Effects of chronic N fertilization on foliar membranes, cold tolerance, and carbon storage in montane red spruce

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Abstract: We evaluated the influence of protracted low-level nitrogen (N) fertilization on foliar membrane-associated calcium (mCa), sugar and starch concentrations, membrane stability, winter cold tolerance, and freezing injury of red spruce (*Picea rubens* Sarg.) trees growing in six experimental plots on Mount Ascutney, Vermont. For 12 consecutive years before this evaluation, each plot received one of three treatments: 0, 15.7, or 31.4 kg $N \cdot ha^{-1} \cdot year^{-1}$ supplied as NH_4Cl. In comparison with trees from control plots, the current-year foliage of trees from N-addition plots had lower mCa concentrations, higher levels of electrolyte leakage, reduced cold tolerance, and greater freezing injury. Levels of mCa, membrane stability, and cold tolerance did not differ between N treatments, but trees in high-N treated plots experienced greater freezing injury. Although no differences in carbohydrate nutrition were detected in September, foliar sugar and starch concentrations from trees in N-treated plots were higher than control plot trees in January. We propose that foliar mCa deficiencies reduced cell membrane stability, decreased cold tolerance, and increased freezing injury for trees in N addition plots relative to controls. Declines in mCa may also help account for increases in respiration previously measured. Because soil, root, and mycorryhizal conditions were not evaluated, it is unknown how treatment-induced changes in these compartments may have influenced the alterations in foliar mCa and physiological parameters measured in this study.

Résumé : Nous avons évalué l'influence d'une fertilisation azotée (N) prolongée à faible dose sur le calcium associé à la membrane (mCa) dans les aiguilles, la concentration de sucre et d'amidon, la stabilité de la membrane, la tolérance au froid et les dommages causés par le gel chez des épinettes rouges (*Picea rubens* Sarg.) croissant dans six parcelles expérimentales sur le mont Ascutney dans l'État du Vermont. Avant de procéder à l'évaluation, chaque parcelle avait reçu soit 0, 15,7 ou 31,4 kg $N \cdot ha^{-1} \cdot an^{-1}$ à chaque année pendant 12 ans sous forme de NH_4Cl. Comparativement aux arbres des parcelles témoins, les aiguilles de l'année des arbres dans les parcelles fertilisées avaient une concentration de mCa plus faible, un degré plus élevé de fuite d'électrolytes, une tolérance au froid réduite et davantage de dommages causés par le gel. Le niveau de mCa, la stabilité de la membrane et la tolérance au froid ne différaient pas en fonction des traitements de fertilisation azotée mais les arbres dans les parcelles traitées avec la plus forte dose d'azote avaient subi plus de dommages dus au gel. Quoique aucune différence dans la nutrition en hydrate de carbone n'ait été détectée en septembre, la concentration de sucre et d'amidon dans les aiguilles était plus élevée chez les arbres dans les parcelles fertilisées que dans les parcelles témoins en janvier. Nous émettons l'hypothèse que les déficiences en mCa dans les aiguilles réduisent la stabilité de la membrane cellulaire, réduisent la tolérance au froid et entraînent une augmentation des dommages causés par le gel dans les parcelles fertilisées à l'azote comparativement aux parcelles témoins. La diminution du mCa peut également aider à expliquer l'augmentation de la respiration mesurée antérieurement. Étant donné que les conditions relatives au sol, aux racines et aux mycorhizes n'ont pas été évaluées, on ne sait pas comment les changements provoqués par la fertilisation dans ces composantes auraient pu influencer les altérations dans le mCa des aiguilles et les paramètres physiologiques mesurés dans cette étude.

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Introduction

Recent research indicates that acidic deposition-induced disruption of foliar calcium (Ca) pools is an important predisposing factor in the well-documented decline of red spruce in northeastern North America (DeHayes et al. 1999; Schaberg et al. 2000*a*). Acidic deposition leaches Ca specifically associated with plasma membranes of mesophyll cells. This loss of membrane-associated Ca (mCa) destabilizes cells, depletes a pool of Ca needed by plant stress response systems, and increases the susceptibility of mesophyll cells to freezing injury that leads to foliar necrosis, crown deterio-

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ration, reductions in growth, and increased tree mortality (DeHayes et al. 1999; Schaberg et al. 2000*a*).

This work has highlighted the impact of direct foliar leaching of mCa on red spruce (*Picea rubens* Sarg.) health and sustainability. However, numerous anthropogenic factors, such as acidic deposition (Likens et al. 1996), nitrogen (N) saturation (Aber et al. 1995), soil aluminum (Al) mobilization (Lawrence et al. 1995), declines in atmospheric base cation deposition (Hedin et al. 1994), intensive forest harvesting (Mann et al. 1988), and potential climate change (Tomlinson 1993), may also deplete Ca from forest soils. Because soil Ca is the ultimate source of mCa, soil Ca depletion (through any combination of anthropogenic drivers) could limit mCa accrual. Considering the documented importance of mCa to cell stability and stress response, the impact of soil Ca depletion on mCa levels could be a fundamental mechanism that threatens forest health (DeHayes et al. 1999; Schaberg and DeHayes 2000).

Of the various factors that may contribute to soil Ca depletion, N saturation provides one of the strongest empirical linkages to forest decline. For example, several studies conducted along regional N deposition gradients have documented increased mortality in spruce–fir forests consistent with increases in N deposition (Rock et al. 1986; McNulty et al. 1991). Furthermore, controlled additions of N to a montane spruce forest on Mount Ascutney, Vermont, have specifically associated chronic N inputs to reduced growth and vigor (McNulty et al. 1996). The inhibitory influence of long-term N additions has also been inferred through the recuperative response of forests to reduced N deposition; increases in tree growth have been reported for two European NITREX sites following experimental reductions in ambient N inputs (Boxman et al. 1998).

Among the studies that indicate protracted N additions impair tree health and productivity, the one that provides the most focused information on the mechanism of decline is the fertilization study on Mount Ascutney, Vermont. Although N concentrations in the forest floor have been unaltered by treatment at this site, protracted N fertilization has resulted in greater N leaching from the forest floor, increased foliar N concentrations, and reductions in foliar Ca and magnesium (Mg) concentrations to near-deficient levels (McNulty and Aber 1993; McNulty et al. 1996; Schaberg et al. 1997). Long-term N fertilization has also resulted in numerous indicators of physiological degradation, including elevated rates of foliar respiration (Schaberg et al. 1997), winter freezing injury (Perkins et al. 2000), reduced tree growth, and increased mortality (McNulty and Aber 1993; McNulty et al. 1996). Importantly, significant correlations between foliar Ca concentrations and measures of physiological disruption have raised speculation of a causal link between N-induced alterations in cation nutrition and tree decline (McNulty et al. 1996; Schaberg et al. 1997; Perkins et al. 2000). Despite this theoretical linkage, the mechanism of decline at Mount Ascutney has not been experimentally verified.

Past work has shown that chronic N fertilization has reduced the foliar Ca concentrations for red spruce on Mount Ascutney (Schaberg et al. 1997). However, the majority of Ca in spruce needles exists as insoluble extracellular Ca oxalate and pectate crystals, which are of little physiological importance (Fink 1991; DeHayes et al. 1997). In contrast, mCa is of great physiological importance and is needed for cell membrane stability (Davies and Monk-Talbot 1990) and stress response (Atkinson et al. 1990). Although it has never been documented, depletion of mCa may even account for the increased respiration and presumed alteration in carbon storage associated with red spruce decline (McLaughlin and Kohut 1992). Overall, it is likely that mCa depletion would have a more direct impact on physiology than would perturbations of more generalized Ca pools.

We hypothesized that N-treatment-induced changes in foliar physiology on Mount Ascutney are the result of reduced Ca incorporation on cell membranes. We proposed that resulting mCa deficiencies would destabilize cells, alter net carbon gain, and reduce foliar cold tolerance. We tested this hypothesis by measuring and comparing foliar mCa, sugar and starch concentrations, membrane stability, cold tolerance, and winter injury levels of red spruce trees at the Mount Ascutney study site.

Materials and methods

Study plots and N treatments

In June 1988, ten 15 × 15 m plots were established at an elevation of approximately 760 m on Mount Ascutney, Vermont, in an area where red spruce comprised >80% of the total basal area (McNulty and Aber 1993; McNulty et al. 1996). Since establishment, two control plots have received no N fertilizer. Of the other eight plots, two each have received yearly applications of the following four treatments: (*i*) 15.7 kg $N \cdot ha^{-1} \cdot year^{-1}$ in the form of NH_4Cl; (*ii*) 19.8 kg $N \cdot ha^{-1} \cdot year^{-1}$ in the form of $NaNO_3$; (*iii*) 25.6 kg $N \cdot ha^{-1} \cdot year^{-1}$ from a combination of NH_4Cl and $NaNO_3$; and (*iv*) 31.4 kg $N \cdot ha^{-1} \cdot year^{-1}$ in the form of NH_4Cl. Fertilizers were applied during June, July, and August from 1988 to 1999. Rates of N application were comparable with annual N deposition rates recorded for spruce–fir forests within industrialized regions of the United States (e.g., 16 kg $N \cdot ha^{-1} \cdot year^{-1}$; Friedland et al. 1991) and Germany (e.g., 30–40 kg $N \cdot ha^{-1} \cdot year^{-1}$; Grennfelt and Hultberg 1986). In addition to N treatments, bulk precipitation collectors located in open areas adjacent to research plots measured ambient additions of 5.4 kg $N \cdot ha^{-1} \cdot year^{-1}$ (McNulty and Aber 1993). Fertilizer treatments included cationic and anionic forms of N so that potential differences in leaching, uptake, and ecological impact could be assessed. However, past work at the site has shown that treatment differences in foliar cation nutrition, tree physiology, growth, and mortality better reflect absolute N-treatment additions than they do the form of N applied (McNulty et al. 1996; Schaberg et al. 1997). Considering this and to accommodate the intensive sampling required for mCa analysis, we simplified this study by removing the confounding effect of fertilizer form and N level and, instead, focused only on the impact of differential NH_4 addition (0, 15.7, or 31.4 kg $N \cdot ha^{-1} \cdot year^{-1}$) on red spruce mineral nutrition and physiology.

Foliar sampling

Ten randomly selected dominant red spruce trees were tagged on each plot prior to the start of physiological assessments. Five trees per plot were sampled in September 1999 so that the influence of N treatment on growing season mCa and carbon relations could be assessed. The remaining five trees per plot were sampled in January 2000 to evaluate possible impacts on winter physiology and nutrition. Three randomly oriented, sunlit branches (≤30 cm long) from the middle to upper crown of each tree were removed using a pruning pole. Because the most recent cohort of red spruce foliage appears uniquely vulnerable to Ca depletion and associated physiological disruption (DeHayes et al. 1999; Schaberg and DeHayes 2000), only current-year shoots were saved for subsequent analysis. Excised shoots were sealed in plastic bags, packed in ice, transported to the laboratory, and immediately prepared for physiological or nutritional assessment. Needles used in carbohydrate analyses were flash frozen in the field using liquid N to limit any loss or conversion of sugars and starch.

Soluble sugar and starch concentrations

Needle tissue was frozen in liquid N, freeze-dried, ground, and stored at –60°C prior to carbohydrate analysis. Cuticular waxes were extracted using hexane, and then soluble sugars were extracted using 80% ethanol (Hinesley et al. 1992). Chlorophyll was removed from the soluble sugar ethanol supernatant using a Waters C_{18} Sep-Pak Plus Cartridge (Millipore Corporation, Milford, Mass.). A subsample of the filtered supernatant was dried at 37°C in a limited volume insert, reconstituted in 200 µL 0.1 mM Ca EDTA, and filtered through a 0.45 µm syringe filter. Samples were analyzed for glucose, fructose, sucrose, stachyose, raffinose, and xylose using a Waters HPLC consisting of a 510 pump, a 410 differential refractometer, and a Waters Sugar-Pak column. The column was maintained at 90°C and 0.1 mM Ca EDTA was used as the solvent at a flow rate of 0.6 $mL \cdot min^{-1}$. Sugar concentrations were quantified using Waters Millennium™ 2000 software and expressed as milligrams per gram dry mass.

The pellet of the ethanol extract was gelatinized with 0.2 M KOH, boiled for 30 min in a water bath, and neutralized with 1 M acetic acidic. This solubilized starch was hydrolyzed to glucose with amyloglucosidase (No. 10115, Fluka Chemical Co., Milwaukee, Wis.) in 0.1 M acetate buffer (pH 4.5) and incubated at 55°C for 30 min. The reaction was terminated by boiling the sample for 4 min. The supernatant was centrifuged for 10 min at 3000 rpm. Starch content was quantified by assaying for glucose using the INT assay (glucose assay No. 115-A; Sigma Chemical Co., St. Louis, Mo.) as described by Hendrix (1993). Samples and glucose standards were read with an Inter-Med TIM-200 ELISA plate reader at 492 nm. Starch concentration was calculated using glucose standard curves and was expressed as milligrams per gram dry mass.

Membrane-associated calcium

On each date, four needles per tree were collected and sectioned to produce three sections per needle for mCa evaluation. Sectioning, staining, microscopy, and image analysis procedures used are described in detail by Borer et al. (1997) and DeHayes et al. (1997). These procedures incorporate epifluorescence microscopy using the fluorescent probe chlorotetracycline (CTC) with computer image processing to quantify the intensity of mCa-specific fluorescent emissions. Chlorotetracycline is a probe that selectively binds to divalent cations associated with biological membranes. Chelation to divalent cations in close proximity to apolar environments (e.g., biological membranes) causes a conformational change in CTC, resulting in an enhanced affinity for these ions and a marked increase in fluorescence of the molecule over that in entirely aqueous solutions (Caswell and Hutchinson 1971). A previous study with red spruce mesophyll cells verified that Ca is the cation associated with CTC fluorescence (Borer et al. 1997).

Cold tolerance and visible freezing injury

In January, current-year foliage from each tree was rinsed in iced distilled water and then chopped into 5-mm sections to prepare a single bulk sample. Subsamples were measured volumetrically (approximately 0.3 mL or 0.1 to 0.2 g) and transferred to 64-cell styrene trays for freezing treatment in a computer-controlled liquid nitrogen freezer (Model KRYO 10, TS Scientific, Perkasie, Pa.). The freezing rate was $6°C \cdot h^{-1}$. Trays were held at 8–12 preselected test temperatures, then removed from the freezer and placed in precooled styrene foam containers to thaw slowly to 4°C.

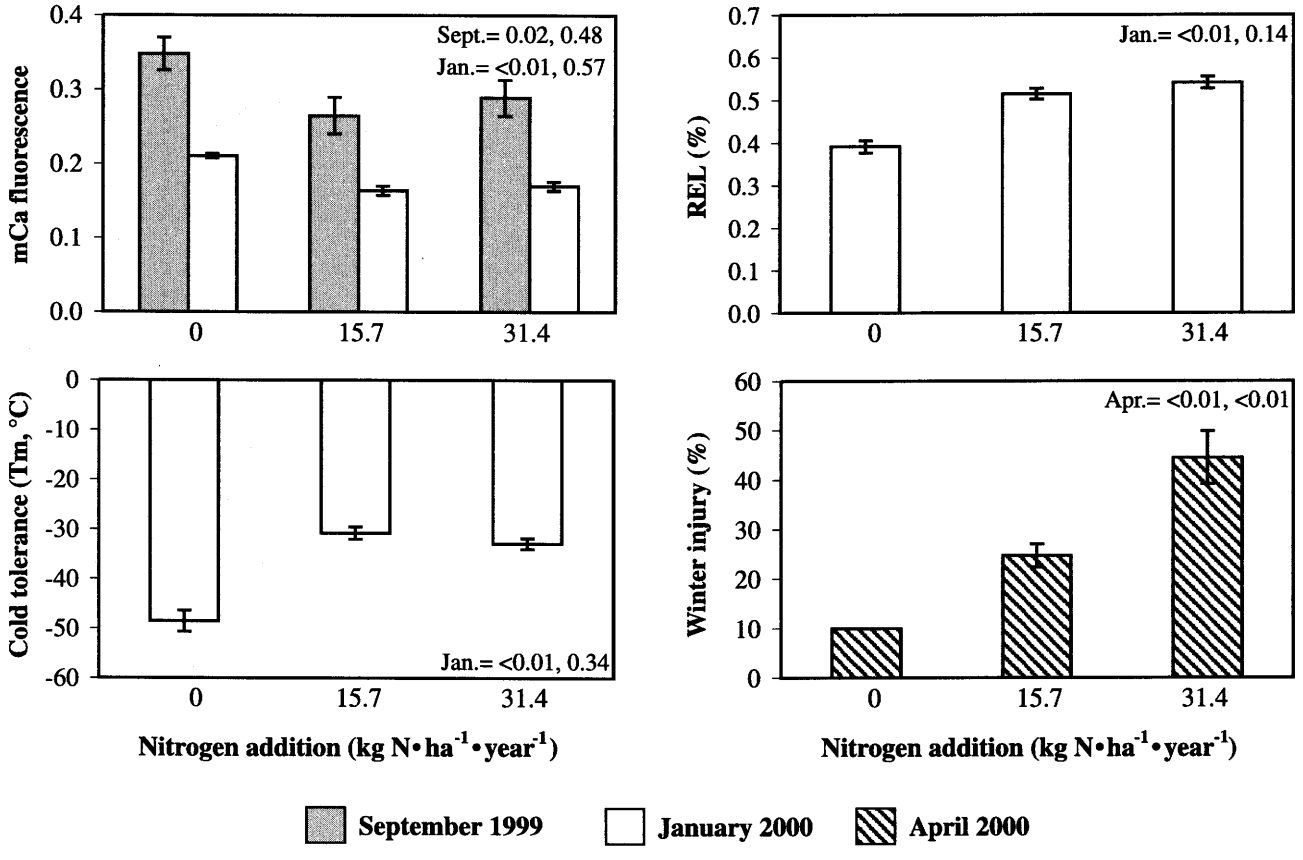
Freezing tolerance was assessed by electrolyte leakage as determined by conductivity measurements. Conductivity data were used to calculate T_m, the temperature at the midpoint of a sigmoid curve fit to electrolyte leakage data of each bulk sample per tree (Strimbeck and DeHayes 2000; Schaberg et al. 2000*c*). Electrolyte leakage assessments effectively distinguish trees or treatments with more or less cold tolerance than others and accurately discern increases or decreases in cold tolerance over time or in response to environmental disturbance or manipulation (Schaberg and DeHayes 2000).

In addition to measuring midwinter foliar cold tolerance, cumulative winter freezing injury was visually assessed in April 2000. All mature red spruce trees within sample plots were scored for visible foliar freezing injury using an injury assessment scale of 1 (1–10% of current-year shoots injured) to 10 (91–100% of current-year shoots injured).

Membrane integrity

Electrolyte leakage from plant cells is an effective method to detect changes in membrane permeability and integrity associated with osmotic (Zwiazek and Blake 1991) and water stress (Vasquez-Tello et al. 1990), high temperatures (Ruter 1996), altered mineral nutrition (Branquinho et al. 1997), cell senescence (van Bilsen and Hoekstra 1993), and exposure to acidic deposition (Schaberg et al. 2000*a*). Using the methods outlined by Schaberg et al. (2000*a*), relative electrolyte loss from nonfrozen controls used in cold-tolerance assessments (conductivity levels of needle leachates prior to freezing stress divided by the total conductivity of leachates after sample autoclaving; DeHayes and Williams 1989) was used to evaluate the effect of long-term soil N addition on membrane stability. The proportional expression of electro-

Fig. 1. Treatment means of physiological measurements for the current-year foliage of red spruce trees from the Mount Ascutney, Vermont, study site. Error bars are SEs. Statistical probabilities ($P > |t|$) of treatment differences for the September, January, and April sampling dates are listed for two orthogonal contrasts: 0 kg N·ha^{-1}·year^{-1} versus both N additions (first value), and 15.7 versus 31.4 kg N·ha^{-1}·year^{-1} (second value). Membrane-associated Ca (mCa) was measured using computer image analysis to quantify the intensity of mCa-specific fluorescent emissions. Relative electrolyte leakage (REL) from cells was used as a measure of membrane integrity. Cold tolerance was measured as T_m, the temperature at the midpoint of the sigmoid curve fit to electrolyte leakage data. Winter injury was assessed visually in the field.



lyte leakage relative to the complete electrolyte content of killed cells effectively tares out the influence of treatment-induced ion buildup and provides a specific measure of membrane integrity (Schaberg et al. 2000*a*).

Statistical analyses

Analyses of variance were used to test for differences among N treatment groups. The study followed a nested design that included the factors N treatment, plot within N treatment, and tree within plot within N treatment. Previous studies at the Mount Ascutney site have shown that the mineral nutrition and health of red spruce were influenced more by the amount of N added than by the form of N applied (McNulty and Aber 1993; McNulty et al. 1996; Schaberg et al. 1997). To further assess the impact of N additions, differences between treatment means were evaluated using two mutually exclusive orthogonal contrasts: 0 versus 15.7 and 31.4 kg N·ha^{-1}·year^{-1} and 15.7 versus 31.4 kg N·ha^{-1}·year^{-1}. These contrasts maximized the statistical power for evaluating issues that we felt were of greatest scientific concern: (*i*) the influence of long-term N fertilization relative to a control and (*ii*) the comparative impact of low versus high levels of N addition. The low level of replication (two plots per N treatment) provided limited power for detecting treatment impacts. Despite this, differences were considered statistically significant if $P \leq 0.05$.

Results

Membrane Ca, membrane stability, cold tolerance, and winter injury

Significant differences in mCa accrual associated with N treatment were found for both sample dates (Fig. 1). In September and January, trees in N addition plots had lower mCa levels than trees in control plots. Treatment-associated changes in membrane stability and cold tolerance mirrored those noted for mCa. Needles of trees from N-addition plots experienced greater electrolyte leakage (i.e., had leakier membranes) and had reduced cold tolerance relative to needles from control plot trees (Fig. 1). No differences in mCa concentration, membrane stability, or cold tolerance were detected between N addition treatments. Visible winter freezing injury was greater for trees in treatment plots relative to the control, and there was greater injury in plots that received high N relative to those given low N (Fig. 1). For all treatments, mCa concentrations fell from September to January. Past work has shown that mCa levels in red spruce current-year foliage reach a seasonal peak in the fall, de-

Table 1. September 1999 treatment means (±SE) of starch, total sugar, and component sugar concentrations ($mg \cdot g^{-1}$) in the current-year foliage of red spruce trees collected from the Mount Ascutney, Vermont, study site.

	Treatment means (kg $N \cdot ha^{-1} \cdot year^{-1}$)			$P > \|t\|$	
Carbohydrate	0	15.7	31.4	0 vs. N treatments	15.7 vs. 31.4
Starch	4.89±0.30	5.25±0.45	5.71±0.63	0.33	0.51
Total sugars	54.09±5.56	63.67±7.35	71.89±6.22	0.09	0.37
Fructose	19.18±1.93	22.85±2.50	25.51±2.17	0.08	0.40
Glucose	31.67±3.16	36.58±4.07	41.29±3.67	0.12	0.37
Raffinose	3.85±0.25	4.37±0.23	3.99±0.28	0.30	0.30
Stachyose	0.23±0.10	0.21±0.05	0.21±0.04	0.85	0.96
Sucrose	0.02±0.01	0.05±0.02	0.04±0.02	0.17	0.55
Xylose	0.28±0.05	0.33±0.04	0.52±0.11	0.10	0.06

Note: Statistical significance levels ($P > |t|$) presented are for two orthogonal contrasts: 0 kg $N \cdot ha^{-1} \cdot year^{-1}$ versus both N treatments, and 15.7 versus 31.4 kg $N \cdot ha^{-1} \cdot year^{-1}$.

Table 2. January 2000 treatment means (±SE) of starch, total sugar, and component sugar concentrations ($mg \cdot g^{-1}$) in the current-year foliage of red spruce trees collected from the Mount Ascutney, Vermont, study site.

	Treatment means (kg $N \cdot ha^{-1} \cdot year^{-1}$)			$P > \|t\|$	
Carbohydrate	0	15.7	31.4	0 vs. N treatments	15.7 vs. 31.4
Starch	0.60±0.08	1.04±0.08	0.96±0.11	0.0017	0.5313
Total sugars	71.44±2.99	91.25±5.55	85.87±2.80	0.0023	0.3489
Fructose	21.65±1.10	27.98±1.82	27.01±1.63	0.0046	0.6582
Glucose	40.45±1.82	46.31±2.40	45.82±1.83	0.0323	0.8667
Raffinose	5.91±0.95	9.90±1.27	6.71±0.83	0.0703	0.0382
Stachyose	3.26±0.76	5.10±0.96	3.06±0.50	0.3890	0.0687
Sucrose	4.15±0.42	7.05±1.07	6.33±0.72	0.0174	0.5251
Xylose	1.06±0.08	1.46±0.30	0.87±0.15	0.6578	0.0397

Note: Statistical significance levels ($P > |t|$) presented are for two orthogonal contrasts: 0 kg $N \cdot ha^{-1} \cdot year^{-1}$ versus both N treatments, and 15.7 versus 31.4 kg $N \cdot ha^{-1} \cdot year^{-1}$.

crease precipitously with the first frost, and then remain at a lower level throughout the late fall and winter (DeHayes et al. 1997).

Carbohydrate concentrations

There were no discernable treatment-associated differences in foliar starch or sugar concentrations evident in September (Table 1). In contrast, numerous differences in foliar carbohydrate concentration were detected in January (Table 2), when trees in control plots had significantly lower starch, fructose, glucose, sucrose, and total sugar concentrations than those within N treatment plots. Differences between N addition treatments were limited to raffinose and xylose concentrations; trees in the 15.7 kg $N \cdot ha^{-1} \cdot year^{-1}$ treatment had higher concentrations of both these sugars.

Discussion

Past work at Mount Ascutney showed that chronic N fertilization resulted in significant alterations of foliar nutrition and physiology, including a nearly twofold increase in N content, reductions in Ca and Mg concentrations to near deficiency levels, and increases in respiration (Schaberg et al. 1997). Treatment differences in foliar nutrition and physiology were not related to the form of N (NH_4^+ vs. NO_3^-) or anion (Cl^- vs. NO_3^-) applied but were attributed to the direct or secondary influences of N addition (Schaberg et al. 1997). Although differences in cold tolerance were not detected, trees in N-addition plots also exhibited greater winter freezing injury (Perkins et al. 2000). Differences in freezing injury could have resulted from treatment-induced alterations in foliar nutrition (Perkins et al. 2000). It was previously hypothesized that N supplements, especially late-season additions, might delay autumn cold acclimation in red spruce and result in increased winter injury (Friedland et al. 1984). However, most evidence has revealed a neutral or positive influence of N additions to red spruce cold tolerance in both seedlings (DeHayes et al. 1989; Klein et al. 1989) and mature trees (White 1996). Similarly, although soil Mg depletion has been implicated in pollution-induced forest decline (Schulze 1989), changes in Mg nutrition do not appear to alter conifer cold tolerance levels (Bigras et al. 2001). In contrast to N and Mg, Ca nutrition has been closely linked to the development and maintenance of cold tolerance in a range of plant species (Arora and Palta 1988; Monroy et al. 1993; Knight 2000) including red spruce (DeHayes et al. 1999; Schaberg et al. 2000*a*). In the current study, the influence of N addition on foliar freezing injury

was reassessed, but for the first-time measurements of physiologically available cellular Ca (i.e., mCa), membrane integrity, and cold tolerance were also made to better define how alterations in freezing injury might have occurred.

The majority of Ca in spruce needles exists as insoluble Ca oxalate and pectate crystals in cell walls and extracellular spaces (Fink 1991). In contrast to these large reserves, ions in equilibrium within the plasma membrane region (including some free and displaced apoplastic Ca from the cell wall) are biologically available and of major physiological importance. This mCa pool, although a relatively small fraction of total foliar Ca concentration, is a distinct compartment that influences membrane structural stability and the response of cells to changing environmental conditions and numerous stress signals (Palta and Li 1978; Legge et al. 1982; Davies and Monk-Talbot 1990; Atkinson et al. 1990; DeHayes et al. 1997). In accordance with its structural role, reductions in mCa within N-addition plots were accompanied by membrane destabilization and reductions in cold tolerance (Fig. 1). Relative electrolyte leakage was negatively correlated with mCa concentration on a tree-to-tree basis across treatments ($r = -0.67$, $P < 0.0001$, $n = 29$), probably because at low mCa concentrations, Ca bridges between the phosphate and carboxylate groups of membrane phospholipids and proteins (Palta and Li 1978; Legge et al. 1982; Davies and Monk-Talbot 1990) were insufficient to fully stabilize membranes and control electrolyte loss. By altering membrane architecture and reducing membrane stability, mCa depletion (or a lack of accrual) would diminish physiological control over the movement of solutions and ions across membranes, a process that enables cells to resist freezing stress.

In addition to altering membrane stability and control, treatment-induced reductions in mCa could have disrupted cellular freezing tolerance by decreasing the availability of messenger Ca. Calcium serves as an important second messenger in the perception and transduction of environmental and stress signals, including low temperatures (Hepler and Wayne 1985; Roberts and Harmon 1992; Bush 1995; Sanders et al. 1999; Pandey et al. 2000; Roos 2000). If mCa deficiency reduced the capacity of cells to sense and transduce low temperature signals, then freezing injury would likely be exacerbated as the response physiology that normally helps cells acclimate to and survive freezing stress was effectively suppressed (Schaberg et al. 2001).

Reduced foliar cold tolerance within N treatment plots likely predisposed trees to increased winter freezing injury (Fig. 1). However, levels of visible injury did not fully correspond to the pattern of treatment-induced alterations of foliar cold tolerance. Although some variation in biological response to the level of N-addition was evident during the early years of treatment (McNulty and Aber 1993), differences among N treatments diminished with time as all fertilized plots showed evidence of N saturation (McNulty et al. 1996; Schaberg et al. 1997). Treatment differences in freezing injury were an exception to this trend. Trees in high N addition plots exhibited greater foliar freezing injury, despite having cold tolerance levels indistinguishable from low-N-treated trees (Fig. 1). We were better able to discern freezing injury in the field than in laboratory-based assessments, perhaps because environmental conditions in the field are more extreme and include rapid freezing following solar warming (Perkins and Adams 1995) and repeating cycles of freezing and thawing (Lund and Livingston 1998) that can exacerbate tissue damage. In addition, whereas laboratory cold tolerance measurements assess injury following a one-time exposure to damaging low temperatures, freezing injury in the field integrates cumulative damage over the course of the winter season.

Treatment-induced changes in mCa incorporation, membrane stability, cold tolerance, and freezing injury are consistent with our hypothesis regarding the mechanism of physiological decline in the Mount Ascutney plots. However, results of carbohydrate assessments did not meet our a priori expectations. No differences in foliar carbohydrate concentrations were found at the end of the growing season (Table 1), and N treatment was associated with higher foliar concentrations of starch, sucrose, fructose, glucose, and total sugars in January (Table 2). We had expected that increased rates of carbon loss via respiration previously noted (Schaberg et al. 1997) would have reduced foliar carbohydrate concentrations for trees in N addition plots. However, this did not occur (Tables 1 and 2). Because N additions did not influence photosynthetic carbon gain (Schaberg et al. 1997), N-induced increases in respiration must have been compensated by other factors, such as reduced growth (McNulty et al. 1996), that lowered carbohydrate utilization and increased net storage. Nitrogen-induced freezing injury (Fig. 1) and foliar loss (McNulty et al. 1996) could also have raised carbohydrate concentrations in remaining foliage as fall increases in carbohydrates (Schaberg et al. 2000*b*) were concentrated into fewer and younger needles. The loss of needles in past winters would have reduced the storage potential of older foliar age-classes that are typically a dominant storage site for carbohydrates in red spruce (Schaberg et al. 2000*b*). With the capacity of distal sinks reduced, carbohydrate accumulation in current-year foliage would be favored. However they developed, it seems unlikely that foliar carbohydrate increases were causally associated with N-induced reductions in cold tolerance or increases in freezing injury. Indeed, elevated concentrations of foliar sugars, especially sucrose and raffinose, are usually associated with enhanced cold tolerance in red spruce (Schaberg et al. 2000*a*), probably because these sugars limit cellular freeze dehydration through their bulk colligative effects (Levitt 1980) or by promoting vitrification (Hirsh 1987). In N addition plots, the protective benefits of high foliar sugar concentrations were apparently offset by other changes in physiology (e.g., reduced mCa and membrane stability) that had a more fundamental influence on cold tolerance and freezing injury susceptibility.

In addition to influencing cold tolerance, low mCa accrual could also be mechanistically related to alterations in respiration previously documented for trees in N addition plots on Mount Ascutney (Schaberg et al. 1997). Increases in respiration have been noted for a range of plant types and tissues suffering from Ca deficiencies (see review by McLaughlin and Wimmer 1999) and may result from associated reductions in membrane integrity (Bangerth 1979). Cells presumably use more energy to maintain ionic parti-

tioning when mCa deficiencies disrupt membrane structure and associated control capacities.

The focus of this study was an examination of how protracted N fertilization altered foliar membranes and associated physiology. Our results documented for the first time that chronic N additions specifically depleted foliar mCa pools and simultaneously reduced membrane integrity and cold tolerance levels. Although these findings provide new insights into the possible causes of treatment-induced increases in freezing injury (Perkins et al. 2000) and decline (McNulty et al. 1996) on Mount Ascutney, the mechanism of mCa disruption remains unresolved. One possibility is that chronic treatment disrupted soil Ca supplies that are the ultimate source for foliar mCa stores. A reduction in the availability of soil cations is a hallmark of current hypotheses concerning the influence of N saturation on forest ecosystems (e.g., Aber et al. 1998; Fenn et al. 1998). Indeed, numerous mechanisms of soil cation depletion have been proposed. Within the Mount Ascutney N-treatment plots, NH_4^+ uptake by roots may have had an antagonistic effect on the uptake of base cations (Schulze 1989). The Cl^- component of N treatments likely accelerated cation leaching from soils, further reducing possible cation uptake (Reuss and Johnson 1986). Treatment-induced increases in soil nitrification (McNulty et al. 1996) would have provided another anion (NO_3^-) and released H^+ that may have exacerbated cation leaching. Anionic additions could have also increased the proportion of Al^{3+} in the soil solution, which would have reduced the uptake of other cations through competitive inhibition (Reuss and Johnson 1986). This last alternative seems unlikely for the Mount Ascutney plots, however, because foliar analyses have revealed no differences in foliar Al concentrations among treatments (Schaberg et al. 1997).

Another possibility is that chronic N additions disrupted root and (or) mycorrhizae physiology, thereby diminishing the Ca uptake capacity of trees. Evidence from European NITREX studies indicates that protracted N inputs can reduce soil densities of both fine roots (Boxman et al. 1995; Boxman et al. 1998) and mycorrhizal fungi (Boxman et al. 1998). However, because evaluations of belowground processes for Mount Ascutney plots are not yet complete, the role of treatment-induced perturbations of soil, root, and mycorrizal processes in instigating foliar mCa deficiencies, dysfunction, and decline remains unknown.

Even though carbohydrate results differed from those expected, our overall findings show a clear parallel between the nutritional and physiological consequences of chronic N fertilization and the well-documented impacts of acidic deposition exposure to red spruce foliage (DeHayes et al. 1999; Schaberg et al. 2000*a*). Whether the result of soil-based N additions (this study) or acidic deposition-induced foliar leaching (DeHayes et al. 1999; Schaberg et al. 2000*a*), treatment-induced mCa limitations were accompanied by enhanced membrane destabilization, reduced foliar cold tolerance, and an increased susceptibility to foliar freezing injury and decline. Although experimental verification of physiological impacts has been independent, both soil- and foliar-based processes are likely to be important drivers of Ca depletion within native forests. Indeed, given that many anthropogenic factors can deplete Ca from forest ecosystems, the development of biologically meaningful Ca deficiencies and associated health declines may be most likely when multiple drivers of Ca depletion (e.g., N saturation, acidic deposition, and intensive harvesting) coexist.

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