, International Equipment Company, Needham Heights, MA, USA) to separate the soluble-sugar-containing supernatant from the pellet. The supernatant was analyzed for sucrose, glucose, fructose, raffinose and stachyose using a Waters (Milford, MA, USA) Alliance HPLC system with a Waters Sugar-pak column and solvent (0.1 mmol<sup>-1</sup> Ca EDTA) at 90 °C ([Wong et al. 2003](#)). Sugars were detected with a Waters 2414 refractive index detector and Waters PC-based Empower software. The separated soluble sugars were identified and quantified with known standards and converted to milligrams of sugar per gram dry weight of tissue (mg g<sup>-1</sup>).

The partitioning of C resources to storage (e.g., soluble sugars) as opposed to woody growth (e.g., xylem increment growth) has been suggested as a strategy for trees experiencing stress to maintain C pools available for physiological responses that could increase the likelihood of survival ([Kobe 1997](#)). To evaluate the influence of treatment on C partitioning, we calculated a growth : storage ratio that combined our soluble sugar data and existing stem increment growth data (J.M. Halman, unpublished data). To compare growth and sugar concentrations in an appropriate temporal context, we summed xylem increment growth expressed as basal area increment (BAI; % of total basal area)

for 5 years (the mean age of branches assayed for sugar concentrations as determined by growth rings) and divided this by the soluble sugar concentrations in shoots for this same period. We found this to be an appropriate comparison due to the long residence time (7 years or more) of soluble sugars in woody tissue (Richardson et al. 2013).

### Foliar cation and antioxidant activity

Foliage from sun-lit crowns was collected using shotguns to assess the impacts of treatment on cation concentrations and antioxidant enzyme activity in early August 2006. Samples used for cation analysis were bagged, transported to the laboratory, and processed and analyzed using previously described methods (see 'Root collection and electrolyte leakage testing').

Foliar antioxidant activity is sensitive to Ca nutrition (Halman et al. 2008) because Ca is a major component of the signal transduction pathways that increase the biogenesis and capacity of antioxidant enzymes such as ascorbate peroxidase (APX) and glutathione reductase (GR) (Jiang and Huang 2001, Jiang and Zhang 2003). However, Al is an oxidative stressor that can initiate antioxidant activity (Richards et al. 1998, Boscolo et al. 2003); thus, elevated antioxidant activity may help reduce oxidative damage when Al bioavailability is high. Samples to be assayed for antioxidant enzyme activity were kept on dry ice, and transported back to the laboratory and stored at  $-80^{\circ}\text{C}$ . Samples were homogenized in extraction buffer modified from Pell et al. (1999), containing 90 mM potassium phosphate (pH 7.8), 1% v/v Triton X-100, 5 mM ascorbate, 4% PVPP, 1.5% PVP, 8% glycerol and 1 mM EDTA, and stored at  $-80^{\circ}\text{C}$  until ready for assay. Enzyme activity was monitored spectrophotometrically with a Beckman DU 800 (Beckman Coulter, Inc., Fullerton, CA, USA). Total soluble protein was analyzed with a brilliant-blue total protein kit (TPO100, Sigma-Aldrich Co., St Louis, MO, USA), and enzyme activity was expressed per unit protein.

The methods of Nakano and Asada (1981) were followed for APX analysis. The 1.0 ml reaction mixture contained 50 mM potassium phosphate, 0.5 mM ascorbate, 0.15 mM  $\text{H}_2\text{O}_2$ , 0.1 mM EDTA and 0.01 ml of sample. This was spectrophotometrically analyzed at 290 nm, and the linear decrease in absorbance for 2 min was recorded to determine the activity of APX as ascorbate scavenged  $\text{H}_2\text{O}_2$ . Ascorbate oxidase (AO) activity was measured via the same methods with the omission of  $\text{H}_2\text{O}_2$ , and subtracted from APX activity to yield APX-specific activity.

Glutathione reductase activity ( $\mu\text{mol TNB min}^{-1} \text{mg}^{-1}$ ) was quantified according to the methods of Smith et al. (1988) and Pell et al. (1999). Here the 1 ml of reaction mixture consisted of 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), 0.2 glutathione oxidoreductase (GSSG) and 10  $\mu\text{l}$  of sample extract. Linear increases in absorbance of DTNB reduced to GSH were

measured at 412 nm (extinction coefficient  $14.15 \text{ mM}^{-1} \text{ cm}^{-1}$ ) for 120 s.

### Spring frost assessments

Adequate Ca nutrition is important in supporting tree responses to a variety of stressors including low temperatures (DeHayes et al. 1999, Halman et al. 2008). A severe spring frost occurred from 9 to 11 May 2010 throughout the region that resulted in widespread foliar injury or mortality to sugar maple trees (Hufkens et al. 2012). To assess the influence of treatment on the severity of this injury and the degree to which trees recovered from this event, we visually quantified both initial injury and subsequent second flushing of foliage from all sample trees.

Initial injury was monitored with binoculars by groups of two researchers per tree on 18 May 2010 using an eight-point scale to quantify the proportion of the crown injured, and second flushing was monitored on 5 June 2010 using a 10-point scale (see Table 2 for specific rating criteria). A ratio of injury : second flush scores was used to integrate individual tree response to frost injury.

### Flowering, seed yield and viability

Calcium is important to flower and seed development (Brewbaker and Kwack 1963, Tretyn et al. 1994). Furthermore, seed production is thought to increase in response to stress (Lee 1988), so it

Table 2. Rating criteria for field evaluations of sugar maple foliar frost injury, second foliar flush production and flowering.

Measure	Score	Description of score
Frost injury	0	No expanded leaves (no injury)
	1	No foliar damage
	1.5	Minor necrosis at leaf tips
	2	<25% of Leaf necrotic
	2.5	25–50% Necrotic
	3	51–75% Necrotic
	3.5	76–90% Necrotic
Second flush	4	91–100% Necrotic
	0	No second flush
	1	1–10% Crown re-flushed
	2	11–20% Crown re-flushed
	3	21–30% Crown re-flushed
	4	31–40% Crown re-flushed
	5	41–50% Crown re-flushed
Flowering	6	51–60% Crown re-flushed
	7	61–70% Crown re-flushed
	8	71–80% Crown re-flushed
	9	81–90% Crown re-flushed
	10	91–100% Crown re-flushed
	1	No flowering
2	1–20% Crown flowering	
3	21–40% Crown flowering	
4	41–60% Crown flowering	
5	61–80% Crown flowering	
6	81–100% Crown flowering	

could be enhanced by elevated Al bioavailability. Sugar maple flowering was abundant in May 2011 across the region. To determine the effect of treatment on flowering ability, we visually assessed sugar maple on the NuPert plots on 25 May 2011 using a six-point scale (Table 2). Seed was collected using ten  $1 \times 1$  m mats randomly distributed through each plot in July 2011 and fixed to the ground using landscaping stakes. In early November 2011, mats were collected and transported back to the laboratory and seed was separated from leaves. The total number of seeds was counted, and viable (filled) versus non-viable (aborted) seeds were determined by assessing whether seeds floated in *n*-pentane. Filled seeds were dried to 10% fresh weight, and stratified at 4 °C for 80 days prior to germination tests. Germination tests employed standard germination boxes and moistened paper at 4 °C, and were assessed daily for 60 days. After each seed germinated, it was recorded and removed from its box. At the end of 60 days, the number of seed germinated was divided by the initial number of seed present in each box to assess germinative success. An estimated maximum number of germinants per m<sup>2</sup> on each plot was calculated by multiplying the number of filled seeds (m<sup>-2</sup>) by the mean percent of seed germinated for each respective treatment.

### Statistical analysis

Treatment differences among means were tested using analyses of variance (ANOVA) and Tukey–Kramer HSD tests. Significance tests utilized a nested design (Montgomery 2001) that tested treatment differences by dividing the mean square for treatment by the mean square for plot within treatment, and tested plot differences by dividing the mean square for plot within treatment by the mean square for tree within plot. If assumptions necessary for ANOVA testing were not met and data transformation failed, treatment differences among means were tested using Wilcoxon Each-Pair non-parametric analyses. For all tests, differences were considered statistically significant at the  $P \leq 0.05$  level.

## Results

### Root physiology

Fine root concentrations of Ca and Al were highest in plots treated with Ca and Al, respectively (Table 3). Coincident with increases in root Al concentration, root REL was highest in trees on Al-treated plots compared with those growing on Ca-addition plots, indicating significantly greater membrane damage associated with elevated Al concentrations (Table 3). Although treatment differences existed for REL, the extent of injury was not high enough to be considered severe cellular damage (REL  $\geq 50\%$ ; Strimbeck et al. 2008).

### Stem-based C allocation

No differences were found among treatments for individual sugars or total shoot sugar concentrations, nor for radial growth (Table 4).

Table 3. Mean ( $\pm$  SE) nutrient concentrations and REL by treatment of dominant sugar maple fine roots in the NuPert plots.

Treatment	Root nutrient concentrations		REL (%)
	Ca (mg kg <sup>-1</sup> )	Al (mg kg <sup>-1</sup> )	
Al addition	3138 $\pm$ 213 <sup>b</sup>	1634 $\pm$ 203 <sup>a</sup>	38 $\pm$ 2 <sup>a</sup>
Control	3761 $\pm$ 365 <sup>ab</sup>	752 $\pm$ 119 <sup>b</sup>	36 $\pm$ 2 <sup>ab</sup>
Ca addition	4502 $\pm$ 496 <sup>a</sup>	701 $\pm$ 104 <sup>b</sup>	32 $\pm$ 2 <sup>b</sup>

Means within columns with different letters are significantly different based on Tukey–Kramer HSD tests ( $P < 0.05$ ).

However, comparisons of radial growth (summed from 2004 to 2008) and C storage (i.e., soluble sugars in shoots of the same age) revealed that trees growing on Ca-addition plots devoted significantly more C to growth relative to storage (Table 4). However, this ratio of growth : storage was not significantly different between trees on the Al-addition and control plots (Table 4).

### Foliar cations and antioxidant activity

Foliar Ca concentrations were greatest in trees on Ca-addition plots, and were nearly twice the level found in leaves of trees from both Al-addition and control plots (Table 5). No significant differences among treatments were found in foliar Al concentrations, even for trees in Al-addition plots. However, both antioxidant enzyme systems measured showed significantly greater activity in trees growing on Al-addition plots than those in other treatments (Table 5). Glutathione reductase activity was significantly greater in Al-addition trees than trees on both Ca-addition and control plots, which were not different from one another. Ascorbate peroxidase activity was significantly higher for trees on Al-addition plots; APX activity was also greater on control plots than on Ca-addition plots (Table 5). Reported values are within, though at the low end of, the range expected for antioxidant enzyme activity in sugar maple foliage (St Clair et al. 2005).

### Foliar frost injury and response

The response of sugar maple trees to the late spring frost of 2010 was at least partially influenced by treatment. Frost injury, however, was not significantly different among treatments (Table 6). In addition, the extent of secondary foliar flush did not differ significantly among the treatments. However, when the ability to produce a second flush of foliage following the injury was assessed as a function of the degree of initial injury (see Materials and methods), trees growing on Al-addition plots had a significant disadvantage compared with trees growing on control and Ca-addition plots (Table 6).

### Reproductive capacity

Flowering of sugar maples on Ca-addition plots was greater than that of trees on both control and Al-addition plots (Table 7). Sugar maple trees on Ca-addition plots exhibited ~50% greater flowering than trees on Al-addition plots

Table 4. Mean ( $\pm$  SE) soluble sugar concentrations of branch tissues and 5-year sums of radial growth by treatment for dominant sugar maple trees in the NuPert plots.

Treatment	Soluble sugar concentrations ( $\text{mg g}^{-1}$ dry mass)						Total sugars	2004–2008 BAI (%)	Growth : storage
	Stachyose	Raffinose	Sucrose	Glucose	Xylose	Fructose			
Al addition	1.19 $\pm$ 0.06	0.85 $\pm$ 0.07	18.03 $\pm$ 0.9	10.29 $\pm$ 0.47	0.16 $\pm$ 0.03	8.97 $\pm$ 0.42	39.48 $\pm$ 1.50	3.84 $\pm$ 0.56	0.96 $\pm$ 0.13 <sup>b</sup>
Control	1.09 $\pm$ 0.09	0.92 $\pm$ 0.11	17.32 $\pm$ 1.24	8.58 $\pm$ 0.56	0.13 $\pm$ 0.03	7.75 $\pm$ 0.62	35.79 $\pm$ 2.23	3.96 $\pm$ 0.59	1.02 $\pm$ 0.10 <sup>b</sup>
Ca addition	0.98 $\pm$ 0.06	0.66 $\pm$ 0.07	15.88 $\pm$ 0.64	8.23 $\pm$ 0.44	0.64 $\pm$ 0.24	7.12 $\pm$ 0.39	33.51 $\pm$ 1.32	5.93 $\pm$ 0.90	1.66 $\pm$ 0.23 <sup>a</sup>

Growth : storage ratios were calculated by dividing 2004–2008 BAI by total sugar concentrations for each sample. Means within columns with different letters are significantly different based on Wilcoxon each-pair analyses ( $P < 0.05$ ).

Table 5. Mean ( $\pm$  SE) foliar nutrient concentrations and antioxidant activity by treatment for dominant sugar maple trees in the NuPert plots.

Treatment	Ca ( $\text{mg kg}^{-1}$ )	Al ( $\text{mg kg}^{-1}$ )	Antioxidant enzyme activity	
			GR ( $\mu\text{mol TNB min}^{-1} \text{mg protein}^{-1}$ )	APX ( $\mu\text{mol Asc min}^{-1} \text{mg protein}^{-1}$ )
Al addition	4754.2 $\pm$ 437.8 <sup>b</sup>	21.3 $\pm$ 1.8	0.121 $\pm$ 0.026 <sup>a</sup>	1.102 $\pm$ 0.115 <sup>a</sup>
Control	4835.1 $\pm$ 229.3 <sup>b</sup>	18.5 $\pm$ 1.0	0.012 $\pm$ 0.001 <sup>b</sup>	0.673 $\pm$ 0.090 <sup>b</sup>
Ca addition	8362.9 $\pm$ 538.7 <sup>a</sup>	16.4 $\pm$ 1.2	0.019 $\pm$ 0.002 <sup>b</sup>	0.172 $\pm$ 0.052 <sup>c</sup>

Glutathione reductase and APX activities were expressed on a protein basis. Means within columns with different letters are significantly different based on Tukey–Kramer HSD tests ( $P < 0.05$ ).

Table 6. Mean ( $\pm$  SE) re-flush/frost injury scores by treatment for dominant sugar maple trees in the NuPert plots.

Treatment	Injury score	Re-flush score	Re-flush : injury
Al addition	3.0 $\pm$ 0.2	5.1 $\pm$ 0.6	1.6 $\pm$ 0.2 <sup>b</sup>
Control	3.2 $\pm$ 0.2	7.1 $\pm$ 0.4	2.2 $\pm$ 0.1 <sup>a</sup>
Ca addition	3.2 $\pm$ 0.1	6.8 $\pm$ 0.3	2.1 $\pm$ 0.1 <sup>a</sup>

Foliar re-flush and injury scales are described in Table 2. Means within columns with different letters are significantly different based on Tukey–Kramer HSD tests ( $P < 0.05$ ).

(Table 7). Flowering differences were associated with significantly greater seed production and filled seeds on Ca-addition plots compared with both Al-addition and control plots (Table 7). Seed production on Ca-addition plots was nearly sixfold greater than on Al-addition plots, and more than threefold greater than on control plots. Among the seeds collected, those from the Ca-addition plots contained eight times more filled seeds than those from the Al-addition plots. Al-addition and control plots did not differ in the total number of seed produced, but were different in terms of number of filled seeds, with control plots containing higher numbers. Treatment did not affect the germination of filled seeds, but did significantly impact the estimated maximum number of germinants per plot (Table 7). Maximum germinants on Ca-addition plots were nearly seven times greater than those on Al-addition plots, and twice as large as those on control plots (Table 7).

## Discussion

### Root physiology

Root Ca and Al concentrations were significantly different and coincident with treatment for both Ca and Al additions (Table 3),

whereas foliage only exhibited treatment differences for Ca concentrations (Table 5). We note that the effects of Al addition on tissue chemistry developed gradually, as earlier sampling in these plots revealed no significant differences (Huggett et al. 2007; P.G. Schaberg, unpublished data); thus, Al responses reflected some cumulative effects. The contrast in response between roots and foliage presumably results from the fact that Al is often excluded from or tightly bound in roots to functionally limit transport and toxicity in above-ground tissues (Schaedle et al. 1989, Rengel 1996); whether chronic Al loading eventually can overwhelm this Al exclusion may be evident with continued monitoring. Furthermore, Kelly et al. (1990) found that root-Al concentrations were more reliable for predicting physiological dysfunction than were foliar-Al concentrations. With direct exposure to Al-addition, roots would likely be the tissue most sensitive to Al toxicity. Indeed, we found higher membrane disruption for roots of trees on Al-addition relative to Ca-addition plots (Table 3).

Despite higher levels of electrolyte leakage, REL values in the range measured are typically not high enough to suggest severe cellular damage. However, these levels do indicate that tree roots from Al-addition plots showed signs of elevated stress. Notably, the molar ratios of Ca : Al in the Al treatment (1.3) remained above the threshold level (0.2) indicative of severe stress in forest trees (Cronan and Grigal 1995). We sampled tree roots in mid-summer during a period when soils were relatively moist (e.g., no drought present) and growing conditions were generally favorable (see <http://www.hubbardbrook.org/data/dataset.php?id=58>); thus, any stress present resulted from the treatment. It would be an interesting extension of this work to sample roots at times of known duress during either summer or winter (e.g., drought or cold exposure and soil freezing) to evaluate the interaction of nutrient and known environmental stresses.

Table 7. Mean ( $\pm$  SE) flowering scores and seed production values by treatment for dominant sugar maples in the NuPert plots.

Treatment	Flowering score	Total seeds (m <sup>-2</sup> )	Filled seeds (m <sup>-2</sup> )	Germinated seeds (%)	Estimated germinants (m <sup>-2</sup> )
Al addition	2.9 $\pm$ 0.4 <sup>b</sup>	45.6 $\pm$ 4.9 <sup>b</sup>	6.9 $\pm$ 0.8 <sup>c</sup>	74.1 $\pm$ 7.0	5.1 $\pm$ 0.6 <sup>b</sup>
Control	3.3 $\pm$ 0.4 <sup>b</sup>	76.2 $\pm$ 7.9 <sup>b</sup>	23.2 $\pm$ 2.6 <sup>b</sup>	63.8 $\pm$ 9.6	11.7 $\pm$ 1.5 <sup>b</sup>
Ca addition	4.5 $\pm$ 0.3 <sup>a</sup>	276.2 $\pm$ 16.8 <sup>a</sup>	60.0 $\pm$ 4.8 <sup>a</sup>	62.1 $\pm$ 9.0	37.3 $\pm$ 3.0 <sup>a</sup>

Flowering score is based on a six-point scale for the percentage of crown flowering as described in Table 2. Means within columns with different letters are significantly different based on Tukey–Kramer HSD tests ( $P < 0.05$ ). Flowering score data did not satisfy assumptions of normality, and significant differences between means were analyzed with a Wilcoxon each-pair test ( $P < 0.05$ ).

### Carbon allocation

Nutrient additions at NuPert likely influence individual physiological processes differently. Because Ca has previously been shown to increase growth and reproductive success of sugar maple (Juice et al. 2006, Huggett et al. 2007), we hypothesized that trees would devote more C to growth and seed production under Ca treatment, and our data support this hypothesis.

Storage of carbohydrates and tissue growth are two major C pools within trees (Richardson et al. 2013), and the distribution of C to these pools can be used as a proxy for the health of trees, including sugar maple. High sugar concentrations and reductions in radial growth in sugar maple have been associated with increased stress and decline of the species (Renaud and Mauffette 1991, McLaughlin et al. 1996). We found two pieces of evidence that indicate that trees growing on our Ca-treated plots devoted significantly more C to growth relative to storage (Table 4). First, the ratio of wood growth : storage was significantly higher on the Ca plots; and second, the ability of trees to grow a second flush of foliage after a spring frost was enhanced relative to Al-treated trees (Table 6). Increased concentrations of soluble sugars may serve as a C pool from which trees can fuel growth or defense during times of stress (Kobe 1997). However, trees from Al-treated plots that devoted more resources to C storage than growth were just as stressed in 2010 by spring frost injury (i.e., lost similar amounts of foliage), yet were unable to recover as well as trees on other treatments (Table 6). It does not appear that insufficient sugar concentrations inhibited the ability of these trees to respond, since their sugar levels were similar to those of trees on other treatments that were able to produce a more abundant second flush of foliage (Table 4). Instead, other unmeasured processes were likely impaired by Al addition and constrained the recovery of trees from the frost event.

### Foliar stress response

We hypothesized that trees would devote more C to defense physiology under potentially phytotoxic Al addition. Although the binding of Al in soils and roots may have limited its ascent to above-ground tree tissues, impacts of Al addition manifested themselves in tissues as distant as upper-canopy leaves. Foliage from the sunlit upper portions of crowns exhibited higher antioxidant activity (Table 5) and an impaired ability to recover from frost injury (Table 6) on Al-addition plots. Foliar APX and GR activities in trees from Al-addition plots were as

much as 10-fold greater than trees from Ca-addition plots (Table 5). In particular, in sugar maple, the activity of these enzymes has been used as a biomarker to indicate oxidative stress resulting from factors such as nutrient limitations (St Clair et al. 2005, 2008).

Considering sugar maple resiliency to foliar frost injury, it is informative that effects associated with Al addition (e.g., a delayed or insufficient second flush of foliage) was evident despite the lack of differences in foliar Al concentrations (Table 6). Delays in recovery from events such as these may prove more significant in future years as the climate changes. Indeed, the underlying cause of the foliar freezing injury in 2010 was early leaf-out of sugar maples (~3 weeks ahead of average) throughout the region followed by a widespread frost (Hufkens et al. 2012). Climate models for northeastern North America predict a generalized increase in winter and early spring air temperatures in future years (Hayhoe et al. 2007). Warming trends appear related to the early budbreak for some tree species including sugar maple (Groffman et al. 2012). However, because frost events may occur episodically despite overall warming trends, early budbreak could put species like sugar maple at a competitive disadvantage, increasing potential injury and altering C relations for these species relative to those (e.g., American beech) that break bud later (Richardson et al. 2006). The influence of Ca and Al nutrition on the capacity of trees to leaf-out again following spring frost damage could interact with and complicate the competitive responses of species to perturbations like the 2010 frost injury event.

### Reproductive capacity

Nutrient perturbation also significantly altered tree reproductive capacity. The role of Ca in plant flowering has long been recognized (Brewbaker and Kwack 1963), but studies documenting the effect of Ca on the flowering of forest trees are lacking. One exception to this is work presented by Long et al. (1997) from the Allegheny plateau in Pennsylvania in which limed sugar maple displayed consistently greater flower and seed crops than control sites over a 5-year period. In that study, the addition of large amounts of dolomitic limestone (which supplies both Ca and Mg) did not increase the frequency of large flower or seed crops, but did increase the proportion of the crown that flowered by as much as three times that of unlimed sites, and increased the amount of seed

produced by as much as four times (Long et al. 1997). Our data show a similar relationship between remediation of Ca lost due to acid deposition (i.e., Ca addition designed to approach pre-depletion levels, rather than excessive liming) and flower and seed crops, with greater flowering occurring in trees growing on Ca-addition plots when compared with Al-addition or control plots. One would expect higher seed production to coincide with greater flowering, and indeed that was the case. In addition to greater overall seed production, Ca-addition resulted in increased numbers of filled seeds (Table 7). A recent study at HBEF found no differences in filled (likely viable) seeds associated with Ca addition (Cleavitt et al. 2011). However, the authors did not quantify flowering of sugar maples and, thus, comparisons between flower abundance and filled seeds could not be made. Regardless, it is clear that Ca addition in the NuPert plots greatly enhanced the flowering, seed production and number of filled seeds per plot (Table 7).

Seed germination rates in the laboratory were not statistically different among the treatments (Table 7). Thus, as long as seeds were filled, potential germination was unaffected by soil cation manipulation. Cleavitt et al. (2011) also observed only slight maternal effects on seed germination in a reciprocal seed transplant study between reference and Ca-treated sites at the HBEF. Their field emergence trials indicated much stronger elevation effects across the experimental watersheds at the HBEF rather than Ca treatment effects. In the present study when numbers of filled seeds and germination rate were combined to estimate the maximum number of germinants per unit area, Ca addition resulted in the greatest number of potential seedlings (Table 7). These data support previous findings at the HBEF that showed an increase in seedling density with Ca addition (Juice et al. 2006). The large differences between treatments in terms of flower and seed crops, and estimated germinants, suggest that Ca nutrition plays a key role in reproduction within native sugar maple forests, and that soil Ca depletion during the 20th century has greatly impaired the reproductive success of the species. Despite the pervasive influences of Ca addition alone, to our knowledge, these are the first data to indicate that both Ca and Al availability influence the reproductive success of sugar maple trees.

### Similarities between control and treated plots

The control plots at NuPert represent ambient levels of soil Ca depletion in the northern hardwood forest: the Ca-addition plots represent pre-pollution conditions; and the Al-addition plots represent an extreme level of soil acidification. As such, similarities between control and treated plots provide insight into trends in native forests relative to these extremes. Our data show that trees growing on control plots appeared to be resilient to frost injury and experience relatively low foliar oxidative stress (Tables 5 and 6). This suggests that sugar maple health is not seriously impaired with respect to these indicators

of stress under current conditions. However, trees on control plots contained levels of foliar Ca very close to those in Al-addition plots and devoted similar amounts of C to growth relative to storage (Tables 4 and 5), whereas these measures were strongly altered by remediation of soil Ca. Furthermore, flower and seed crops from control plots were similar to those on Al plots, whereas they were greatly increased on Ca-addition plots (Table 7). These results suggest that baseline Ca nutrition and C partitioning may be impaired relative to those estimated for sugar maples prior to acid-induced cation perturbations. The similarity between seed crops for control and Al-treated plots further emphasizes the possibility that native sugar maples are currently more stressed than generally perceived. Adequate Ca nutrition, C partitioning and reproductive capacity are important to sustaining sugar maple forests under static climatic conditions, and are likely to be of even greater significance as the climate changes.

### Conclusions

Our study tracked differences in sugar maple physiology from roots through crowns, and highlighted the differential response of tissues to long-term soil Ca and Al manipulation. In general, Ca addition increased Ca nutrition, the ratio of C allocation to growth : storage, flowering, total and filled seeds. In contrast, Al addition increased root Al concentrations with signs of coincident root cell membrane disturbance, increased APX and GR activity (indicators of oxidative stress), reduced foliar re-flush following frost injury and reduced the number of filled (viable) seeds. Trees on control plots that reflect ambient Ca depletion conditions mirrored trees on Ca-addition plots for some parameters assessed (e.g., resilience to freezing injury and foliar oxidative stress), but more closely resembled trees on Al-addition plots for other measurements (e.g., C partitioning, flowering and seed production). Similarities between control and Al-addition plots are particularly notable, because these suggest that some physiological impairment exists under ambient conditions. Although some increases in sugar maple biomass have been reported in recent years (van Doorn et al. 2011), trends among control and treatment plots in the current study show that Ca nutrition, and in some cases Al availability, continue to be important modulators of the health, productivity and reproduction of sugar maple trees in the northern hardwood forest.

### Acknowledgments

The authors are grateful to Paula Murakami, Christopher Hansen, Ali Kosiba, Ben Engel, Homer Elliott, William Young, Nick Huntington and Hannah Wahlstrom for their assistance in both the field and laboratory. We thank Drs Samuel St Clair, Kimberly Wallin, Shelly Rayback and Donald Ross for providing helpful feedback on an earlier version of this manuscript. We



further thank the staff at the Hubbard Brook Experimental Forest for their generous help in all aspects of our sample collections. This manuscript is a contribution of the Hubbard Brook Ecosystem Study. Hubbard Brook is part of the Long-Term Ecological Research (LTER) network, which is supported by the National Science Foundation. The Hubbard Brook Experimental Forest is operated and maintained by the USDA Forest Service, Northern Research Station, Newtown Square, PA, USA.

## Conflict of interest

None declared.

## Funding

This research was supported by funds provided by the Forest Service Northern Research Station, USDA CSREES McIntire-Stennis Forest Research Program, and the Northeastern States Research Cooperative.

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