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Calcium addition at the Hubbard Brook Experimental Forest increases sugar storage, antioxidant activity and cold tolerance in native red spruce (*Picea rubens*)

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Summary In fall (November 2005) and winter (February 2006), we collected current-year foliage of native red spruce (*Picea rubens* Sarg.) growing in a reference watershed and in a watershed treated in 1999 with wollastonite ($CaSiO_3$, a slow-release calcium source) to simulate preindustrial soil calcium concentrations (Ca-addition watershed) at the Hubbard Brook Experimental Forest (Thornton, NH). We analyzed nutrition, soluble sugar concentrations, ascorbate peroxidase (APX) activity and cold tolerance, to evaluate the basis of recent (2003) differences between watersheds in red spruce foliar winter injury. Foliar Ca and total sugar concentrations were significantly higher in trees in the Ca-addition watershed than in trees in the reference watershed during both fall ($P = 0.037$ and 0.035, respectively) and winter ($P = 0.055$ and 0.036, respectively). The Ca-addition treatment significantly increased foliar fructose and glucose concentrations in November ($P = 0.013$ and 0.007, respectively) and foliar sucrose concentrations in winter ($P = 0.040$). Foliar APX activity was similar in trees in both watersheds during fall ($P = 0.28$), but higher in trees in the Ca-addition watershed during winter ($P = 0.063$). Cold tolerance of foliage was significantly greater in trees in the Ca-addition watershed than in trees in the reference watershed ($P < 0.001$). Our results suggest that low foliar sugar concentrations and APX activity, and reduced cold tolerance in trees in the reference watershed contributed to their high vulnerability to winter injury in 2003. Because the reference watershed reflects forest conditions in the region, the consequences of impaired physiological function caused by soil Ca depletion may have widespread implications for forest health.

Keywords: ascorbate peroxidase, calcium depletion, cations, freezing tolerance, New Hampshire, soluble sugars, wollastonite.

Introduction

There is considerable evidence that anthropogenic factors are disrupting natural nutrient cycles and depleting cation stores, notably calcium (Ca), from forest ecosystems (Likens et al. 1998, Bailey et al. 2005, Lawrence et al. 2005). A consistent and well-documented explanation for anthropogenically induced Ca leaching from northern temperate forests is high acid loading in the form of rain, snow or mist (Likens et al. 1996, 1998). Other factors contributing to cation depletion of forest soils include intensive forest harvesting (Mann et al. 1988, Federer et al. 1989), declines in atmospheric base cation inputs (Hedin et al. 1994), soil aluminum (Al) mobilization (Shortle and Smith 1988, Lawrence et al. 1995), nitrogen (N) saturation (Aber et al. 1998, Schaberg et al. 2002) and changing climatic conditions that may speed natural acidification (Tomlinson 1993). Because Ca is critical to plant health (Marschner 1995), anthropogenic impacts on its availability may have consequences at the plant, population and ecosystem scales.

Many studies have shown that the movement of labile Ca among specific cellular compartments acts as a signal mediating physiological responses to a wide array of environmental stresses including drought, cold, heat, salinity, fungal pathogens, and oxidative and mechanical stresses (e.g., Roberts and Harmon 1992, Bush 1995, Sanders et al. 1999, Knight 2000, Pandey et al. 2000). Given the role of Ca in signal transduction, Ca deficiency may affect the ability of plants to sense and respond adaptively to their environment.

Foliar winter injury in red spruce (*Picea rubens* Sarg.) causes reddening and subsequent loss of current-year foliage due to freezing damage (Perkins et al. 1991, DeHayes 1992), the consequences of which include reductions in carbon assimilation and storage, and growth (Tobi et al. 1995, Schaberg et al. 2000*a*). Exposure to acid rain reduces red spruce cold tolerance through a reduction in Ca availability, therby increasing the risk of winter injury and crown deterioration (Schaberg and DeHayes 2000, DeHayes et al. 2001). Recent field data confirm the link between soil Ca depletion and increased winter injury in red spruce forests. In 2003, there was severe winter injury to red spruce foliage in the northeastern USA that re-

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sulted in large losses of current-year foliage. Lazarus et al. (2004) found that, for 28 sites in the states of New York, Massachusetts, Vermont and New Hampshire, nearly 90% of trees sampled exhibited symptoms of winter injury and lost on average 46% percent of their current-year foliage. Analysis of the pattern of injury across the region indicated that damage was greatest in western locations, on west-facing slopes, and at higher elevations—where acidic deposition and associated Ca depletion were greatest (Lazarus et al. 2006). Hawley et al. (2006) found that Ca addition, in the form of slow-release wollastonite ($CaSiO_3$), to a watershed in the Hubbard Brook Experimental Forest (HBEF, Thornton, NH) in 1999 reduced winter injury in red spruce by a factor of three during the high-injury year of 2003. However, the physiological basis for reduced winter injury by Ca addition to the soil at HBEF has yet to be determined and may involve one or more Ca-mediated stress responses.

Cold tolerance of red spruce is readily compromised by experimental exposure to acid deposition (Sheppard et al. 1993), which leaches membrane-bound Ca (mCa) from foliar cells and reduces the Ca pool needed to sense and respond to cold-stress signals (DeHayes et al. 1999, Schaberg and DeHayes 2000, Schaberg et al. 2000*a*, 2000*b*, Borer et al. 2005). Other Ca-mediated processes that support foliar stress responses and cold tolerance may also be influenced by soil Ca depletion, including carbohydrate storage and antioxidant enzyme activity.

Calcium nutrition is critical to photosystem function and carbohydrate metabolism (Bowler and Chua 1994, McLaughlin and Wimmer 1999, Snedden and Fromm 2001). Adequate sugar storage, in particular, is necessary for the maintenance of foliar cold tolerance in red spruce (Schaberg et al. 2000*c*). Antioxidant systems target reactive oxygen species (ROS), often generated by environmental stress such as photooxidative damage at low temperatures (Wise and Naylor 1987, Polle and Rennenburg 1994). Left unscavenged, excess ROS can result in severe cellular dysfunction (Foyer et al. 1994). Calcium is a major component of the signal transduction pathways required for antioxidant activity and increases the activity of many antioxidant enzymes, including ascorbate peroxidase (APX) (Jiang and Huang 2001, Jiang and Zhang 2003). In conifer species, APX activity is vital to tree health, especially in winter months (Anderson et al. 1992).

To evaluate the physiological basis of the decreased foliar winter injury documented for red spruce in a Ca-addition watershed at HBEF compared with untreated watersheds in the region, we measured foliar nutrition, carbohydrate storage, activity of a Ca-dependent antioxidant enzyme (APX) and cold tolerance of current-year foliage of mature trees in a reference and a Ca-addition watershed. Examining the physiology of winter injury of foliage may help identify the role that Ca deficiency plays in red spruce decline.

Materials and methods

Site characteristics and sampling

The Hubbard Brook Experimental Forest (HBEF) in Thornton, NH is divided into multiple small 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 of the soil has been documented (hereafter the reference watershed) (Likens et al. 1996, 1998). Watershed 1 was fertilized in 1999 with $CaSiO_3$, wollastonite, a slow-releasing form of Ca, to increase soil Ca availability to preindustrial concentrations (hereafter the Ca-addition watershed) (Groffman et al. 2004). Within each watershed, six south-facing plots were established, each containing five mature dominant or codominant red spruce (n = 30 per watershed). Current-year foliage was collected in the fall (November 4, 2005) and winter (February 21, 2006), from branches shot down from the upper sunlit crowns, and processed for specific analyses.

Foliar nutrition

Samples were sealed in plastic bags for transport to the laboratory, where they were dried for 2 weeks at 65 °C. Dried samples were ground to pass a 2-mm sieve, digested by heating with nitric acid and hydrogen peroxide in a block digester (adapted from Jones and Case 1990), and analyzed for total foliar cations (Ca, Al, K, Mg and Mn) by inductively coupled plasma atomic emission spectroscopy (ICP-AES, PlasmaSpec 2.5, Leeman Labs, Lowell, MA). Eastern white pine needles from the National Bureau of Standards and Technology (SRM 1575), sample duplicates and blanks were analyzed for procedural verification. Tissue standards were within 5% of certified values.

Soluble sugar analysis

Samples to be analyzed for soluble sugar concentration were frozen in liquid nitrogen in the field, stored on dry ice for transport to the laboratory, freeze-dried, ground and stored at –80 °C until analyzed. Cuticular waxes were removed with hexane, and sugars were extracted with 80% ethanol (Hinesley et al. 1992). Concentrations of stachyose, glucose, sucrose, xylose, fructose and raffinose were determined as described by Schaberg et al. (2002) with a Waters HPLC equipped with an Alliance 2695 separations module, a 2414 differential refractometer and a Waters Sugar-Pak column. Data were analyzed for individual and total sugar concentrations with Waters Empower Pro software, and expressed as mg g^{-1} dry mass.

Ascorbate peroxidase activity

Current-year foliage was collected and frozen in liquid nitrogen in the field, transported on dry ice to the laboratory and stored at –80 °C. Samples were homogenized in an extraction buffer modified from Schwanz and Polle (1998), containing 100 mM KPO_4 (pH 7.8), 1% (v/v) Triton X-100, 5 mM ascorbate, 400 mg polyvinylpolypyrrolidone and 2 mM EDTA and stored at –80 °C until assayed by the methods of Nakano and Asada (1981). To determine APX activity as ascorbate scavenged H_2O_2, each 1.0-ml reaction mixture contained 50 mM potassium phosphate, 0.5 mM ascorbate, 0.15 mM H_2O_2, 0.1 mM EDTA, and 0.01 ml of sample. The linear de-

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crease in absorbance at 290 nm was recorded for 2 min with a Beckman DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA). Ascorbate oxidase (AO) activity was measured by the same method with the omission of H_2O_2, and subtracted from the APX activity to yield APX specific activity. Total soluble protein was analyzed with a brilliant-blue total protein kit (TP0100, Sigma-Aldrich, St. Louis, MO).

Cold tolerance

To measure cold tolerance when red spruce is most vulnerable to freezing injury, current-year foliage was collected on February 21, 2006. Samples were bagged and stored overnight at 4 °C. The following day, samples were rinsed in distilled water and chopped into 5-mm sections to produce one bulk sample per tree. Subsamples of 0.3 ml were transferred to 64-cell styrene trays and exposed to decreasing temperatures at a rate of −6 °C h^{-1} and held at 14 preselected test temperatures ranging from −15 to −90 °C for 30 min, transferred to pre-chilled styrene foam containers, stored at −5 °C, and then slowly brought to 4 °C. Three ml of detergent solution (0.01%, v/v, Triton X-100 in deionized water at 4 °C) was added to each cell, and sample trays were shaken in high humidity chambers for 8 h at room temperature. Initial conductivity was measured with a multi-electrode instrument (Wavefront Technology, Ann Arbor, MI), samples were then dried for 72 h at 45 °C, soaked in fresh detergent solution for 24 h, and final conductivity measured. Relative electrolyte leakage (REL), a measure of membrane permeability inferred from the proportion of initial to final conductivity at a given test temperature, was used to calculate the temperature at the midpoint of a sigmoid curve fit to the REL data for all test temperatures (T_m; Schaberg et al. 2000*c*, Strimbeck and DeHayes 2000).

Membrane integrity

Relative electrolyte leakage measurements have been used to elucidate changes in membrane integrity associated with various environmental stresses (e.g., freezing, osmotic and heat stress, perturbations to mineral nutrition and acid mist exposure; DeHayes and Williams 1989, Zwiazek and Blake 1991, David et al. 1994, Strimbeck et al. 1995, Ruter 1996, DeHayes et al. 1999, Schaberg et al. 2001). We used foliar REL data from tissues not exposed to experimental freezing (i.e., initial conductivity data for the highest test temperature used in cold tolerance tests, REL_i) to estimate baseline membrane integrity and incipient field injury. Visibly injured tissues were excluded from these tests, thus, the REL_i data are estimates of in situ damage to visibly uninjured tissues.

Statistical analysis

Treatment differences among means were tested by analysis 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. Hawley et al. (2006) previously employed this design in a paired-watershed study. For all tests, differences were considered statistically significant at $P \leq 0.05$ (**) or $P \leq 0.10$ (*).

Results

Foliar nutrient concentration

Cation concentrations of current-year foliage are shown in Table 1. Current-year foliage from the Ca-addition watershed had significantly higher Ca concentrations on a mass basis than current-year foliage from the reference watershed on both collection dates (November, $P = 0.037$; February, $P = 0.055$). Foliar Mn concentrations also differed significantly between treatments for each collection (November, $P = 0.053$; February, $P = 0.024$) with concentrations from reference trees being about 50% (November) and 62% (February) higher than those from Ca-addition trees.

Foliar soluble sugar concentration

Foliage of trees in the Ca-addition watershed maintained significantly higher total sugar concentrations than foliage of trees in the reference watershed on both collection dates (November, $P = 0.035$; February, $P = 0.036$; Table 2). Significantly greater amounts of fructose, glucose and stachyose were found in foliage from Ca-addition trees than from reference trees in November ($P = 0.013$, 0.007 and 0.081, respectively). Glucose, but not fructose or stachyose, concentrations

Table 1. Mean (± SE) cation concentrations of current-year red spruce (*Picea rubens*) foliage from the Ca-addition and reference watersheds at the Hubbard Brook Experimental Forest on two measurement dates. Significant differences between watershed means based on ANOVA are indicated by asterisks: *, $P < 0.10$; and **, $P < 0.05$.

Watershed	Foliar cation concentration (mg kg^{-1} dry mass)				
	Ca	Al	K	Mg	Mn
November 2005					
Reference	1796.3 ± 77.0 **	36.0 ± 1.6	5242 ± 178	681.0 ± 19.8	1330.8 ± 106.0 *
Ca-addition	2035.4 ± 78.2 **	35.4 ± 1.8	5385 ± 193	715.4 ± 17.1	881.8 ± 64.0 *
February 2006					
Reference	1848.2 ± 81.2 *	32.0 ± 1.8	5400 ± 199	808.2 ± 23.1	1418.0 ± 111.5 **
Ca-addition	2119.3 ± 90.3 *	30.2 ± 1.8	5647 ± 183	825.6 ± 24.2	873.0 ± 57.7 **

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Table 2. Mean (± SE) soluble sugar concentrations of current-year foliage of *Picea rubens* from the Ca-addition and reference watersheds at the Hubbard Brook Experimental Forest on two measurement dates. Significant differences between watershed means based on ANOVA are indicated by asterisks: *, $P < 0.10$; and **, $P < 0.05$.

Watershed	Foliar soluble sugar concentration (mg g^{-1} dry mass)						
	Fructose	Sucrose	Glucose	Stachyose	Raffinose	Xylose	Total
November 2005							
Reference	24.56 ± 0.76 **	4.17 ± 0.39	39.62 ± 0.83 **	0.23 ± 0.03 *	1.95 ± 0.20	0.53 ± 0.04	69.15 ± 3.07 **
Ca-addition	27.75 ± 0.73 **	5.23 ± 0.71	43.47 ± 0.78 **	0.31 ± 0.03 *	1.96 ± 0.16	0.52 ± 0.04	79.82 ± 2.22 **
February 2006							
Reference	25.74 ± 1.20	2.68 ± 0.29 **	39.85 ± 1.48 *	3.41 ± 0.19	3.01 ± 0.22	2.05 ± 1.03	74.85 ± 3.05 **
Ca-addition	28.01 ± 1.18	4.00 ± 0.30 **	44.18 ± 1.42 *	3.56 ± 0.19	3.08 ± 0.16	1.49 ± 0.72	84.65 ± 2.34 **

were significantly higher in Ca-addition foliage than in reference foliage in February ($P = 0.061$). Foliar sucrose concentrations did not differ significantly between treatments in November, although in the winter collection, sucrose concentration was 42% greater in Ca-addition foliage than in reference foliage ($P = 0.040$, Table 2). Foliar raffinose and xylose concentrations did not differ significantly between treatments for either collection date.

Foliar antioxidant enzyme activity

There were no significant differences between watersheds in mean APX activity of samples collected in November: mean APX activity in foliage from the reference and Ca-addition watersheds was 16.4 and 19.3 µmol ascorbate min^{-1} mg^{-1} protein, respectively ($P = 0.28$). Samples collected in February, however, showed significant differences in mean APX activity ($P = 0.063$) between watersheds. Mean APX activity of reference foliage in February was similar to the mean value in November (15.1 µmol ascorbate min^{-1} mg^{-1} protein), whereas mean APX activity of foliage from the Ca-addition watershed increased to 25.8 µmol ascorbate min^{-1} mg^{-1} protein in February (Table 3).

Table 3. Mean (± SE) ascorbate peroxidase (APX) activity (expressed per mg of protein), membrane integrity (REL_i) and cold tolerance (T_m) of current-year foliage of *Picea rubens* from the Ca-addition and reference watersheds at the Hubbard Brook Experimental Forest. Values of REL_i compare injury before freeze-induced damage, and T_m means were generated after exposure to increasingly lower temperatures to determine the maximum cold tolerance of each sample. Significant differences between watershed means based on ANOVA are indicated by asterisks: *, $P < 0.10$; and **, $P < 0.05$.

Watershed	APX activity (µmol mg^{-1} min^{-1})	REL_i (%)	T_m (°C)
November 2005			
Reference	16.4 ± 1.8	–	–
Ca-addition	19.3 ± 2.6	–	–
February 2006			
Reference	15.1 ± 2.8 *	27.9 ± 1.1	−32.2 ± 1.1 **
Ca-addition	25.8 ± 3.9 *	24.8 ± 0.6	−43.5 ± 1.4 **

Cold tolerance and membrane integrity

Foliage collected in February was assessed for both initial membrane damage (REL_i), and cold tolerance (T_m) of tissues calculated from the change in REL following exposure to progressively lower temperatures. Based on T_m values generated for each tree, the mean cold tolerance of current-year foliage was 11 °C higher in trees in the Ca-addition watershed than in trees in the reference watershed ($P = 0.05$, Table 3). Although large differences were found in maximum cold tolerance of foliage between treatments, REL_i values did not differ significantly between watersheds, with mean leakage values of 28 and 25% for foliage from the reference and Ca-addition watersheds, respectively ($P = 0.40$), indicating that there were no differences in membrane damage attributable to Ca treatment before experimental freezing tests.

Discussion

The application of wollastonite to Watershed 1 in 1999 was expected to increase concentrations of exchangeable Ca in the soil to preindustrial values over a 10–20-year period (Peters et al. 2004). Just 3 years after fertilization, foliar Ca concentrations in mid-canopy sugar maples had nearly doubled (Juice et al. 2006). Our data collected in fall 2005 and winter 2006 suggest that additional Ca was also incorporated into current-year foliage of red spruce trees in the Ca-addition watershed, where foliar Ca concentrations were significantly higher than in the reference watershed (Table 1). Current-year foliage of red spruce has high photosynthetic activity (Andersen et al. 1991) and is a key site for carbohydrate storage (Schaberg et al. 2000*b*). Therefore, the loss of this tissue could compromise tree health. In addition, it is the current-year foliage that is exposed to the greatest extremes in solar radiation and air temperature. Thus, there must be adequate tissue Ca for stress signal transduction and stress responses to occur.

Red spruce current-year foliage can accrue Ca throughout the fall and early winter, but more slowly than during the growing season (DeHayes et al. 1997). Although foliar Ca concentrations differed between watersheds, all mean values were higher than previously established Ca-deficiency thresholds for current-year foliage of red spruce (cf. Swan 1971, Joslin and Wolfe 1994). Nevertheless, despite seemingly ade-

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quate Ca concentrations in current-year foliage of trees in the reference watershed, we found marked differences in Ca-associated physiological processes between foliage from the Ca-addition and reference watersheds. This suggests that, even when total foliar Ca concentrations appear sufficient, certain process-specific pools may be insufficient for optimal physiological function.

We found higher Mn concentrations in current-year foliage of reference trees than of Ca-addition trees. It has been reported that high foliar Mn concentrations can be phytotoxic (St. Clair and Lynch 2004, 2005), giving rise to decline symptoms in sugar maple (*Acer saccharum* Marsh.) in the eastern USA (Horsley et al. 2000). Field observations have shown that soil Mn concentrations increase as soil Ca concentrations decrease (Kogelmann and Sharpe 2006), and Juice et al. (2006) found that Ca-addition at Hubbard Brook reduced foliar Mn concentrations in sugar maple in the 4th and 5th years after treatment. The role of Mn in the decline and winter injury of red spruce is poorly understood, and no standard threshold for Mn toxicity in red spruce has been established. Documented concentrations of foliar Mn in red spruce range from 389 to 4500 mg kg^{-1} (Robarge et al. 1989), and our Mn values are at the low end of this range. Huntington et al. (1990) reported that foliar Mn concentration and crown condition in red spruce were not significantly correlated. Furthermore, we found no literature connecting Mn toxicity to reduced cold tolerance in any species, suggesting that other nutritional influences (e.g., Ca-nutrition) are more important modulators of cold hardiness.

Total soluble sugar concentrations of current-year red spruce foliage from the Ca-addition watershed were significantly greater than those of foliage from trees in the reference watershed on both collection dates, suggesting a link between Ca and carbohydrate availability, regardless of season. Calcium has been linked through its second messenger functions to the development of chloroplasts (Bowler and Chua 1994), thereby indirectly but importantly affecting the synthesis of carbohydrates through photosynthesis.

In addition to its influence on photosynthetic activity, Ca plays important roles in carbohydrate metabolism by binding to calmodulin (CaM), which activates key effector enzymes that regulate the formation or breakdown of carbohydrates (Snedden and Fromm 2001). Calcium has been implicated in regulating the synthesis and metabolism of sucrose in the cytosol (Brauer et al. 1990). The specific influence of Ca nutrition on the sugar stores of current-year foliage has important implications for tree health, because current-year foliage represents a significant store of a tree's carbohydrate reserves (Schaberg et al. 2000*a*). A reduction in carbohydrates could ultimately affect overall carbon storage in trees. Additionally, individual sugars have been shown to influence the cold tolerance of plants. Sucrose has a particularly important role in the maintenance of cold tolerance, because it acts as a cryoprotectant in the cell, occupying cytosolic space and preventing solute leakage and dehydration (Sakai and Larcher 1987, Hinesley et al. 1992, Koster and Lynch 1992). In winter, when sucrose is most necessary, red spruce trees fertilized with Ca had significantly higher foliar sucrose concentrations than trees in the reference watershed (Table 2).

Despite treatment differences, concentrations of sucrose and raffinose—another cryoprotectant sugar (Hinesley et al. 1992)—were less than those previously reported in current-year red spruce foliage in winter (Schaberg et al. 2000*c*), whereas fructose and glucose concentrations were elevated compared with previously reported values, resulting in comparable total soluble sugar concentrations. Elevated sucrose and raffinose concentrations have been linked to increased cold tolerance in red spruce foliage (Schaberg et al. 2000*c*), which accords with our finding that low concentrations of these sugars were associated with limited foliar cold tolerance. The significantly lower concentration of total soluble sugars in foliage of reference watershed trees is consistent with the documented relationship between reduced Ca concentration and elevated respiration (McLaughlin et al. 1991, 1993), which can result in reduced carbon storage.

Although elevated soluble sugar concentrations can help protect tissues from freezing injury, antioxidant activity may help repair tissue following photooxidative damage at low temperatures. When temperatures begin to decrease but irradiance remains high, photooxidative damage is likely, leading to an increase in ROS, and thus a need for increased antioxidant activity (Gilles and Vidaver 1990, Taiz and Zeiger 2002). In November, foliar APX activity was comparable between treatments, but in February, activity was higher in Ca-addition foliage than in reference foliage. There were no significant differences between seasons in APX activity within treatments, but treatment differences in foliar APX activity in winter suggest that, in the presence of ample Ca stores, APX activity contributes to the abatement of ROS when photooxidative stress peaks (Foyer et al. 1994, Polle and Rennenburg 1994). In the case of the 2003 region-wide winter injury event, insufficient APX activity in current-year foliage of red spruce trees from the reference watershed may have made trees of the reference watershed more susceptible to winter injury than trees of the Ca-addition watershed.

Maximum winter hardiness achieved by current-year red spruce foliage is barely sufficient to protect the tissue from damage at low ambient temperatures (DeHayes et al. 2001). Thus, any factor that reduces cold tolerance of current-year foliage can significantly increase the risk of freezing injury. Foliage from trees fertilized with Ca withstood lower temperatures than trees from the reference watershed (Table 3). However, before experimental exposure to decreasing temperatures, foliage from the two watersheds did not differ significantly in membrane integrity, as indicated by REL_i values (Table 3). The T_m values reported here fall within the documented range for red spruce cold tolerance (Schaberg et al. 2000*c*), with mean values in the low and mid regions of the range for the reference and Ca-addition watersheds, respectively. Laboratory studies have shown that reductions in cold tolerance are attributable to experimentally induced reductions in available Ca (DeHayes et al. 2001), but our data are the first to show that ambient soil-Ca depletion is associated with impaired cold tolerance.

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The roles Ca plays in plant cell function can be divided into two categories: (1) structural—maintaining both cell wall architecture and plasma membrane integrity; and (2) labile—serving as a messenger in signaling cascades that allow cells to sense and respond to environmental stimuli. In red spruce, foliar leaching of Ca by acidic mist application significantly reduces membrane-associated Ca and increases electrolyte leakage from cells—an indication of diminished membrane stability (DeHayes et al. 1999, Schaberg et al. 2000*b*). This particular loss of membrane integrity occurred at foliar Ca concentrations below reported deficiency values for the species (DeHayes et al. 1999, Schaberg et al. 2000*b*). In our study, although foliar Ca concentrations differed between watersheds, overall values exceeded typical deficiency thresholds (Swan 1971). At these foliar Ca concentrations, we detected no differences in foliar electrolyte leakage during winter before freeze-induced damage (REL_i, Table 3). However, in winter, total sugar storage, sucrose concentrations and antioxidant activity—all labile-Ca mediated measures—were significantly greater in foliage from the Ca-addition watershed than from the reference watershed. This suggests that, although Ca within foliage was sufficient to maintain adequate membrane integrity, labile-Ca pools and associated physiology of trees from the reference watershed were preferentially disrupted as a result of soil Ca depletion. Our results are the first to implicate reductions in labile Ca and associated physiology to Ca depletion of the soil at foliar concentrations often considered adequate for normal growth in red spruce.

In conclusion, red spruce trees exposed to soil Ca loss had lower foliar Ca concentrations than trees from a watershed treated to provide preindustrial concentrations of soil Ca. Lower foliar Ca concentrations were associated with reduced concentrations of sucrose, reduced APX activity and lower foliar cold tolerance in winter. The observed differences in Ca nutrition and Ca-dependent physiology contribute to a mechanistic understanding of the differences in winter injury previously reported for this study site (Hawley et al. 2006). Our data provide examples of the physiological disruptions currently induced by Ca depletion of soils under ambient conditions.

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