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Elevation dependent sensitivity of northern hardwoods to Ca addition at Hubbard Brook Experimental Forest, NH, USA

Rakesh Minocha[a,*], Stephanie Long[a], Palaniswamy Thangavel[a,1], Subhash C. Minocha[b], Christopher Eagar[a], Charles T. Driscoll[c]

[a] Forest Service, U.S. Department of Agriculture, Northern Research Station, 271 Mast Road, Durham, NH 03824, USA
[b] Department of Biological Sciences, University of New Hampshire, Durham, NH 03824, USA
[c] Department of Civil and Environmental Engineering, Syracuse University, Syracuse, NY 13244, USA

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ABSTRACT

Acidic deposition has caused a depletion of calcium (Ca) in the northeastern forest soils. Wollastonite (Ca silicate) was added to watershed 1 (WS1) at the Hubbard Brook Experimental Forest (HBEF) in 1999 to evaluate its effects on various functions of the HBEF ecosystem. The effects of Ca addition on foliar soluble (extractable in 5% $HClO_4$) ions, chlorophyll, polyamines, and amino acids were studied in three hardwood species, namely sugar maple, yellow birch, and American beech. We further analyzed these effects in relation to elevation at Ca-supplemented WS1 and reference WS3 watersheds. Foliar soluble Ca increased significantly in all species at mid and high elevations at Ca-supplemented WS1. This was accompanied by increases in soluble P, chlorophyll, and two amino acids, glutamate and glycine. A decrease in known metabolic indicators of physiological stress (i.e., the amino acids, arginine and γ-aminobutyric acid (GABA), and the diamine, putrescine) was also observed. In general, these changes were species-specific and occurred in an elevation dependent manner. Despite an observed increase in Ca at high elevation for all three species, only sugar maple exhibited a decrease in foliar putrescine at this elevation indicating possible remediation from Ca deficiency. At higher elevations of the reference WS3 site, foliar concentrations of Ca and Mg, as well as Ca:Mn ratios were lower, whereas Al, putrescine, spermidine, and GABA were generally higher. Comparison of metabolic data from these three species reinforces the earlier findings that sugar maple is the most sensitive and American beech the least sensitive species to soil Ca limitation. Furthermore, there was an increase in sensitivity with an increase in elevation.

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1. Introduction

Calcium (Ca) plays a vital role in the growth and productivity of forest trees (Tomlinson, 2003; St. Clair et al., 2008). It modulates many cellular activities including ionic balance needed for stomatal closure, turgor pressure regulation, gene expression, carbohydrate metabolism, and cell division (Ma et al., 2005; Boudsocq and Sheen, 2010). Several studies have demonstrated that Ca and other essential cations are available in less-than-adequate quantities in various forest ecosystems in the United States (Huntington, 2005; Johnson et al., 2008; Warby et al., 2009). Over the past 4 decades, this trend has also been observed at the Hubbard Brook Experimental Forest (HBEF), a Long Term Ecological Research (LTER) site in New Hampshire, USA (Likens et al., 1998). The lowering of soil pH due to chronic N and S inputs (Aber et al., 1995; Minocha et al., 2000) and the mobilization of soil Al (Shortle and Smith, 1988; Minocha et al., 1997) are factors related to Ca depletion in these soils. Furthermore, it is well established that susceptibility of soils to acidification increases with increasing elevation (Lawrence, 2002; Bailey et al., 2004). The natural acidification processes are also more pronounced at higher elevations.

The aforementioned changes in pH and concentrations of Ca and Al add to environmental stress in the forest ecosystem which causes metabolic changes in trees that would ultimately lead to less than optimum growth. Trees have complex metabolic processes (biochemical reactions) that enable them to detect, respond to, and survive multiple simultaneous environmental stresses. The effects of multiple stresses on metabolism are often additive. Many metabolic pathways affected by stress are interconnected via one or more common signal transduction pathways that use Ca as a secondary messenger (Hetherington and Brownlee, 2004); these pathways often share common metabolic precursors (Minocha et

* Corresponding author. Tel.: +1 603 868 7622; fax: +1 603 868 7604.
E-mail addresses: rminocha@unh.edu, rminocha@fs.fed.us (R. Minocha).
[1] Present address: School of Environmental Science and Engineering, Sun Yat-Sen University, Guangzhou 510 275, PR China.

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al., 2004). Because of such biochemical interactions, changes in one metabolite will have pleiotropic effects on several other metabolites within the same and related pathways (Page et al., 2007; Mohapatra et al., 2009). Some of these metabolites (e.g., polyamines and amino acids), in combination with other cellular components (e.g., chlorophyll and total soluble proteins), have been used as indicators of environmental stress in trees before the morphological symptoms of stress are visible (Dohmen et al., 1990; Näsholm and Ericsson, 1990; Bauer et al., 2004). In addition, molar ratios of foliar total ions (e.g., Ca:Mn and Mg:Mn) have also been suggested as indicators of stress (Kogelmann and Sharpe, 2006).

Polyamines (PAs) are nitrogen (N)-rich compounds that are present in all living organisms and are essential for growth and development. Three common PAs in plants are putrescine (Put), spermidine (Spd) and spermine (Spm). Cellular contents of PAs, specifically Put, are indicative of the physiological response of forest trees to an array of environmental stress conditions including a shortage of soil available Ca, excess Al, and chronic N accumulation (Minocha et al., 1997, 2000; Wargo et al., 2002). It has been suggested that under conditions of stress, PAs also play roles in directly imparting stress tolerance in plants (e.g., through lowering NH_3 toxicity and scavenging free radicals). In addition, PAs act as signal molecules to regulate gene activity related to cellular N metabolism and the metabolism of several amino acids: proline (Pro), arginine (Arg), γ-aminobutyric acid (GABA) and glutamic acid (Glu), all of which play important roles in plant responses to higher N exposure (Näsholm and Ericsson, 1990; Bauer et al., 2004; Bouché and Fromm, 2004).

Application of Ca fertilizer in plot-level studies in sugar maple stands suffering from a deficiency in soil available Ca improved growth and vigor and reduced cellular concentrations of Put (Ouimet and Fortin, 1992; Long et al., 1997; Moore et al., 2000; Wargo et al., 2002; Ouimet et al., 2008). In the present watershed-level experiment, wollastonite (Ca silicate; a slow-release Ca source) was applied to watershed 1 (WS1) at the HBEF in 1999. Since this Ca application, WS1 has been studied extensively to evaluate changes in soil solution, stream water and foliar chemistry, Ca and Sr uptake into tree foliage, and N mineralization and cycling in the soil (Dasch et al., 2006; Groffman et al., 2006; Cho et al., 2010). An increase in tolerance to cold was shown to be accompanied by higher foliar Ca concentrations in red spruce (*Picea rubens*) growing on the ridge top of Ca-supplemented WS1 as compared to the ones growing at ridge top of untreated watershed WS6 (Hawley et al., 2006; Halman et al., 2008). Calcium addition at WS1 at the HBEF was also associated with enhanced foliar chlorophyll and growth and survival of sugar maple seedlings compared to reference WS6 (Juice et al., 2006). The present study complements earlier work by providing an assessment of the effects of soil Ca addition on forest health as indicated by changes in stress-related organic marker metabolites and inorganic nutrition in the foliage of mature trees. We examined these factors in three hardwood species further extending the analysis to three different elevations in a paired watershed experiment using Ca-treated WS1 and reference WS3.

2. Materials and methods

2.1. Site description

The HBEF is a deciduous second-growth forest located within the boundaries of the White Mountain National Forest, West Thornton, NH, USA. Climate, hydrology, topography, and vegetation of this site are described in Juice et al. (2006). American beech (*Fagus grandifolia* Ehrh.), sugar maple (*Acer saccharum* Marsh.), yellow birch (*Betula alleghaniensis* Britt.), and paper birch (*Betula papyrifera* Marsh.) are the dominant hardwood species at this site; red spruce (*P. rubens* Sarg.) and balsam fir (*Abies balsamea* (L.) Mill.) are dominant conifer species at the ridge top. Soils at HBEF are formed from glacial till and are moderately well drained, Spodosols (Haplorthods) of sandy-loam to loamy-sand texture. The pH of the thick organic horizon (average 6.9 cm) that overlies bouldery mineral soil ranges from 3.4 to 3.8. Continuous snowpack normally develops early each winter to a depth of about 1.5 m.

Of the nine gauged watersheds at HBEF (Fig. 1), the present study was conducted on Ca-supplemented WS1 (Area: 11.8 ha, Slope: 18.6°, Aspect: S22°E and Elevation: 488–747 m) and along the eastern edge of reference WS3 (Area: 42.4 ha, Slope: 12.1°, Aspect: S23°W and Elevation: 527–732 m). These watersheds are in close proximity (less than 1.5 km apart) with similar geology, temperature, soils, and overall climate (Fig. 1).

Fifty-five tons of powdered and pelletized wollastonite ($CaSiO_3$ – 1.2 mg ha^{-1} of Ca) was applied by helicopter to WS1 in October 1999, soon after leaf fall (Peters et al., 2004). This application rate was intended to increase the base saturation of the soil from 10% to approximately 19%, and therefore, increase soil pH to estimated levels of 50 years ago (www.hubbardbrook.org). At WS1, no other treatments have been applied since 1956. Watershed WS3 has been the hydrological reference since 1957 and not subjected to any experimental treatments.

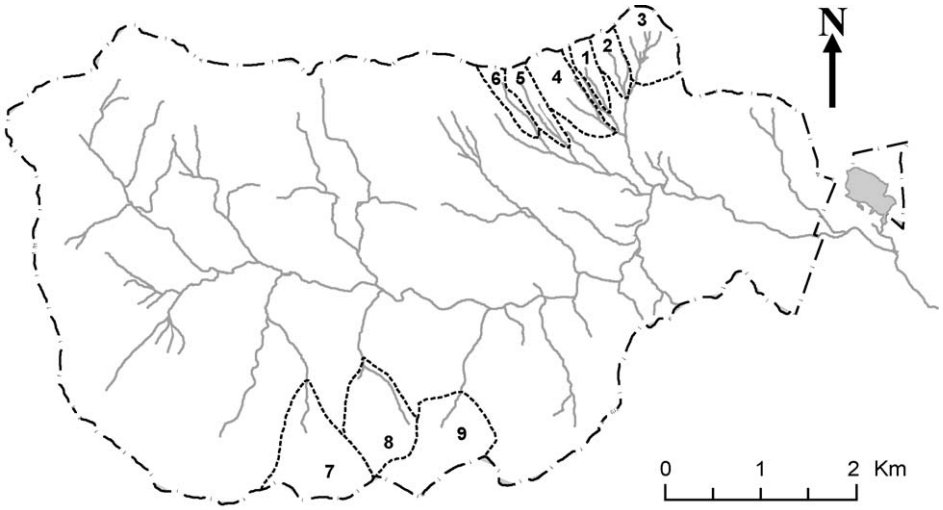
Pre-treatment Ca concentrations in stream flows from WS1 and WS3 were similar at 0.87 ± 0.01 and 1.0 ± 0.01 mg^{-L}, respectively (data averaged from 1993–1999 for each WS). After the addition of wollastonite, stream flow Ca concentrations from WS1 rose to 1.47 ± 0.05 mg^{-L} while for WS3 Ca concentrations in stream flow declined slightly to 0.88 ± 0.01 mg^{-L} (data averaged from 1999–2005 for each watershed). In addition, chemical analyses of mid elevation soil samples in 2006 from both watersheds showed up to a five-fold increase in soil Ca at WS1 (R. Minocha et al., unpublished data).

2.2. Foliar sampling

Foliage samples were collected from three elevation zones (a proxy for changing soil chemistry, landscape, and climate) designated as low (500–550 m), mid (550–650 m), and high (650–740 m) at both WS1 and WS3. Samples were collected in mid-July/early August in 2004 and 2005 from mid to upper tree canopies using shotguns to drop small branches. Visually healthy leaves were collected from a population of dominant or co-dominant 80–90-year-old trees. With few exceptions, the same trees were sampled for each of the two years of this study. The number of samples collected at Ca-supplemented WS1 was: maple = 60 (10 trees, 3 elevations, and 2 years), birch = 40 (5 trees at low, 10 at mid, and 5 at high elevation, and 2 years) and beech = 40 samples (10 trees each at mid and high elevations, and 2 years; no beech trees were sampled at low elevation). At reference WS3, 30 samples each of maple, birch, and beech (5 trees, 3 elevations, and 2 years) were collected. Whereas foliar samples have been collected each year since 1999 to evaluate the effects of Ca addition on total inorganic ion analyses at WS1, during 2004–2005, samples were collected at both WS1 and WS3 for comparison of foliar soluble ($HClO_4$ extractable) inorganic ions and stress-related metabolites.

2.2.1. Sample processing

For analysis of total inorganic ion content, 20–60 leaves per tree were placed in paper bags for transport to the laboratory. For soluble (5% $HClO_4$ extractable) inorganic ions and other biochemical analyses, a pool of leaf disks (6.35 mm diameter) was collected from 2 to 4 leaves from each sample using a paper punch, avoiding main veins. From this pool, two sub-samples were collected: one (approximately 200 mg FW) was placed in a pre-weighed



WS	Area (ha)	Slope* (°)	Aspect	Elevation (m)	Initial Year	Treatment
1	11.8	18.6	S22°E	488-747	1956	$CaSiO_3$ in 1999
3	42.4	12.1	S23°W	527-732	1957	None

* Slope measurements are the slope of a plane fitted to the circumference.

Fig. 1. Map of the Hubbard Brook Experimental Forest (HBEF) showing the location of Ca-supplemented WS1 and reference WS3.

microfuge tube containing 1.0 mL of 5% $HClO_4$, and the other was placed in an empty microfuge tube. All samples were transported to the laboratory on ice and stored at −20 °C until further analysis. Before analyses, the samples in $HClO_4$ were weighed, frozen and thawed (3×) to break cell membranes and to release all soluble ions and metabolites, and centrifuged at 13,000 × *g* for 10 min. The supernatant was used for analysis of $HClO_4$-extractable (free) PAs, amino acids, and soluble inorganic ions (Minocha et al., 1994). The other set of samples was used for analysis of soluble proteins and total chlorophyll. Samples from each tree were analyzed individually without pooling for PAs, amino acids, inorganic ions, chlorophyll, and soluble proteins.

2.3. Analyses of foliar total and soluble inorganic ions

Total and soluble inorganic ions were quantified using a simultaneous axial inductively coupled plasma emission spectrophotometer (Vista CCD, Varian, Palo Alto, CA, USA) using Vista Pro software (Version 4.0). For total inorganic ions, leaves were oven-dried at 70 °C, ground in a Wiley mill to pass a 0.85 mm sieve (#20 mesh), and digested by heating with H_2SO_4, H_2O_2, and H_2SeO_3 using a digestion block (Issac and Johnson, 1976). Eastern white pine needles (SRM 1575A), (SRM 1515), and peach (SRM 1547) leaf samples from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) were analyzed in duplicate for procedure verification. Tissue standards were within 5% of the certified values. Foliar total N was analyzed according to QuikChem Method 13-107-06-2-G, "Determination of Total Kjeldahl Nitrogen in Plant Digest by Flow Injection Analysis Colorimetry," (Block Digestion Method), Revision Date: 6/07/2002.

2.4. Biochemical analyses

The supernatant from $HClO_4$-extracted samples was subjected to dansylation for quantitation of PAs and amino acids according to Minocha and Long (2004) with a minor modification in that the reaction was terminated using 50 μL of L-asparagine (20 mg mL^{-1} in water). Since the HPLC column could not always separate the peaks of Arg and threonine (Thr), the areas for these two amino acids were pooled for each standard to derive a combined calibration curve for their quantitation.

For soluble proteins, 50 mg of leaf disks were placed in 0.5 mL of extraction buffer [100 mM Tris–HCl, pH 8.0, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA, and 10% (v/v) glycerol], frozen and thawed three times, and the supernatant used for protein analysis according to Bradford (1976). For analysis of chlorophyll, two leaf disks were placed in 1 mL of 95% ethanol and incubated in the dark at 65 °C for 16 h (Minocha et al., 2009). Following centrifugation (13,000 × *g* for 5 min), the supernatant was scanned for absorbance (350–710 nm) in a Hitachi U-2010 (Hitachi Ltd., Tokyo, Japan) spectrophotometer equipped with Hitachi UV Solutions 2.0 software.

2.5. Statistical analyses

Two types of statistical comparisons were made: (1) between the treatments at watershed level, i.e., Ca-supplemented WS1 vs. reference WS3, and (2) post-treatment vs. pre-treatment at WS1. The data for each dependent parameter were subjected to analysis using repeated measures ANOVA with two main factors: treatment with two levels (reference and Ca-supplemented) and elevations with three levels (low, mid, and high) and one repeated measures factor for year of sampling (2004 and 2005). If the *F* values were significant for interaction between treatment and elevation, the data were not used further for watershed level comparisons. Since several dependent variables in the data showed significant interaction between treatment and elevation ($\alpha \leq 0.05$), we conducted individual analyses for each elevation using repeated measures ANOVA with treatment (two levels, reference and Ca-supplemented) as the main factor and one repeated measures factor for year of sampling (two levels, 2004 and 2005). Each sample was analyzed individ-

Table 1
Comparison of pre- and post-Ca-addition total ions in the foliage of trees growing at Ca-supplemented WS1 of the HBEF. SM = Sugar maple; YB = Yellow birch; AB = American beech. $N = 60$ for SM (10 trees, 3 elevations, 2 years), $N = 40$ for YB (5 trees at low, 10 at mid, and 5 at high elevation for 2 years) and $N = 40$ for AB (10 trees each at mid and high elevations and 2 years). No collections for AB were made at low elevation. The 1999 data for each tree were compared against the mean data for the corresponding trees in 2004 and 2005 (repeat measure). Soluble ions (for 2004 and 2005) are given as a % of total ions. Mean percent moisture content was calculated only from 2000 to 2001 data from the same trees and applied to soluble ions data for 2004 to 2005 for conversion from fresh weight to dry weight. Data are mean ± SE. Total Al data have been multiplied by 10 for the ease of presentation.

Total nitrogen (%) and total ions (g kg^{-1} DW)							Soluble ions as % of total ions	Corresponding soluble ions with total ions
Species and ions	Pre-Ca (1999)	Post-Ca (04–05)	Pre-Ca (1999)	Post-Ca (04–05)	Pre-Ca (1999)	Post-Ca (04–05)		
	Low elevation		Mid elevation		High elevation			
SM								
N	2.01 ± 0.05	1.91 ± 0.03*	1.98 ± 0.05	1.89 ± 0.03	2.19 ± 0.08	↓1.85 ± 0.06*		
Ca	5.38 ± 0.37	↑7.57 ± 0.39*	5.45 ± 0.44	↑8.99 ± 0.53*	5.50 ± 0.48	↑8.77 ± 0.92*	53.9 ± 1.2	0.828†
K	8.40 ± 0.29	8.93 ± 0.29	8.31 ± 0.49	↓6.94 ± 0.33*	8.45 ± 0.46	↓7.23 ± 0.23	55.0 ± 1.4	0.361†
Mg	0.95 ± 0.07	1.02 ± 0.06	0.93 ± 0.05	0.86 ± 0.05	1.05 ± 0.12	↓0.68 ± 0.04*	62.7 ± 1.7	0.837†
Mn	1.11 ± 0.08	1.05 ± 0.07	1.22 ± 0.12	1.20 ± 0.06	2.54 ± 0.26	↓1.40 ± 0.05*	62.9 ± 1.5	0.818†
P	1.07 ± 0.03	1.09 ± 0.02	1.13 ± 0.03	↓1.02 ± 0.04*	1.83 ± 0.10	↓1.43 ± 0.11*	48.2 ± 1.9	0.776†
Al × 10	0.36 ± 0.03	↓0.13 ± 0.01*	0.33 ± 0.05	↓0.16 ± 0 02*	0.61 ± 0.04	↓0.13 ± 0.00*	54.0 ± 3.6	0.268†
YB								
N	2.36 ± 0.06	2.43 ± 0.10	2.41 ± 0.07	2.32 ± 0.04	2.61 ± 0.10	↓2.31 ± 0.06*		
Ca	8.51 ± 0.26	7.43 ± 2.41	7.62 ± 0.80	↑11.08 ± 0.51*	6.05 ± 0.29	↑8.93 ± 1.26*	53.5 ± 2.3	0.592†
K	11.89 ± 0.85	6.68 ± 2.11	9.58 ± 1.27	9.04 ± 0.58	7.50 ± 1.02	6.69 ± 1.05	52.6 ± 1.8	0.808†
Mg	1.91 ± 0.02	↓1.01 ± 0.34	1.48 ± 0.11	↑1.76 ± 0.05*	1.98 ± 0.26	1.15 ± 0.32	66.4 ± 2.0	0.801†
Mn	1.97 ± 0.15	↓0.52 ± 0.17*	1.65 ± 0.20	↓1.18 ± 0.07*	3.22 ± 0.61	↓1.32 ± 0.24*	61.5 ± 2.0	0.928†
P	1.03 ± 0.02	↑1.55 ± 0.17*	1.24 ± 0.03	1.26 ± 0.03	1.93 ± 0.10	↓1.53 ± 0.19*	48.6 ± 1.6	0.751†
Al × 10	0.28 ± 0.04	↓0.07 ± 0.04*	0.34 ± 0.05	↓0.17 ± 0.06	0.31 ± 0.05	↓0.16 ± 0.04*	45.5 ± 6.0	−0.013
AB								
N	NA	NA	2.27 ± 0.07	2.18 ± 0.02	2.18 ± 0.10	2.12 ± 0.03		
Ca	NA	NA	4.86 ± 0.43	↑6.65 ± 0.42*	4.69 ± 0.20	↑6.99 ± 0.54*	58.6 ± 1.8	0.660†
K	NA	NA	7.34 ± 0.32	↓6.03 ± 0.42	5.87 ± 0.42	6.50 ± 0.27	57.1 ± 2.0	0.428†
Mg	NA	NA	1.18 ± 0.14	1.39 ± 0.11	0.81 ± 0.04	↑0.92 ± 0.06*	63.4 ± 1.5	0.857†
Mn	NA	NA	0.82 ± 0.08	0.74 ± 0.03	2.55 ± 0.19	↓1.38 ± 0.10*	64.9 ± 1.6	0.937†
P	NA	NA	1.26 ± 0.07	1.16 ± 0.07	1.85 ± 0.25	↓1.40 ± 0.08*	54.6 ± 1.7	0.750†
Al × 10	NA	NA	0.25 ± 0.04	0.15 ± 0.03	0.32 ± 0.06	0.23 ± 0.07	50.7 ± 11.3	−0.294†

NA = data not available.
↑ or ↓ arrows indicate significant increase or decrease relative to its pre-treatment concentration.
* ($P \leq 0.05$) indicates pre- and post-treatment differences.
† Denotes $P \leq 0.05$ for significance of correlation.

ually for each metabolite and the two data points per year per tree were averaged for statistical significances using repeated measures analyses. Pearson correlation coefficients were used to assess the significance of relationship between various parameters studied. Analyses were done using SYSTAT Version 10.2 for Windows (SYSTAT, Richmond, CA, USA) and Microsoft Excel (Version 2003); $P \leq 0.01$ (**) or $P \leq 0.05$ (*) indicate significant differences unless specified otherwise. Low elevation data were compared with mid or high elevation data and are shown as $P \leq 0.01$ (††) and $P \leq 0.05$ (†). Since there was significant interaction between treatment and elevation at WS1 at $P \leq 0.05$, effects of elevation by itself on foliar stress-related metabolites were evaluated using the data from WS3.

2.6. Justification of study design

Replication and scale are important elements to consider in designing an experiment for ecological research (Binkley, 2008). Whereas, a small well-replicated study design easily meets statistical requirements, a watershed-level study (although hard to replicate) provides a larger scale, which is often needed to ask relevant questions under a specific set of conditions (Hurlbert, 1984; Oksanen, 2001; Cottenie and De Meester, 2003). The present study was conducted as a follow up to our earlier small-scale well replicated plot level studies (Minocha et al., 2000; Wargo et al., 2002; Minocha et al., unpublished data). Using large-size paired watersheds, we hypothesized that Ca addition to Ca-depleted soil would not only have species-specific effects, but also the tree responses would be influenced by environmental factors associated with elevation changes. Even though a true evaluation of the effects of soil Ca addition on forest health would require replication of this experiment at more than one location, replicating such a large-scale Ca fertilization within the same timeframe would not be feasible for many reasons. The HBEF spans a range of topographic factors (elevation and slope position) covering varying levels of soil development and growth conditions. Thus, the sampling of 180 trees each from three different species growing at three different elevations of WS1 and WS3 should provide a robust evaluation of the response to Ca addition by elevation at this site. Individual trees were used as a unit of replication in this study since genetic variability among trees of the same species and the micro site variations in the soil are major factors potentially responsible for differences in physiological responses of individual trees to Ca addition. Benowicz et al. (2001), for example, showed that genetic differences among populations of paper birch in British Columbia, Canada, accounted for a significant portion of variability among the study groups in terms of germination parameters, frost hardiness, biomass, etc.

3. Results

3.1. Comparison of foliar total inorganic ions by elevation (pre- and post-Ca addition)

A comparison of total ions in the foliage between pre-treatment (1999) and post-treatment (2004–2005) samples from WS1 showed significantly higher Ca in post-treatment maple at all elevations and in beech and birch at mid and high elevations

Table 2
Ca:Mn and Mg:Mn molar ratios of total ions by elevation. Ratios were calculated using pre-treatment and post-treatment total ions from Ca-supplemented WS1. Sample collection protocol is described in Table 1. ANOVA was used to compare the ratios of totals of pre-treatment (1999) against post treatment (mean of 2004 and 2005 for each individual year, repeat measure) data for WS1. The numbers in bold indicate post-Ca-addition molar ratios (total) at the low elevation (mid for beech) that were considered as benchmark for the relative recovery from Ca depletion at higher elevations. Data are mean ± SE.

	Total ions molar ratio		Total ions molar ratio	
Species and elevation	Pre-Ca-treat Ca:Mn	Post-Ca-treat Ca:Mn	Pre-Ca-treat Mg:Mn	Post-Ca-treat Mg:Mn
SM				
Low	5.08 ± 0.53	**7.48 ± 0.61***	0.91 ± 0.10	**1.01 ± 0.09**
Mid	4.67 ± 0.30	7.58 ± 0.44*	0.84 ± 0.09	0.74 ± 0.06
High	2.33 ± 0.24	6.20 ± 0.51*	0.44 ± 0.05	0.49 ± 0.03
YB				
Low	4.39 ± 0.25	**11.59 ± 3.47**	0.99 ± 0.06	**1.56 ± 0.46**
Mid	4.74 ± 0.23	9.56 ± 0.45*	0.99 ± 0.10	1.54 ± 0.09*
High	2.10 ± 0.30	7.09 ± 0.59*	0.70 ± 0.15	1.18 ± 0.14
AB				
Mid	6.04 ± 0.49	**9.04 ± 0.63***	1.45 ± 0.16	**1.87 ± 0.12***
High	1.89 ± 0.09	5.38 ± 0.63*	0.34 ± 0.04	0.70 ± 0.06*

* ($P \leq 0.05$) indicates significant differences.

(Table 1). Lower total Mg was evident at high elevation in maple; however, birch at mid and beech at high elevations had higher Mg. Total Mn decreased by ≃50% at high elevation in all three species; in birch, this decrease was seen at low and mid elevations as well. Total P decreased in response to Ca treatment in the foliage of all three species growing at high elevation. Whereas, total P also decreased at mid elevation in maple, in birch it increased at low elevation. Foliar Al accumulation was significantly lower after Ca-addition at all three elevations in maple and at low and high elevations in birch, with no significant change in beech. Total N (%) in the leaves did not change in most cases with Ca addition; the only exception was a significant decrease in maple and birch at high elevation. Depending on the species and the ion, 5% $HClO_4$-soluble ions were 45–66% of the total ions (Table 1). The percent $HClO_4$-soluble ions did not vary when sorted by elevation (data not shown).

3.1.1. Molar ratios of foliar total Ca:Mn and Mg:Mn

A comparison of molar ratios of total Ca:Mn at each elevation based on pre- and post-treatment total ions (Table 2) showed that Ca addition caused a significant increase in Ca:Mn ratio in all species at all elevations. The numbers in bold (Table 2) indicate post-Ca-addition molar ratios at the low (mid for beech) elevation which were considered as benchmarks for the relative recovery at higher elevations. Even though the highest percent increase in Ca:Mn ratios was observed at high elevation, the absolute values for mid elevation ratios were higher and equaled those at low elevation. Molar ratios of total Mg:Mn did not change in maple but increased in birch at mid and beech at mid and high elevations.

3.2. Comparison of WS1 and WS3 foliar soluble inorganic ions by elevation

At reference WS3 site, soluble foliar Ca declined in birch and beech with increasing elevation but was present in similar amounts in maple at low and high elevations; the lowest amount was seen at mid elevation (Fig. 2A). Significantly higher soluble Ca was found in the leaves of all three species at WS1 at higher elevations in response to the wollastonite treatment; the magnitude of increase was highest for maple (Fig. 2A). The extent of this increase in soluble Ca did vary by species and elevation. While a significant increase in Ca was observed at all elevations in maple, birch and beech exhibited significant increases only at mid and high elevations.

Lower amounts of soluble Mg were seen in the leaves of all species at higher elevations at reference WS3 (Fig. 2B). The addition of Ca to the soil caused no significant change in soluble Mg content in the foliage of all three species (Fig. 2B).

At reference WS3, a significantly higher concentration of soluble Mn was seen at higher elevation as compared to low elevation for all species (Fig. 2C). Higher concentrations of Mn were observed at mid elevation for all three species at Ca-supplemented WS1 compared to WS3. However, in birch at high elevation, soluble Mn was significantly lower at the WS1 after Ca addition (Fig. 2C).

The concentrations of soluble P were higher at high elevation as compared to low elevation for all species at reference WS3 (Fig. 2D). Significantly higher concentrations of soluble P were seen in maple and beech ($P \leq 0.06$) trees at mid elevation at supplemented WS1, but no change was observed in soluble P in birch (Fig. 2D).

At reference WS3, significantly higher concentrations of soluble Al were observed at mid elevation only in maple (Fig. 2E). A significant decrease in Al was seen in maple leaves at all elevations and in beech leaves at mid and high elevations in samples collected from Ca-supplemented WS1 (Fig. 2E). There were no treatment-related differences observed in birch leaves.

3.3. Foliar polyamines

Free ($HClO_4$-soluble) Put was the dominant PA in the foliage of all three species (Fig. 3A). On a fresh weight basis, maple leaves had the lowest amount of both Put (Fig. 3A) and Spd (Fig. 3B) as compared to birch and beech. At reference WS3, foliar Put was lower in maple and birch at low elevation as compared to mid elevation (Fig. 3A); neither the elevation nor the Ca treatment-related changes in Put were observed in beech.

Foliar Put was significantly lower in maple at Ca-supplemented WS1 at mid and high elevations compared to Put concentrations at the same elevation at reference WS3. At low elevation, however, no Ca effect was seen in any species. Birch at mid elevation had a significant decrease in Put in response to Ca-addition (Fig. 3A).

Like Put, lower concentrations of foliar Spd were observed in maple and birch at low elevation as compared to high elevation at WS3; there was no effect of elevation on Spd in beech leaves (Fig. 3B). Calcium addition had no significant effect on Spd in any species at any elevation (Fig. 3B).

3.4. Total chlorophyll and soluble proteins

At reference WS3, chlorophyll content did not vary much with elevation in maple, birch, or beech leaves. Total chlorophyll in the leaves of maple, beech and birch ($P \leq 0.06$) was significantly higher for trees at Ca-supplemented WS1 compared with reference WS3;

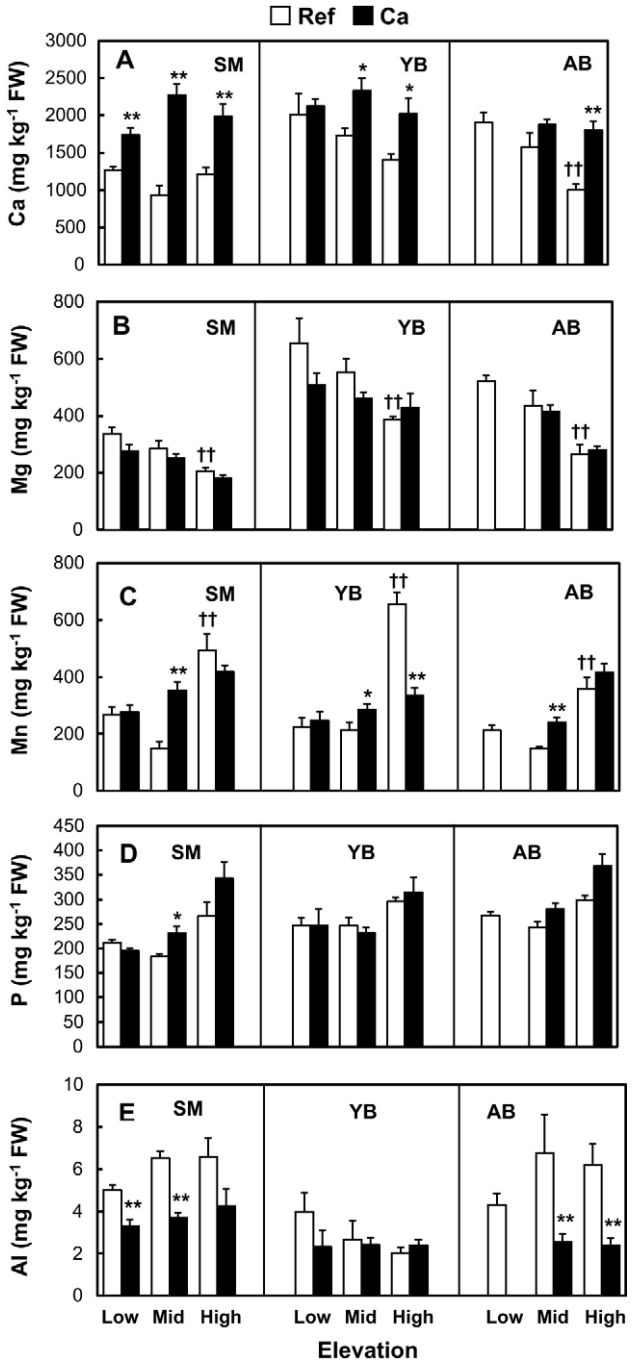


Fig. 2. Concentrations of soluble ions: Ca (A), Mg (B), Mn (C), P (D) and Al (E) in the foliage of trees growing at different elevations at WS3 and WS1 at HBEF. SM = Sugar maple; YB = Yellow birch; AB = American beech. The number of samples collected from WS3 was: 30 for each species (5 trees, 3 elevations for 2 years). For WS1 numbers, refer to Table 1. With a few exceptions, the same trees were sampled in 2004 and 2005 at both watersheds. Therefore, repeated measures ANOVA was used to evaluate significant differences in treatments at each elevation. Data are mean ± SE. ** ($P \leq 0.01$) and * ($P \leq 0.05$) indicate treatment differences. Elevation-related differences were compared only for reference site since there was a significant interaction observed between treatment and elevation at Ca-supplemented WS1. Symbol FW in the *Y*-axis legend stands for fresh weight. †† ($P \leq 0.01$) and † ($P \leq 0.05$) indicate differences of low elevation from mid and high elevation.

the response was different in each species depending upon the elevation (Fig. 4A).

At reference WS3, soluble protein content in the foliage was not affected by elevation in any species; however, the leaves of different species had different amounts of protein per gram FW, e.g.,

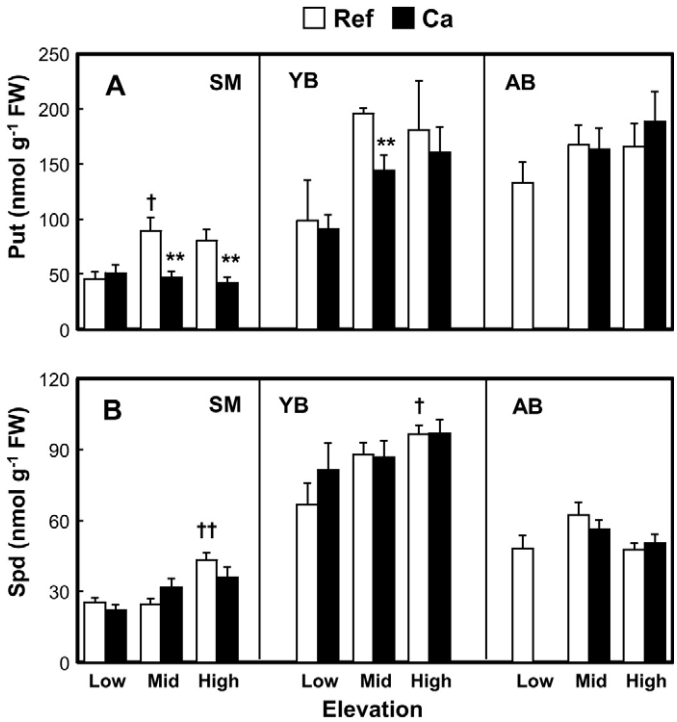


Fig. 3. Concentrations of free putrescine (A) and spermidine (B) in the foliage of trees growing at different elevations at reference WS3 and Ca-supplemented WS1 at HBEF. See Fig. 2 legend for more details.

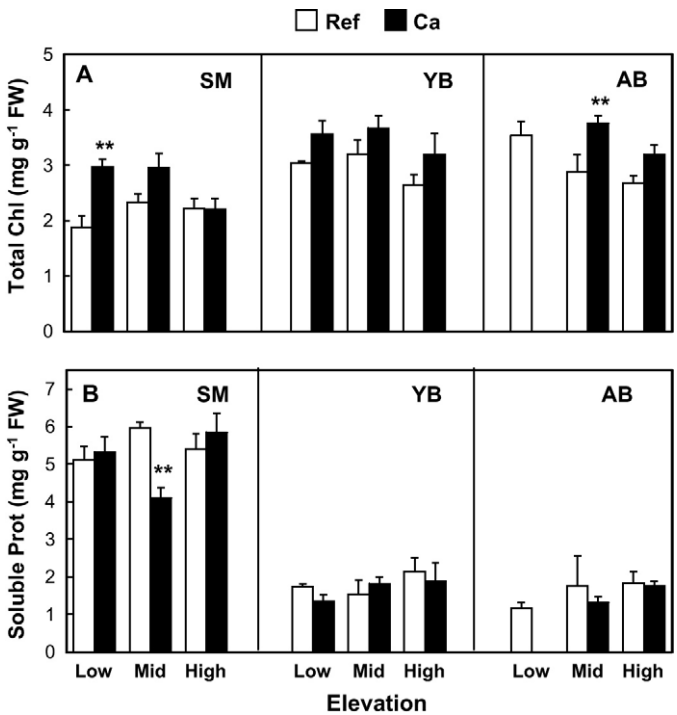


Fig. 4. Concentrations of total chlorophyll (A) and soluble proteins (B) in the foliage of trees growing at different elevations at reference WS3 and Ca-supplemented WS1 at HBEF. See Fig. 2 legend for more details.

maple leaves had almost twice as much soluble protein as birch and beech (Fig. 4B). The only significant difference observed in soluble proteins in response to wollastonite treatment was a decrease in protein content in maple leaves at mid elevation (Fig. 4B).

3.5. Foliar amino acids

Of the twenty-two amino acids separated in our HPLC system, only the ones known to be related with stress (Glu, Gly, Arg + Thr, and GABA) showed consistent and significant differences between the reference and the Ca-supplemented sites in 2004 and 2005 (Fig. 5A–D). The concentrations of all amino acids varied among species with each having a different dominant amino acid (detailed data for other amino acids not shown).

Glu, which was the predominant amino acid in birch, did not vary by elevation at reference site WS3 in any species. At Ca-supplemented WS1, the Glu content was significantly higher in the foliage of maple at low and mid elevation and birch at low elevation ($P \leq 0.06$) as compared to reference WS3 (Fig. 5A).

Foliar Arg + Thr concentrations were lower at high elevation compared to low elevation in maple at the reference site and did not change in response to Ca treatment at any elevation (Fig. 5B). In contrast, a decrease in Arg + Thr was seen in response to Ca treatment in beech; there was little variation due to elevation at WS3 (Fig. 5B).

At reference WS3, Gly did not vary with elevation in any species; however, its response to Ca treatment was both species and elevation-dependent. It was higher in maple at low and mid elevations and in beech at high elevation at Ca-supplemented WS1 in comparison with reference WS3 (Fig. 5C).

At WS3, GABA concentrations were significantly higher at mid elevation in both birch and beech. Except for a significant decrease in GABA in beech at mid elevation, no major difference was observed in this amino acid between the two watersheds (Fig. 5D). The concentration of GABA in beech was approximately three-fold higher than the two other species. Contents of other free amino acids either did not show significant differences between the two watersheds or were too low to be accurately quantified.

4. Discussion

It is well known that mid and high elevation watersheds are more prone to soil Ca depletion due to acidic deposition and Ca leaching either down slope or into deeper soil layers (Bailey et al., 2004). The high elevation trees sampled in the present study were growing at the upper limit of northern hardwood distribution in the White Mountains of New Hampshire, USA. Soil characteristics at HBEF change significantly within a 240-meter elevation span (Likens et al., 1998). The soil Ca concentrations available to plants were lower at higher elevations as reflected by the presence of lower foliar Ca in trees. This was also accompanied by lower Mg and higher Mn; all these factors contribute to stress at high elevation. However, the extent of stress differs with species and depends on a combination of species-specific factors such as threshold Ca concentrations needed for growth (Joslin and Wolfe, 1994; Hallett et al., 2006) and root uptake efficiency (Högberg et al., 1998). This stress is often reflected in the cellular concentrations of Put and/or other related amino acids (Minocha et al., 2000; Alcázar et al., 2006).

Watershed WS1 has been investigated extensively with respect to the effectiveness of wollastonite treatment on the restoration of Ca in the soil and the foliage of trees, on growth of sugar maple seedlings and mature trees, and on tolerance of red spruce to winter injury (Groffman et al., 2006; Hawley et al., 2006; Juice et al., 2006; Halman et al., 2008; Cho et al., 2010). Studies to date lead to the general conclusion that this treatment was quite effective in the overall improvement of forest health at this site. The results of the present study, while supporting this conclusion, provide further refinement of the analysis with respect to species specificity and the role of elevation (a proxy for changing soil chemistry, landscape, and climate) in the response of trees to Ca supplementation.

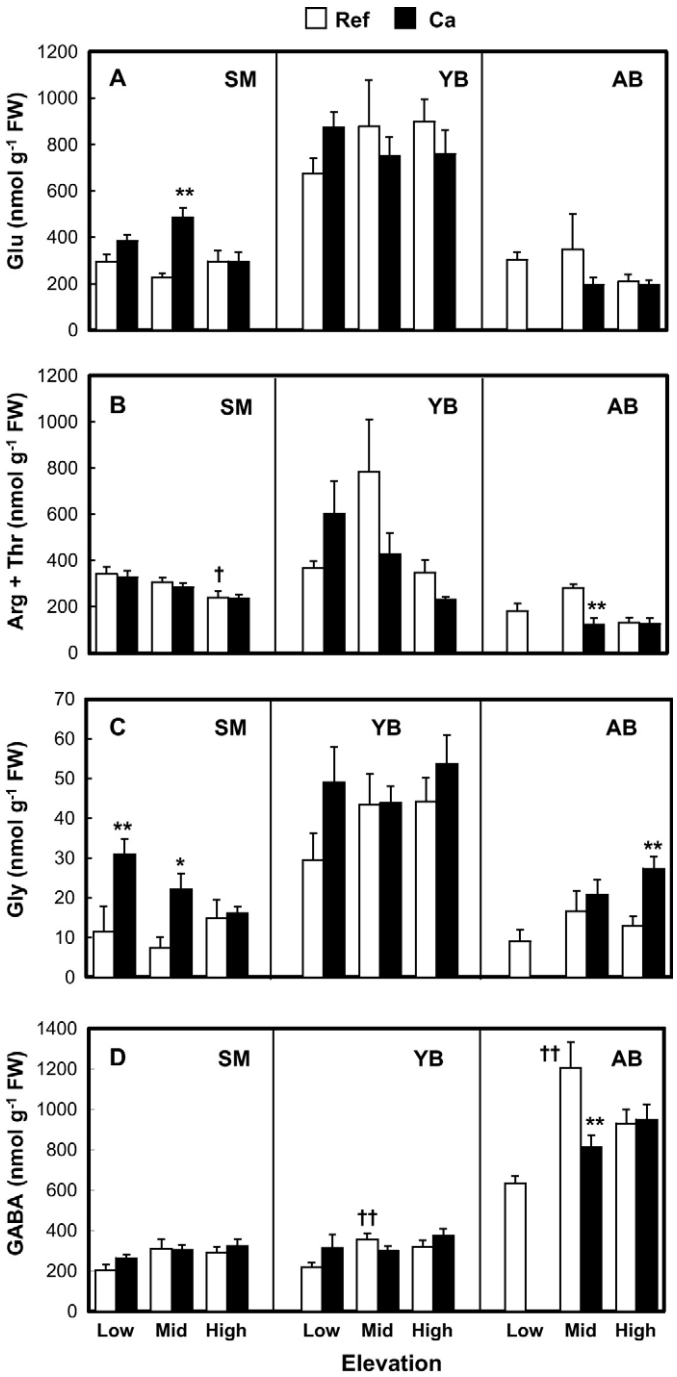


Fig. 5. Concentrations of free amino acids: glutamic acid (A), arginine + threonine (B), glycine (C), and γ-aminobutyric acid (D) in the foliage of trees growing at different elevations at reference WS3 and Ca-supplemented WS1 at HBEF. See Fig. 2 legend for more details.

4.1. Wollastonite treatment changed foliar chemistry

Calcium plays a key role in the physiological responses of cells to a changing environment. Its measured content in living tissues can vary depending upon the extraction protocol used. The fraction

of Ca that is easily extracted in water or dilute acid is referred to as "soluble" or "labile" Ca; this is presumably the most important physiological form of Ca and is considered to be a better indicator of soil Ca availability as compared to total foliar Ca under the same conditions (DeHayes et al., 1997; Wargo et al., 2002; Borer et al., 2004). In the present study, the foliar soluble Ca content is defined as the 5% $HClO_4$-extractable fraction of total Ca.

The increases seen in foliar soluble Ca at WS1 compared to WS3 (Fig. 2A) as well as in total Ca in 2004–2005 as compared to pre-treatment Ca levels in 1999 at WS1 (Table 1) are consistent with earlier reports on the effectiveness of wollastonite in enhancing the soil Ca available to plants. The magnitude of increase in soluble foliar Ca was greatest in maple, a species purported to be the most sensitive to low Ca (Bailey et al., 2004). The positive physiological effects of Ca addition were further reflected in the foliage of maple at mid and high elevation (where Ca deficiency is most likely) by lower cellular Put, which often shows an inverse relationship with soil Ca under conditions of Ca deficiency (Long et al., 1997; Minocha et al., 1997, 2000; Wargo et al., 2002). This was accompanied by an increase in soil solution pH and Ca along with a decrease in Al at high elevation during 2002–2003 as compared to 1999 (Cho et al., 2010). Organic and mineral soils collected in 2006 from mid elevation also showed significantly higher Ca at WS1 as compared to WS3 (data not shown here). An increase in soil P was also observed in the organic horizon at mid elevation of Ca-supplemented WS1 compared to the reference WS3 (Minocha et al., unpublished data).

Despite the observed decrease in the total foliar concentrations of Mn and P, the corresponding soluble fractions of these ions increased in maple and beech leaves at the Ca-supplemented watershed. This suggests an intracellular reallocation of total ions between soluble and bound fractions after their uptake from soil; P and Mn are known to be distributed to different sub-cellular compartments (e.g., plastids and vacuoles) in the cell (Versaw and Harrison, 2002; Demirevska-Kepova et al., 2004).

Maple foliage obtained from a glaciated (healthy) site had ratios of >50 for total Ca:Mn and >9 for total Mg:Mn; the corresponding values for plants from an un-glaciated site (with declining growth) were <2.3 for Ca:Mn and <0.7 for Mg:Mn (Kogelmann and Sharpe, 2006). Based on this information, pre-treatment total Ca:Mn ratios of about 2 in the foliage of all three species at high elevation in our study (Table 2) indicate poor health of these trees. LaeBot et al. (1990) had earlier suggested that foliar total Mg:Mn molar ratio of <0.7 in plants could be considered as an indicator of poor health. We chose the post-treatment molar ratios at low (mid for beech) elevation (supposedly less stressed compared to higher elevations; numbers shown in bold in Table 2) for each species to be the benchmark for its relative recovery at higher elevations. Post-treatment mid elevation Ca:Mn ratios rose to either equal to or slightly lower than the benchmark value at low elevation. However, the ratios at high elevation, though higher than those for pre-Ca addition, were still lower as compared to other elevations for all species. Since higher Ca:Mn ratios indicate better growth conditions and possibly less Mn toxicity (Kogelmann and Sharpe, 2006), our findings predict that mid elevation trees should show better overall improvement in response to Ca addition as compared with those at high elevation; this indeed was the case for maple.

In the present study, we observed a ratio of ≤ 1 for the foliar total Mg:Mn ratios for all three species at mid to high elevations in 1999 (pre-treatment) indicating poor health that improved in some cases with Ca addition (Table 2).

4.2. Foliar metabolites are a reliable indicator of Ca stress and its amelioration

While an increase in the foliar Ca in response to Ca addition to Ca-depleted soil may be predictable, this is not an a-priory demonstration of recovery from Ca deficiency unless it is accompanied by changes in growth parameters. Since growth responses, such as changes in stem diameter and crown growth, to Ca addition are rather slow (in years), potentially fast and reliable approaches to evaluate amelioration of Ca deficiency have been proposed which include specific metabolic markers such as foliar concentrations of Put, Arg or GABA. These metabolic markers can be validated with actual growth measurements later. Previous studies have shown strong correlations of Put with soil Ca as well as with growth in a wide variety of tree species (Minocha et al., 1997, 2000; Wargo et al., 2002; Bauer et al., 2004). In these studies, an inverse relationship was observed between foliar Put and foliar and/or soil Ca under conditions of established deficiency in soil Ca available to plants. However, species-specific and elevation dependent effects of Ca depletion have not been evaluated in the earlier studies.

In the present study, at the reference watershed, the lowest foliar Put was observed at low elevation (Fig. 3A) accompanied by the highest foliar Ca among the three elevations studied (Fig. 2A). Akin to the results of Juice et al. (2006), we observed no change in Put in the foliage of mature maple or birch trees at this elevation at WS1 in response to Ca addition. This indicates the absence of low Ca-related stress at this elevation. Foliar Put concentration at low elevation (mid for beech, Fig. 3A) of Ca-supplemented WS1 was thus chosen as an overall benchmark for recovery from Ca deficiency stress at higher elevations. In maple, Put declined at mid and high elevations in response to Ca-addition to concentrations comparable to that of low elevation trees at reference WS3 site. These data suggest that soil Ca depletion was a major (if not the primary) source of stress in maple at mid and high elevations. However, only a partial decrease of Put was observed in birch in post-Ca addition trees at mid elevation; the Put concentrations remained higher than foliar benchmark concentrations for birch at the low elevation WS1 site. This leads us to postulate that birch at mid to high elevations may also be suffering from other stress factors besides Ca depletion that also contribute to similar stress related metabolic changes. Since Ca addition had no effect on foliar Put in beech at either mid or high elevation, it can again be argued that beech too was not suffering from soil Ca-depletion related stress. Along with Ca concentration, light, temperature, other soil nutrients, and biotic infestations also vary with elevation all of which contribute to overall stress in trees; this was reflected in their foliar metabolite concentrations. The lack of change in foliar Spd with Ca addition in these trees is consistent with the notion that Spd is a tightly regulated metabolite, showing only minor changes in plant cells even when its precursor Put exhibits large changes (Minocha et al., 1997; Bhatnagar et al., 2001).

Consistent with the above argument and an earlier study by Juice et al. (2006) on maple seedlings at low elevation at WS1, we found that the restoration of foliar Ca resulted in an increase in chlorophyll concentrations at low to mid elevation in all three species. The increase in chlorophyll in seedlings in response to Ca treatment was accompanied by higher seedling density, increased mycorrhizal colonization of roots, and higher organic horizon soil pH, but with no change in the rate of photosynthesis. This study also reported improved sugar maple crown condition with Ca addition at WS1. At high elevation, regardless of the increase in soluble Ca to comparable concentrations in all three species, no change in chlorophyll was evident. This observation, together with lower Ca:Mn and Mg:Mn ratios in the foliage at high elevation, indicates that overall growth conditions were relatively harsher at this elevation compared to mid and low elevations. Despite no change in foliar Ca, the positive effects observed at lower elevation may be attributed to the indirect effects of Ca addition on soil pH and base cation exchange capacity, among other factors.

In general, total foliar N at WS1 remained unchanged by Ca addition; it also did not vary by elevation except in maple and birch at

high elevation where foliar total N decreased post Ca treatment (Table 1). In soils with low Ca due to acidic deposition, trees face multiple stress factors including Ca deficiency, higher Al and Mn (Wargo et al., 2002), and excess N (Minocha et al., 2000; Minocha et al., unpublished data). Under these conditions, trees expend extra energy to produce one or more of stress-related N rich (i.e., high N:C ratios) metabolites (e.g., Put, Arg, GABA, and Pro) in a species-specific manner for their protection from toxic ammonia. Increased accumulation of Arg, GABA, and Put have all been shown to indicate stress from higher N accumulation, either in response to chronic N application or other forms of environmental pollution (Dohmen et al., 1990; Bauer et al., 2004). In the present study, two of these amino acids (i.e., GABA and Arg) decreased in response to Ca treatment, whereas Glu (the precursor of GABA and Arg) and Gly [required for chlorophyll biosynthesis – (Porra et al., 1983)] increased significantly accompanied by an increase in chlorophyll content. We hypothesize that N that was previously being used to make stress-protective metabolites may now (upon replenishment of soil Ca) become available to support growth via increased photosynthesis, thus resulting in higher productivity and biomass.

The species-specific response to Ca treatment observed in the present study corroborates a previously published seedlings study by Zaccherio and Finzi (2007). In comparison with American beech, a higher requirement for soil nutrients by sugar maple seedlings (Leak, 2005) and mature trees (Finzi et al., 1998) has also been reported.

5. Conclusions

While corroborating the conclusions of earlier reports on the positive effects of Ca addition to soils for sugar maple, the present study advances our understanding of the physiological basis of Ca depletion stress in three hardwood species in relation to changes in elevation (a proxy for changing soil chemistry, landscape, and climate). The results confirm that mid and high elevations are more prone to soil Ca depletion than low elevations as indicated by significant changes in foliar chemistry/metabolism upon Ca addition. The results of this study further substantiate the use of Put, Arg and GABA as biochemical indicators of the presence of physiological stress as well as recovery from it. Consistent with our previous findings with red spruce and sugar maple, also a decrease in foliar Put with Ca addition to soil in this study also indicates the presence of Ca deficiency. The response to Ca addition was species-specific and varied with elevation, perhaps due to species-specific differences in the threshold Ca requirement for growth and the efficiency of root Ca uptake as pointed out by Zaccherio and Finzi (2007). The data further show that of the three species, sugar maple is the most sensitive and American beech is the least sensitive to Ca depletion; yellow birch being moderately affected by Ca depletion. Whereas Ca limitation may be a major factor for stress in sugar maple, birch and beech apparently have other site-specific sensitivities besides Ca at higher elevations. Indirect positive effects of Ca addition (that were unrelated to Ca nutrition), such as an increase in chlorophyll and foliar P and/or a decrease in foliar Al, were observed for all species.

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