Trees (2011) 25:1053–1061
DOI 10.1007/s00468-011-0580-8

ORIGINAL PAPER

Calcium addition at the Hubbard Brook Experimental Forest increases the capacity for stress tolerance and carbon capture in red spruce (*Picea rubens*) trees during the cold season

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Received: 20 December 2010 / Revised: 13 May 2011 / Accepted: 20 May 2011 / Published online: 7 June 2011

Abstract Red spruce (*Picea rubens* Sarg.) trees are uniquely vulnerable to foliar freezing injury during the cold season (fall and winter), but are also capable of photosynthetic activity if temperatures moderate. To evaluate the influence of calcium (Ca) addition on the physiology of red spruce during the cold season, we measured concentrations of foliar polyamines and free amino acids (putative stress-protection compounds), chlorophyll (a key photosystem component), and sapwood area (a proxy for foliar biomass), for trees in Ca-addition ($CaSiO_3$ added) and Ca-depleted (reference) watersheds at the Hubbard Brook Experimental Forest (NH, USA). Ca-addition increased concentrations of the amino acids alanine and γ-aminobutyric acid (GABA) and the polyamines putrescine (Put) and spermidine (Spd) in November, and Put in February relative to foliage from the reference watershed. Consistent with increased stress protection, foliage from the Ca-addition watershed had higher total chlorophyll and chlorophyll *a* concentrations in February than foliage from the reference watershed. In contrast, foliage from the reference watershed had significantly lower glutamic acid (Glu) and higher alanine (Ala) concentrations in February than foliage from the Ca-addition watershed. Imbalances in Ala:Glu have been attributed to cold sensitivity or damage in other species. In addition to concentration-based differences in foliar compounds, trees from the Ca-addition watershed had higher estimated levels of foliar biomass than trees from the reference watershed. Our findings suggest that Ca-addition increased the stress tolerance and productive capacity of red spruce foliage during the cold season, and resulted in greater crown mass compared to trees growing on untreated soils.

Keywords Acidic deposition · Calcium depletion · Polyamines · Free amino acids · Chlorophyll content · Sapwood area

Communicated by F. M. Cánovas.

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Introduction

Laboratory and field-based studies have determined that red spruce (*Picea rubens* Sarg.) foliage attains limited cold tolerance during winter (Strimbeck et al. 1995; Schaberg et al. 1996), but that it is also quick to become photosynthetically active when temperatures moderate (e.g., during winter thaws; Schaberg et al. 1995, 1998). Indeed, carbon (C) gains during winter thaws can significantly augment foliar sugar stores (Schaberg et al. 2000a) and support the possibility that red spruce may rely on protracted, low level photosynthetic activity over many seasons to support its annual C budget (Schaberg 2000). However, increased photosynthetic activity during winter thaws is accompanied by a partial reduction in foliar cold tolerance (Strimbeck et al. 1995; Schaberg et al. 1996) that increases the risk of damage from freezing when more typical ambient low

temperatures return (Schaberg and DeHayes 2000; DeHayes et al. 2001). Consequently, the response of red spruce to winter thaw is a tradeoff between the potential benefit of increased C capture versus the increased risk of damage from freezing dependent on the long-term balance of costs and benefits (Schaberg and DeHayes 2000; DeHayes et al. 2001).

Until recently, red spruce has successfully balanced this physiological tradeoff and occupied even the coldest habitats in the northeastern US (Little 1971). However, the success and survival of red spruce in cold, high elevation forests have been threatened in recent decades as increased foliar freezing injury during winter has reduced crown health and tree growth, and spurred widespread tree mortality (DeHayes 1992; Johnson 1992). Considerable additional research has shown that acidic deposition contributes to environmental calcium (Ca) depletion that limits biological Ca concentrations in red spruce foliage, reduces foliar cold tolerance, and predisposes red spruce trees to winter injury and decline (DeHayes et al. 1999; Schaberg et al. 2000b; Hawley et al. 2006; Halman et al. 2008).

Numerous studies have concluded that Ca plays a critical role in the regulatory response of plants to an array of environmental stresses and stimuli, including low temperature (Monroy et al. 1993; Abassi et al. 2004), and oxidative stress (Schmitz-Eiberger et al. 2002). Thus, it is not surprising that the anthropogenic depletion of available Ca could result in physiological disruption and tree decline. Furthermore, recent work has shown that the controlled partitioning and flow of Ca is an important component of basic biochemical systems that regulate plant growth and energy relations (e.g., Hepler 2005; Lautner and Fromm 2010). Thus, as Ca becomes environmentally depleted, resulting plant Ca limitations would most likely manifest themselves as disruptions in stress response to freezing, high aluminum (Al), manganese (Mn) and nitrogen (N) toxicity (Minocha et al. 1996, 1997, 2000; Wargo et al. 2002; Bauer et al. 2004; Halman et al. 2008), and altered energy relations (e.g., carbon storage and growth; Huggett et al. 2007; Halman et al. 2008).

Detailed laboratory and field studies on red spruce have elucidated the mechanistic influence of Ca depletion on winter-specific alterations in physiology that lead to increases in foliar freezing injury and tree decline. Exposure of red spruce seedlings to acid mist treatment leaches Ca from foliar membranes, destabilizing these membranes and depleting a source of messenger Ca used for stress (e.g., cold) signal transduction (DeHayes et al. 1999; Schaberg et al. 2000b). The resulting depletion of this biologically active Ca store is followed by a significant reduction in foliar cold tolerance that increases the likelihood of freezing injury (DeHayes et al. 1999; Schaberg et al. 2000b). Furthermore, evidence from the field has implicated soil available Ca depletion as the source of Ca deficits that ultimately increase the risk of foliar winter injury within native forests (Hawley et al. 2006). Recently, we have shown that Ca addition to a watershed in a region with documented soil Ca depletion increased the presence of some plant protective and stress response compounds (e.g., foliar antioxidant enzymes and soluble sugars) in red spruce foliage (Halman et al. 2008). This increase of protective compounds in foliage following Ca treatment was also associated with a significant increase in cold tolerance and a reduced risk of freezing injury during winter (Halman et al. 2008). Although these data support the probability that adequate Ca supplies are needed to bolster some stress responses in plants and limit stress-induced injury, it remains uncertain whether Ca (1) is also needed to support other stress responses and (2) also promotes the capacity for physiological function and long-term health despite stress exposure.

Here, we evaluate the influence of Ca-addition at the watershed level on (1) the production of additional classes of stress-related compounds (foliar polyamines and free amino acids including γ-aminobutyric acid (GABA) that may directly protect or provide other benefits to foliage that increase stress tolerance), (2) photosystem integrity (measured as foliar chlorophyll and soluble protein concentrations), and (3) sapwood area, a measure of overall foliar mass. We integrate past data and new measures to explore the influence of Ca on the protection and physiological capacity of red spruce foliage during the cold season (fall through spring).

Methods

Site characteristics and sampling

The Hubbard Brook Experimental Forest (HBEF) in Thornton, NH, USA, is divided into multiple headwater watersheds that serve as either reference or treatment sites. Watershed 6 is the biogeochemical-reference watershed of the forest for which extensive Ca depletion has been documented (Likens et al. 1996, 1998). Watershed 1 was fertilized in 1999 with $CaSiO_3$ (wollastonite, a form of Ca that is slow-releasing) in order to increase the availability of Ca to pre-industrial levels (Groffman et al. 2004). Within each watershed, six south-facing plots were established containing five mature, dominant or codominant red spruce trees ($n = 30$ per watershed, total $n = 60$ for all measures). Current-year foliage was collected twice (4 November 2005, and 21 February 2006) from the upper sun-lit crowns using shotguns, and was sealed in plastic bags stored on dry ice for transport to the laboratory. Samples were then kept at -80°C until the time of analyses. The details of further

processing depended on the specific analysis listed below. Subsamples of the bulked foliar samples collected on these dates were previously assessed for a range of nutritional and physiological parameters, including cation and sugar concentrations (Halman et al. 2008).

Foliar free amino acids and polyamines

A portion of samples stored at −80°C were transported to the USFS laboratory in Durham, NH where they were stored at −20°C for several weeks prior to analyses. Needles were thawed and chopped into 2–3 mm size pieces, from which two sub-samples were taken: one (approximately 200 mg FW) was placed in a pre-weighed microfuge tube and 1 mL of 5% perchloric acid (PCA) was added to it; and the other placed in a separate microfuge tube without PCA, frozen at −20°C and subsequently used for chlorophyll and soluble protein analyses. Samples in PCA were weighed, frozen and thawed three times (Minocha et al. 1994), and centrifuged at 13,000×*g* for 10 min in preparation for analysis of foliar amino acids and polyamines by HPLC (Minocha and Long 2004). The freeze-dried samples were used for analyses of soluble proteins and total chlorophyll.

The supernatant of PCA extracted samples was subjected to dansylation 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). The HPLC system consisted of a PerkinElmer (Waltham, MA, USA) Series 200 pump and autosampler fitted with a 200 µL loop (20 µL injection volume), a Phenomenex Synergi Hydro-RP C18 column (4 µm particle size, 100 × 4.6 mm i.d.) heated to 40°C, a Phenomenex Security Guard C18 cartridge guard column, and a fluorescence detector (Series 200 PerkinElmer). The excitation and emission wavelengths for the detector were set at 340 and 510 nm, respectively. Details of the gradient profile used for the separation of amino acids and polyamines are described in Minocha and Long (2004).

Soluble proteins and chlorophyll

For soluble proteins, 50 mg of thawed and chopped needles were placed in 0.25 mL of extraction buffer [100 mM Tris–HCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA, and 10% (v/v) glycerol, pH 8.0], frozen and thawed three times, and the supernatant used for protein analysis according to Bradford (1976). For analysis of chlorophyll, 15 mg of chopped needles were placed in 1 mL of 95% ethanol and incubated in the dark in a 65°C water bath for 16 h (Minocha et al. 2009). Following centrifugation at 13,000×*g* for 5 min, the supernatant was scanned for absorbance in the range of 350–710 nm using a spectrophotometer (U-2010, Hitachi Ltd., Tokyo, Japan). Chlorophyll concentration was estimated from the absorbance values: Chlorophyll $a = (13.36 \times A_{664}) - (5.19 \times A_{649})$; Chlorophyll $b = (27.43 \times A_{649}) - (8.12 \times A_{664})$; Total chlorophyll $= (22.24 \times A_{649}) + (5.24 \times A_{664})$ (Lichtenthaler 1987). We used chlorophyll concentration as an index of photosystem capacity because reductions in the amount of chlorophyll are seen as a major limitation to photosynthetic activity in fall and winter (e.g., Martin et al. 1978; Öquist et al. 1978; Hansen et al. 1996; Rowbakowski 2005).

Stem sapwood area

Sapwood area was estimated from two increment cores per tree taken at 180° from one another at 1.3 m above ground level, perpendicular to the slope in October 2005. The boundary between the visibly water-soaked sapwood and drier heartwood was marked on the cores as they were extracted (Bond-Lamberty et al. 2002). Cores were stored at −5°C until laboratory analysis. Cores were mounted, sanded, and annual xylem increments were microscopically measured according to methods of Stokes and Smiley (1968). Linear measurements were converted to basal area increment using stem diameter data obtained when cores were collected. Mean sapwood area was calculated as the average proportion of sapwood relative to overall stem basal area for the two cores collected per tree. For coniferous species, sapwood area has long been used to provide a quantitative estimate of the crown supported by stem vasculature because there is a strong and consistent relationship between sapwood area at breast height and the foliar mass of trees (e.g., Grier and Waring 1974; Waring et al. 1980; Marshal and Waring 1986; Robichaud and Methven 1992; Shelburne et al. 1993; Dvorak et al. 1996).

Statistical analysis

Treatment differences among means were tested using analyses of variance (ANOVA). Significance tests employed 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. This sample design was used by Hawley et al. (2006) and Halman et al. (2008) when evaluating other parameters for trees on the Ca-addition and reference watersheds at HBEF. For all tests, differences were considered statistically significant at the $P \leq 0.01$ (***), $P \leq 0.05$ (**), or $P \leq 0.10$ (*) level. MANOVA analyses of within-subject effects across the two sample dates showed that there were no significant differences associated with repeated measures between the seasons.

Results

Foliar free amino acids and polyamines

Differences in foliar free amino acids and polyamines associated with Ca treatment were detected in both November and February, but were more consistent for the autumn sampling (Table 1). In November, concentrations of the amino acids Ala and GABA were higher in foliage from trees on the Ca-addition watershed relative to trees on the reference watershed. Concentrations of two polyamines (Put and Spd) were also higher in the foliage of trees on the Ca-addition watershed than trees on the reference watershed. In February, foliar Put concentrations remained higher for trees on the Ca-addition watershed compared to trees on the reference watershed, whereas trees on the reference watershed had lower Glu concentrations and higher Ala concentrations in their foliage than trees from the Ca-addition watershed (Table 1).

Soluble proteins and chlorophyll

Treatment-associated differences in chlorophyll concentration were only evident in February (Table 2). At this time, foliage from trees on the Ca-addition watershed had significantly higher concentrations of chlorophyll *a* and total chlorophyll (Table 2). No differences in total soluble proteins attributable to Ca treatment were detected on either sample date.

Stem sapwood area

Mean sapwood area was significantly higher for trees on the Ca-addition watershed than trees on the reference watershed (Fig. 1). Because sapwood area is well correlated with the mass of foliage supported by conductive xylem (e.g., $r^2 = 0.96$ or greater; Grier and Waring 1974), the approximately 22% lower sapwood area for trees on the reference watershed relative to trees on the Ca-addition watershed reflected a considerable reduction in canopy mass when Ca availability was low.

Discussion

Treatment verification

An analysis of the influence of wollastonite treatment on the chemistry of soils at the Ca-addition watershed measured in 2000 and 2002 showed an increase in exchangeable Ca, soil pH, effective cation exchange capacity, and effective base saturation in organic horizons, but smaller or no changes in the chemistry of mineral soils relative to pre-treatment levels (Cho et al. 2010). The chemistry of the soil solution also showed a response to treatment, with increases in concentrations of Ca, dissolved silica, pH, and acid neutralizing capacity, and a decrease in inorganic monomeric Al noted for the same time period (Cho et al. 2010). Although treatment had a somewhat wide-ranging influence on soil and soil solution chemistry, alterations in foliar nutrition were more muted and specific for red spruce. The red spruce trees that we assessed on the Ca-addition watershed had significantly higher foliar Ca concentrations than trees on the reference watershed for both sample dates (i.e., 1,796 ± 77 mg kg^{-1} for reference and 2,035 ± 78 mg kg^{-1} for the Ca-addition watershed in November, and 1,848 ± 81 mg kg^{-1} for reference and 2,119 ± 90 mg kg^{-1} for the Ca-addition watershed in February; Halman et al. 2008). Foliar Ca concentrations for trees on the reference watershed were slightly higher than the Ca concentration threshold affecting red spruce growth (cf. Swan 1971; Joslin and Wolfe 1994). Our previous winter data for red spruce at this site showed that treatment-induced increases in foliar Ca concentrations were accompanied by significant increases in a variety of foliar parameters, including soluble sugar concentrations (e.g., sucrose, glucose, and total sugars), the activity of an

Table 1 Mean (±SE) concentrations of free amino acids and polyamines in the current-year foliage of *Picea rubens* from the Ca-addition and the reference watersheds at the Hubbard Brook Experimental Forest on two sample dates

Sample date and treatment	Foliar polyamines (nmol g^{-1} FW)		Foliar amino acids (nmol g^{-1} FW)			
	Putrescine (Put)	Spermidine (Spd)	Glutamic acid (Glu)	Alanine (Ala)	Ala:Glu	γ-aminobutyric acid (GABA)
November 2005						
Reference	110.76 ± 10.01*	33.84 ± 0.93**	62.01 ± 4.64	1.96 ± 0.72***	0.06 ± 0.02***	2.92 ± 0.54***
Ca-addition	137.40 ± 9.18*	38.19 ± 1.82**	67.27 ± 5.77	15.82 ± 2.65***	0.25 ± 0.03***	18.73 ± 3.76***
February 2006						
Reference	87.85 ± 8.61**	27.67 ± 1.96	35.85 ± 2.98**	35.98 ± 4.14***	1.02 ± 0.10***	89.08 ± 11.39
Ca-addition	120.38 ± 9.42**	30.40 ± 2.29	49.65 ± 4.22**	16.23 ± 3.01***	0.26 ± 0.06***	72.73 ± 9.05

Significant differences between watershed means based on ANOVA are indicated by asterisks: * $P \leq 0.10$, ** $P \leq 0.05$, *** $P \leq 0.01$

Table 2 Mean (±SE) concentrations of total chlorophyll, chlorophyll *a*, chlorophyll *b* the ratio of chlorophyll *a* to *b*, and soluble protein in the current-year foliage of *Picea rubens* from the Ca-addition and the reference watersheds at the Hubbard Brook Experimental Forest on two sample dates

Sample date and treatment	Foliar chlorophyll content (μg g^{-1} FW)				Soluble proteins (mg g^{-1} FW)
	Total chlorophyll	Chlorophyll *a*	Chlorophyll *b*	Chl *a*/chl *b*	
November 2005					
Reference	499.0 ± 25.5	360.4 ± 20.4	138.6 ± 9.3	2.64 ± 0.04	0.32 ± 0.02
Ca-addition	498.9 ± 16.0	377.1 ± 15.0	141.9 ± 5.2	2.50 ± 0.04	0.36 ± 0.02
February 2006					
Reference	470.9 ± 26.6**	336.5 ± 18.6**	130.2 ± 9.9	2.58 ± 0.05	0.31 ± 0.02
Ca-addition	570.2 ± 28.4**	412.5 ± 19.4**	153.2 ± 8.2	2.66 ± 0.04	0.37 ± 0.03

Significant differences between watershed means based on ANOVA are indicated by asterisks: ** $P \leq 0.05$

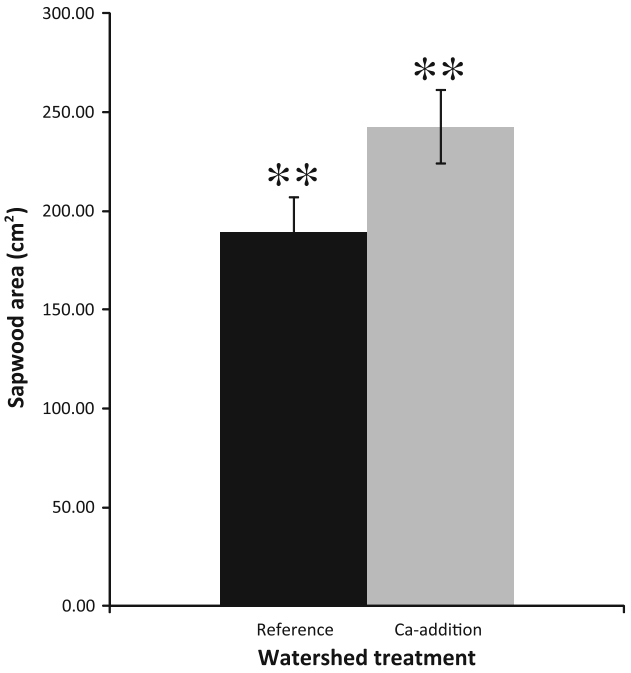

Fig. 1 Mean sapwood area (±SE) of *Picea rubens* trees from the Ca-addition and the reference watersheds at the Hubbard Brook Experimental Forest. Significant differences between watershed means based on ANOVA are indicated by *asterisks*, $^{**}P \leq 0.05$

antioxidant system (ascorbate peroxidase), and winter freezing tolerance that resulted in lower winter injury (Hawley et al. 2006; Halman et al. 2008). This suggests that meaningful Ca limitations can exist for red spruce trees in winter months at foliar Ca concentrations previously thought to be adequate.

Measures of foliar protection

Our new data builds upon past data of the influence of Ca nutrition on foliar protection and physiological capacity during the cold season, and suggests that Ca-induced changes in fall and winter biochemistry are more pervasive than initially documented. For example, Ca-addition was associated with elevated levels of GABA in foliage in November (Table 1). The precocious fall elevation of GABA with Ca treatment is consistent with the biochemistry of GABA production, which is mediated by a Ca-dependent calmodulin binding protein (Bown and Shelp 1997; Shelp et al. 1999), and is triggered by stress signals (including cold stress: Kinnersley and Turano 2000). Furthermore, evidence from the study of *Arabidopsis thaliana* (L.) suggests that GABA helps plants survive stress through (1) direct action as a protective compound (e.g., perhaps functioning as an antioxidant or an osmoregulator), and (2) as an agent that amplifies Ca-signaling and associated stress response (Bouché and Fromm 2004). The difference between the watersheds in the initial timing of GABA increases in fall (when cold acclimation is instigated) is consistent with this possible role in cold signal amplification. However, this speculated association has not been evaluated in native trees and would require direct experimentation to be verified.

Consistent with an enhanced capacity for stress response, foliage of trees from the Ca-addition watershed also had elevated concentrations of Put and Spd relative to foliage from trees on the reference watershed (Table 1). Polyamine accumulation is a long-recognized response of plant cells to stress—particularly abiotic stresses including Al and N toxicity (Minocha et al. 1996, 1997, 2000), Ca depletion (Wargo et al. 2000), and low temperature and oxidative stresses (Alcázar et al. 2006; Kuznetsov et al. 2006) that are prevalent during winter. In particular, recent work with *Arabidopsis* has highlighted a specific increase in Put when plants were experimentally challenged by low temperatures (Cook et al. 2004; Cuevas et al. 2008).

Similar to GABA, polyamines are thought to provide direct protection from stress agents by maintaining functional protein conformation after binding or providing

initial protection from oxidative stress (Kuznetsov et al. 2006). In addition, Put may protect plants through decreased membrane leakage caused by low temperature stress (Kim et al. 2002) or by modulated abscisic acid (ABA) biosynthesis and gene expression (Cuevas et al. 2008). As with this suggested modulation of ABA, polyamines as a group may exert a broader influence on stress protection by influencing the up- or down-regulation of other stress response systems (Kuznetsov et al. 2006).

In accordance with the general importance of GABA and polyamines in protecting plants from cold injury, we found that only trees on the Ca-addition watershed had elevated levels of these protective compounds with the onset of cold temperatures (mean daily temperatures in the watersheds for the week preceding collection ranged from −2 to 11°C; http://www.hubbardbrook.org/data/dataset.php?id=58). Furthermore, it was just for trees on the Ca-addition watershed that one of these compounds (Put) remained high when some of the coldest temperatures of the winter existed in February (mean daily temperatures in the watersheds for the week preceding collection ranged from −17 to 4°C; http://www.hubbardbrook.org/data/dataset.php?id=58). In February, signs of the negative influence of low Ca availability on foliar biochemistry became evident for trees on the reference watershed: they had lower concentrations of Glu and higher concentrations of Ala than foliage from trees on the reference watershed. The ratio of Ala:Glu has been used as an indicator of cold hardiness and injury in plants, with ratios of about 0.2 or lower being typical for cold hardy or uninjured cold sensitive tissues (Patterson et al. 1981). Although Ala:Glu levels were significantly higher for the foliage of trees from the Ca-addition watershed relative to the foliage of trees from the reference watershed in November (Table 1), Ala:Glu levels were near or below the 0.2 threshold for trees from both treatments. By February, Ala:Glu levels for the foliage of trees on the Ca-addition watershed were unchanged from November levels, whereas the foliage of trees from the reference watershed had Ala:Glu ratios over 1.0 (Table 1). Ratios considerably above 0.2 have been detected in other cold sensitive plant tissues and attributed to cold injury, possibly associated with a disruption of glutamate-pyruvate transaminase activity (which catalyzes the inter-conversion of glutamate and alanine) (Patterson et al. 1981).

Chlorophyll content, sapwood area and cold season adaptation

In addition to signs of dysfunction for trees on the reference watershed in February, there were also indications that Ca-addition bolstered foliar physiology at this time. Total chlorophyll, and especially chlorophyll *a*, were significantly higher in foliage from the Ca-addition watershed compared to foliage from trees on the reference watershed (Table 2). Relative to levels in November, foliar chlorophyll concentrations in February were similar for trees on the reference watershed but appeared higher for trees on the Ca-addition watershed. One of the noted protective influences of polyamines such as Spd is the prevention of chlorophyll breakdown (Kuznetsov et al. 2006). However, our data suggest that Ca treatment also supported the buildup of chlorophyll after November, perhaps by assisting chlorophyll recovery from photooxidative damage.

Some of the physiological factors that limit photosynthesis in winter (e.g., stomatal closure) can be readily reversed under favorable environmental conditions (Schaberg et al. 1998). In contrast, cold-induced reductions in foliar chlorophyll content constitute a more durable barrier to photosynthesis (Martin et al. 1978; Öquist et al. 1978). Indeed, the decrease in photosynthetic capacity upon frost hardening measured in Scots pine (*Pinus sylvestris* L.) was largely attributed to a two- to threefold reduction in foliar chlorophyll content (Hansen et al. 1996). Acid deposition-induced Ca limitations can reduce net photosynthesis of red spruce during the growing season (McLaughlin et al. 1993). In particular, red spruce and balsam fir on the Ca-addition watershed at HBEF showed small but significant increases in dark-adapted chlorophyll fluorescence during summer (suggesting improved photosystem function) compared to trees on the reference watershed (Boyce 2007). To our knowledge, the increases in February chlorophyll content that we report are the first indication that enhanced Ca nutrition can benefit the photosynthetic apparatus of red spruce in winter. Increased chlorophyll concentrations would be beneficial during periods of elevated winter and spring temperatures—periods when the enzymatic dark reactions of photosynthesis are less of a limitation and red spruce are known to photosynthesize and store C (Schaberg et al. 1995, 2000a; Schaberg 2000).

In addition to having greater chlorophyll concentrations, measures of sapwood area indicate that red spruce trees on the Ca-addition watershed supported significantly more foliar biomass than trees on the reference watershed (Fig. 1). Greater foliar biomass with Ca addition is also consistent with the almost three-fold greater freezing injury and loss of current-year foliage for dominant and co-dominant spruce on the reference watershed relative to the Ca-addition watershed (Hawley et al. 2006). Overall, Ca treatment was associated with more and better protected foliage that had an increased capacity for photosynthetic function should favorable environmental conditions occur. Red spruce is a species that is capable of photosynthesizing in the traditional cold season during thaws and other periods of milder weather (Schaberg et al. 1995, 1998). However, the species also reduces its cold tolerance in preparation for winter carbon capture, establishing a

potential tradeoff between the protection of existing tissues and carbon stores and the acquisition of new stores needed for competitive growth into the future (Strimbeck et al. 1995; Schaberg et al. 1996).

The combined capacity for adequate protection from freezing damage along with an enhanced capacity for photosynthetic gain during mild winter interludes is an established niche for evergreen conifers in locations where winter climates are historically mild (e.g., Ludlow and Jarvis 1971; Fry and Phillips 1977; Harrington et al. 1994). However, in the traditionally cold northeastern US, this ecological niche may be more unique (Schaberg 2000; Schaberg and DeHayes 2000). Indeed, it has been speculated that the tendency for red spruce to express marginal cold hardiness with enhanced photosynthetic capacity during winter may be a holdover from adaptive qualities favored when the species was in a restricted glacial refugia, likely under a more maritime climate (White and Cogbill 1992; Schaberg and DeHayes 2001). Regardless of its origins, the limited cold tolerance and propensity for cold season C capture have historically put red spruce at no adaptive disadvantage in the northeast, as evidenced by its wide-spread occurrence and generally good health up through the 1960s (White and Cogbill 1992). However, in more recent decades red spruce freezing injury has become more common and has contributed to the regional decline of the species (DeHayes 1992; Johnson 1992).

Impacts of Ca depletion

Recent increases in red spruce freezing injury have been tied to acid deposition-induced leaching of Ca from soils and foliage (DeHayes et al. 1999; Hawley et al. 2006). Resulting foliar Ca limitations destabilize membranes (DeHayes et al. 1999) and lead to reductions in foliar sugars and antioxidant enzyme activities that help bolster foliar cold tolerance (Halman et al. 2008). However, until now, the influence of Ca supplementation on other stress response compounds such as GABA and foliar polyamines in red spruce trees have remained unknown. Data showing that Ca addition can facilitate the accumulation of GABA during cold acclimation and increase polyamine concentrations in foliage during the cold season builds on past results (Hawley et al. 2006; Halman et al. 2008), and suggests that the complex integration of Ca signaling and winter physiology within red spruce foliage can be impaired by ambient Ca depletion. The outcome of this impairment includes an immediate reduction in C storage (e.g., foliar sugar concentrations; Halman et al. 2008), and declines in the functional capacity of foliage to capture more C during winter (exemplified by chlorophyll concentrations in February; Table 2). These consequences are amplified by the influence of Ca on foliar biomass levels (indicated by differences in sapwood area; Fig. 1) that build upon other concentration-based differences in foliar C capacity to more broadly alter tree C relations. Among its other implications, this connection between adequate Ca nutrition and elevated C sequestration capacity at the tree-level highlight how one anthropogenic disruption (Ca depletion) may influence another (C sequestration that helps to mitigate atmospheric CO_2 accumulations and associated climate change).

Based on our new data, we postulate that adequate Ca supply may provide the biochemical means to regulate the balance between cellular protection (e.g., stress response) and physiological activity (e.g., C capture) during winter that allows this species to exploit a niche whereby the transition from greater tissue protection to greater physiological activity can be modulated and controlled. If verified through additional testing, this would refine our understanding of the recent red spruce decline to involve not only a greater propensity toward foliar freezing injury coincident with anthropogenic Ca depletion, but also a shift in C sequestration capacity attributable to Ca limitation. Specific tests are needed to assess the influence of Ca nutrition on red spruce C relations throughout the year, including periods of more moderate temperature during the traditional cold season.

Acknowledgments The authors are grateful to Paula Murakami, Michelle Turner, Kelly Baggett, John Bennink, Kendra Gurney, Brynne Lazarus, Brett Huggett, Chris Hansen, Tammy Coe, and Cathy Borer for their assistance in both the field and laboratory. We further thank the staff at the Hubbard Brook Experimental Forest for their generous help in all aspects of our sample collections. Thanks are also extended to Drs. G. Richard Strimbeck, Jonathan R. Cumming, Timothy D. Perkins and Kevin T. Smith for helpful review comments on an earlier draft of this manuscript. This research was supported by funds provided by the Northeastern States Research Cooperative, the USDA CSREES McIntire-Stennis Forest Research Program, and the U.S. Environmental Protection Agency.

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