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Acid mist and soil Ca and Al alter the mineral nutrition and physiology of red spruce

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Summary We examined the effects and potential interactions of acid mist and soil solution Ca and Al treatments on foliar cation concentrations, membrane-associated Ca (mCa), ion leaching, growth, carbon exchange, and cold tolerance of red spruce (*Picea rubens* Sarg.) saplings. Soil solution Ca additions increased foliar Ca and Zn concentrations, and increased rates of respiration early in the growing season (July). Soil Al treatment had a broad impact, reducing foliar concentrations of Ca, Mg, Mn, P and Zn, and resulting in smaller stem diameters, sapling heights and shoot lengths compared with soil treatments with no added Al. Aluminum treatment also reduced respiration when shoots were elongating in July and decreased net photosynthesis at the end of the growing season (September). Three lines of evidence suggest that Al-induced alterations in growth and physiology were independent of foliar Ca status: (1) Ca concentrations in foliage of Al-treated saplings were within the range of sufficiency established for red spruce; (2) mCa concentrations were unaffected by Al treatment; and (3) no Al × Ca interactions were detected. Acid mist treatment increased foliar Fe and K concentrations and increased leaching of Ca, Mg, Mn, Zn, Fe, and Al from foliage. Leaching losses of Ca were more than twice those of the element with the next highest amount of leaching (Zn), and probably led to the reductions in mCa concentration and membrane stability of acid-treated saplings. Acidic mist resulted in enhanced shoot growth, and consistent reductions in foliar cold tolerance in the fall and winter. Of the few significant interactions among treatments, most involved the influence of mist pH and Al treatment on foliar nutrition. In general, reductions in cation concentration associated with Al addition were greater for pH 5.0-treated saplings than for pH 3.0-treated saplings. We propose that H^+-induced leaching of mCa from mesophyll cells is the mechanism underlying acid-induced reductions in foliar cold tolerance of red spruce.

Keywords: acid precipitation, aluminum, calcium, cations, cold tolerance, foliage, growth, leaching, membrane-associated calcium, photosynthesis, Picea rubens, respiration.

Introduction

Evidence from Europe and North America indicates that acid precipitation can disrupt base cation cycling and nutrition within forest ecosystems (Shortle and Smith 1988, Schulze 1989, Likens et al. 1996). Cationic (H^+) and anionic (NO_3^- and SO_4^{2-}) components of acid precipitation can act to deplete soil pools of base cations and limit the uptake and incorporation of Ca^{2+} and Mg^{2+} by trees (Likens et al. 1996). Soil acidification and NO_3^- and SO_4^{2-} additions can also increase Al availability in the soil solution (Johnson and Fernandez 1992). This Al then competes with and inhibits Ca^{2+} uptake by tree roots (Reuss and Johnson 1986, Shortle and Smith 1988). In addition, acid precipitation can leach cations from foliage (Scherbatskoy and Klein 1983, Joslin et al. 1988, Klemm et al. 1989), with Ca having the greatest rate of efflux (Klemm et al. 1989). When soil availability of Ca is low, acid-induced foliar leaching may contribute to the development of Ca deficiencies (Joslin et al. 1988).

The decline of red spruce (*Picea rubens* Sarg.) in the southern Appalachian Mountains of the United States has been linked to recent changes in Ca availability in these forests. Tree ring chemistry has shown that Ca:Al ratios have declined concurrently with tree vigor, potentially reflecting acid-precipitation-induced reductions in soil Ca availability (Bondietti et al. 1990, McLaughlin and Kohut 1992). Several studies have linked acid-deposition-induced reductions in foliar Ca concentrations to increases in foliar respiration (McLaughlin et al. 1991, McLaughlin and Tjoelker 1992, McLaughlin et al. 1993) and reductions in net photosynthesis (McLaughlin and Tjoelker 1992, McLaughlin et al. 1993). Based on these and other findings, Johnson et al. (1992) concluded that acid-deposition-induced increases in Al availability and disruptions in Ca nutrition have probably contributed to the decline of red spruce in the southern Appalachians.

In northeastern USA, red spruce decline is synonymous with freezing injury, and the link between atmospheric inputs of acid precipitation and freezing injury has been firmly established (DeHayes 1992). Exposure of red spruce foliage to either simulated (Fowler et al. 1989, Kohut et al. 1989, Waite et al. 1994) or ambient acid cloud water (DeHayes et al. 1991, DeHayes 1992, Vann et al. 1992) markedly reduces cold tolerance of current-year needles in autumn and winter. Acid-mist-induced reductions in cold tolerance can result in increases in the frequency and extent of freezing injury (Johnson et al. 1988, DeHayes 1992). Freezing injury and associated loss of foliage can reduce carbon assimilation, basal area, and height growth to values typical of those in declining trees (Wilkinson 1990, Tobi et al. 1995).

If Ca nutrition plays a role in red spruce decline in the northeastern USA, it seems probable that this influence is mediated by some perturbation in cold tolerance, because freezing injury is a major contributor to this decline in northern montane forests (DeHayes 1992, Johnson 1992, Johnson et al. 1992). Studies with crop plants have shown that Ca plays a prominent role in preventing freezing injury (Pomeroy and Andrews 1985, Arora and Palta 1986, Monroy et al. 1993, Crotty and Poole 1995). However, the influence of Ca nutrition on the cold tolerance of red spruce has not been evaluated.

We investigated direct effects and potential interactions among acid mist and soil solution Ca and Al treatments on foliar cations, ion leaching, growth, carbon exchange, and cold tolerance of red spruce. In addition, we assessed the influence of the treatments on the pool of Ca specifically associated with the plasma membrane of mesophyll cells. This pool of membrane-associated Ca (mCa) influences membrane stability and environmental message transduction, and is of particular physiological importance to plants (Atkinson et al. 1990, Roberts and Harmon 1992, Bush 1995, DeHayes et al. 1997).

Materials and methods

Plant material

In September 1992, 208 5-year-old, bare-root red spruce saplings of a Danville, VT seed source were transplanted to 25-liter plastic pots containing a coarse sand and perlite mix (4:1, v/v). The water-holding capacity of this mixture was 400 ml l^{-1} (8 l per pot), and the Ca concentration (52.4 ± 4.1 $\mu g\ g^{-1}$) was relatively low compared with native soils in red spruce forests (e.g., 31 to 123 $\mu g\ g^{-1}$, Taylor et al. 1986). Analysis of foliage before treatment revealed nutrient concentrations within the normal range reported for red spruce seedlings (Swan 1971, Friedland et al. 1988). All plants were kept outdoors at the Northeastern Research Station in Burlington, VT (44°28′ N, 73°09′ W), and received ambient precipitation. Pots were insulated with perlite from November to April of each year to protect roots from low air temperatures.

Treatments

The experimental design was a split plot and included six experimental blocks of 32 saplings each. Within each block, saplings were randomly assigned to one of four Ca (0, 25, 75, or 225 µM) and two Al (0 or 200 µM) soil watering treatments as part of a factorial arrangement, and were split between two mist treatment plots (pH 5.0 or 3.0). Calcium and Al treatments were obtained by adding $CaSO_4$ or $Al_2(SO_4)_3$, or both, to a modified base nutrient solution designed to maintain rhizosphere pH and Al solubility (Cumming and Brown 1994). This modified base solution contained (µM): NH_4NO_3 (200), KH_2PO_4 (50), K_2SO_4 (75), $MgSO_4$ (50), H_3BO_3 (25), $MnSO_4{\cdot}H_2O$ (2), $ZnSO_4{\cdot}7H_2O$ (2), $CuSO_4{\cdot}5H_2O$ (0.5), and Na_2MoO_4 (0.5). All soil solutions were adjusted to pH 4.0 ± 0.1 with H_2SO_4. The soil treatments bracketed the range of Ca concentrations (David and Lawrence 1996) and approached the maximum Al concentrations (Miller et al. 1992, Joslin and Wolfe 1992) reported for soil solutions in red spruce forests. Soil solutions were delivered through individual tubes with a pump-manifold system that automatically provided each plant with 1 l day^{-1} for 7 days a week from July through October 1993 and 1994. Soil applications were made in the morning to recharge soils with the designated Ca or Al treatment following the evening mist additions.

Saplings were misted for 6 h, four nights per week from July through October 1994. During each application, plots received 6 mm of mist. Mist treatments comprised a base mist solution adjusted to pH 3.0 or pH 5.0 with H_2SO_4. Although ambient cloudwater contains HNO_3, this was not included in the mist treatments because differential N additions can themselves alter foliar Ca concentrations in red spruce (Swan 1971, Schaberg et al. 1997). The ionic composition of the base mist solution was patterned after results from a regional analysis of cloud chemistry (Li and Aneja 1992) and consisted of (µM): NH_4NO_3 (150), $(NH_4)_2SO_4$ (12.5), NaCl (10), KCl (5), and $MgSO_4{\cdot}7H_2O$ (2.5).

A second experiment was conducted in 1997–98 to evaluate further the impacts of acid mist on foliar mCa concentrations, membrane stability, and cold tolerance. For this experiment, we used saplings from the same planting, with the same seed source and potting medium described above, but the saplings received only ambient precipitation following transplanting in 1992. This experiment was a completely randomized design with four experimental blocks of four saplings. Within each block, two saplings were randomly assigned to either a pH 3.0 or 5.0 mist treatment. Most aspects of mist treatment (e.g., base mist solution composition, volume and weekly schedule of mist application) followed protocols previously described. However, in this experiment, SO_4^{2-} concentrations of pH 3.0 and 5.0 mist treatments were equalized by titrating both treatments to pH 3.0 with H_2SO_4, and then adding NaOH to the pH 5.0 solution to achieve the target pH. Another difference from the primary study was that mist treatments were applied only during autumn (September through November 1997).

Foliar cation concentrations

Analyses were conducted on current-year needles from the upper third of the crown of one sapling per treatment × block combination (96 saplings) in July and November 1994, and February 1995. Following each collection, samples were oven-dried and then digested with sulfuric acid and hydrogen peroxide as oxidizers, selenium as a catalyst, and lithium sulfate to elevate the boiling point (Parkinson and Allen 1975). Digestions were performed in 75-ml reflux tubes in a AD-4020 block digestor (Westco Scientific Instruments, Danbury, CT). Foliar samples were predigested at room temperature overnight and then digested at 250 °C for 20 min and

450 °C for 1.5–2 h. Digests were diluted to 75 ml with distilled, deionized water and analyzed for cations by inductively coupled plasma atomic emission spectroscopy (ICPAES, PlasmaSpec 2.5, Leeman Labs, Lowell, MA). White pine needles from the National Bureau of Standards and Technology (SRM 1575) were also digested and analyzed as a procedural check.

Collection and analysis of leachate

On five dates in July, August and September 1994, throughfall samples were collected from two saplings per treatment combination within each of two blocks during experimental mist application on days with no ambient precipitation. Throughfall was collected with 55-mm diameter funnels, emptying into 60-ml polypropylene bottles, located beneath current-year shoots within the sapling canopies. Funnels were secured to the shoots by a loop of string at the base of each flush. Two additional funnels per plot collected mist solutions before they came in contact with foliage. Mean cation concentration of incoming mist solutions collected with these funnels was subtracted from measurements of throughfall to estimate actual leaching from shoots. Cation concentrations were measured by ICPAES. Only Ca, Al, Mg, Mn, Zn, K, P and B concentrations were above the detection limits.

Membrane-associated calcium (mCa)

Half of the saplings that received the 0 and 225 μM Ca treatments (48 saplings) were assessed for concentrations of membrane-associated Ca (mCa) in September and November 1994, and January 1995. On each date, three needles per sapling were collected and sectioned to produce three sections per needle for mCa evaluation. In addition, two needles per sapling were collected and processed for mCa assessment in February 1998 as part of the 1997–98 supplemental experiment. 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 when specific excitation and barrier microscope filters are used (Borer et al. 1997).

Cold tolerance

Cold tolerance was assessed in one sapling per treatment × block combination of the main study (96 saplings) in November 1994, and January and February 1995 and in all 16 saplings in the supplemental study in February 1998. Current-year foliage from each plant was rinsed in iced distilled water, then chopped in 5-mm sections to prepare a single bulk sample. Subsamples of approximately 0.3 ml (i.e., 0.1 to 0.2 g) were transferred to 64-cell polystyrene trays for freezing treatment in a computer-controlled liquid nitrogen freezer (Model Kryo 10, TS Scientific, Perkasie, PA). Freezing rate was 6 $^\circ C\ h^{-1}$. Trays were held at eight to 12 pre-selected test temperatures, then removed from the freezer and placed in pre-cooled polystyrene foam containers to thaw slowly to 4 °C.

Freezing injury was assessed by electrolyte leakage as determined by conductivity measurements. Conductivity data were used to calculate critical temperatures (the highest temperature at which freezing injury can be detected) for each bulk sample per sapling. DeHayes (1992) has shown that critical temperatures are correlated with freezing injury in the field and that they are effective in discerning cold tolerance differences among imposed treatments, plant tissues, and needle age classes. Detailed descriptions of freezing procedures and critical temperature calculations have been published by DeHayes and Williams (1989) and DeHayes et al. (1990).

Membrane integrity

Electrolyte leakage from plant cells can be used 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), and cell senescence (van Bilsen and Hoekstra 1993). Cellular electrolyte loss is also useful for evaluating the influence of acid deposition on membrane stability. In February 1998, we obtained conductivity data for non-freeze-stressed foliar samples collected from the 1997–98 supplemental study to evaluate the effect of differential H^+ addition on membrane stability. Mist treatments in this study had equal SO_4^{2-} concentrations, so evaluation of electrolyte loss from these foliar samples allowed us to evaluate H^+ effects on membrane integrity without the confounding influence of differential SO_4^{2-} addition. Because of additional H^+ and the greater conductivity of H^+ relative to other ions, the pH 3.0 mist had a higher conductivity than the pH 5.0 mist even though buffering ions were added to the pH 5.0 mist. Therefore, higher electrolyte leakage from pH 3.0-treated foliage relative to pH 5.0-treated foliage could result from either incomplete surficial removal and foliar uptake of applied H^+, or treatment-induced reductions in membrane integrity and associated increases in electrolyte loss from within cells, or some combination of the two. Our measurements indicated that only about 6.8 ± 2.5% of overall foliar electrolyte leakage following acid treatment resulted from H^+ leaching into solution (authors' unpublished data). In addition, total electrolyte loss from autoclaved foliage subjected to the pH 3.0 and 5.0 treatments in the 1997–98 supplemental study was minimal and did not differ significantly between the treatments ($P = 0.29$, $n = 16$), further suggesting that treatment-associated H^+ residues were small relative to cellular electrolyte concentrations. To account for any small treatment-associated H^+ contributions, we used relative electrolyte loss (conductivity of needle leachates before freezing stress divided by the total conductivity of leachates after autoclaving (DeHayes and Williams 1989)) from each sample to assess acid mist effects on membrane integrity. We found that the relative electrolyte leakage measurements provided a useful indicator of acid-induced differences in membrane stability.

Foliar gas exchange and stem growth

Net photosynthesis and dark respiration were measured for one sapling per treatment × block combination (96 saplings) in July, September and November 1994. Measurements were made on one freshly excised, current-year shoot from the upper third of each crown with an LI-6200 portable photosynthesis system with a 0.25 l cuvette (Li-Cor Inc., Lincoln, NE). The LI-6200 was calibrated each day with a span gas CO_2 concentration of 350 $\mu l\ l^{-1}$, and zero settings of the IRGA and flow meter were adjusted regularly between sample measurements. Measurements were made immediately (usually within 2 min) following branch excision. A 110-130V/300W metal halogen lamp (General Electric Company, Cleveland, OH) was used to provide saturating photon flux densities for photosynthesis measurements ($950 \pm 30\ \mu mol\ m^{-2}\ s^{-1}$, Alexander et al. 1995). Stomatal conductance was measured concomitantly with photosynthesis. Respiration measurements were made after darkening the cuvette with a double-layer of cloth for approximately 1 min (McLaughlin et al. 1990, Schaberg et al. 1996). For each photosynthesis and respiration measurement, three 30-s gas exchange readings were averaged to provide one rate per shoot. For all samples, projected needle areas were measured with a Li-Cor LI-3100 leaf area meter. Rates of photosynthesis and respiration were assessed on a projected needle area and dry weight basis. Both standardizations of gas exchange measurements exhibited the same response to treatments. Only area-based results are presented.

Sapling heights, stem diameters (5 cm above the root collar), leader lengths, and the lengths of current-year lateral shoots in the upper whorl were measured in August 1994 following growth cessation as determined by periodic measurements.

Statistical analyses

Analyses of variance (ANOVA) were used to test for treatment differences among means as well as two- and three-way interactions among treatments. Because of the split-plot design of the experiment, the block × mist mean square error (MSE) was used to test the significance of mist treatment effects. Other treatment effects were tested with the experiment-wise MSE, which included other interactions with block. Differences among soil solution Ca treatments were determined by the Tukey-Kramer HSD test. When interactions between treatments were detected, differences between specific means were determined by partitioning the sums of squares for the interaction term using orthogonal contrasts. We also used ANOVAs to test for differences between means attributable to mist pH in the 1997–98 supplemental study. Differences were considered statistically significant if $P \le 0.10$.

Results

Foliar cation concentrations

Treatment effects on cation concentrations were similar on all three assessment dates, although concentrations increased slightly over time. To simplify presentation, only the November 1994 data are presented (Table 1). Soil solution Ca and Al amendments modified foliar Ca concentrations as well as the concentrations of several other cations. Total foliar Ca concentrations generally increased with increasing soil Ca treatment (Table 1). The Al treatment increased foliar Al concentrations and reduced foliar Ca, Mg, Mn, Zn, and P concentrations (Table 1). The pH 3.0 mist application increased foliar concentrations of Fe and K relative to those of pH 5.0-misted saplings (Table 1).

Of the few significant interactions detected among treatments (Figure 1), most involved the influence of mist pH × Al treatment on foliar nutrition. Although the specific dynamics of the interaction varied among the elements (Ca, Mg, Zn and P), the basic trend was similar; i.e., reductions in cation con-

Table 1. Effects of acid mist and soil solution Ca and Al treatments on element concentrations in the current-year foliage of red spruce seedlings. Data presented are for November 1994. Similar results were found for analyses conducted in July 1994 and February 1995.

Factor	Treatment	Mean foliar element concentration (mg kg^{-1})								
		Al	B	Ca	Fe	K	Mg	Mn	P	Zn
Mist pH	3	181	30	2262	62*[1]	9610***	713	463	2010	31
	5	152	19	2246	7	8459	760	494	1813	34
Soil Ca	0	164	23	1733a[2]***	34	8812	762	461	1865	29a**
	25	162	24	2125b	25	9007	726	469	1835	28a
	75	179	26	2435bc	30	9489	768	539	1990	37b
	225	161	24	2743c	49	8796	688	445	1954	37b
Soil Al	0	138***	22	2562***	25	8953	865***	520***	1978*	39***
	200	196	27	1926	45	9109	600	435	1839	26

[1] Significance values: *,** and *** denote $P \le 0.10$, 0.05 and 0.01, respectively (ANOVA).

[2] Calcium treatment means with the same letter are not significantly different ($P \le 0.10$) based on the Tukey-Kramer HSD test.

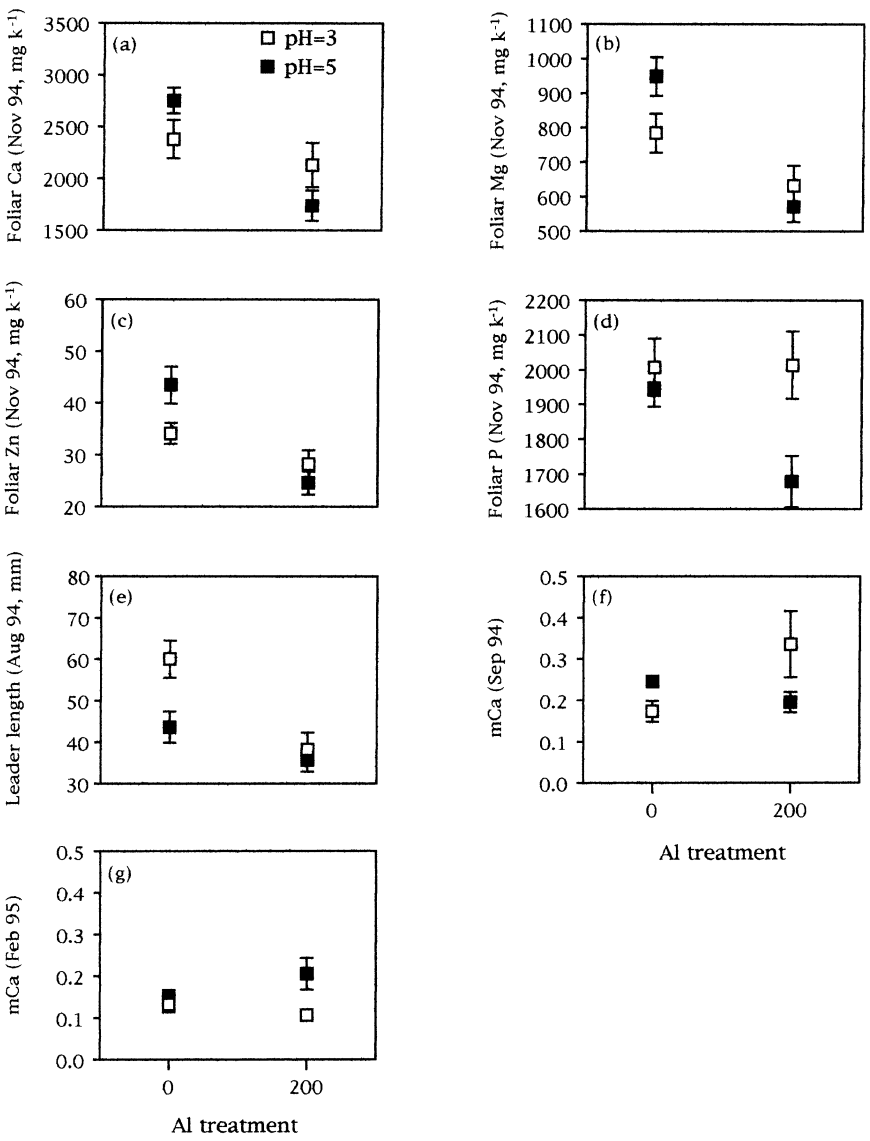

Figure 1. Significant Al × acid mist interactions on foliar Ca (a), Mg (b), Zn (c), and P (d) concentrations, leader length (e), and September 1994 (f) and February 1995 (g) mCa concentrations of red spruce seedlings. Interactions were significant ($P \leq 0.05$) for all parameters depicted based on analysis of variance. Error bars are ± 1 SE from means.

centrations associated with Al addition were greater for pH 5.0-treated saplings than for pH 3.0-treated saplings.

Cation leaching

The pH 3.0 acid mist treatment enhanced leaching of Ca, Zn, Al, Mg, Fe, and Mn from current-year shoots compared with the pH 5.0 acid mist treatment (Table 2). Among the cations for which the pH 3.0 acid mist treatment significantly increased leaching, Ca had the highest loss (approximately 2.25 times the concentration of the element with the next highest amount of leaching, Zn). Leachates from pH 3.0-treated saplings had Ca concentrations comparable with those of throughfall samples from montane red spruce exposed to ambient acidic mists (Joslin et al. 1988). Although Ca treatment had no effect on the extent of cation leaching, treatment with Al was associated with a reduction in Mg concentrations in leachate samples that may have resulted from the Al-induced decrease in foliar Mg concentration (Table 1).

Membrane-associated Ca

Although soil solution Ca and Al additions had no direct effect on foliar mCa concentrations, saplings treated with pH 3.0 mist had lower mCa concentrations than saplings treated with pH 5.0 mist on two of three dates assessed (November 1994 and January 1995) (Table 3). The influence of pH 3.0 mist on mCa was also observed in the supplemental study conducted in 1997–98 (Table 3). Significant acid mist × Al treatment interactions were detected in September 1994 and January 1995 (Figure 1). The pH 3.0 mist treatment decreased foliar mCa concentrations in September for saplings that received 0.0 µM Al, and in January for saplings that received 200 µM Al.

Cold tolerance

Acid mist treatment consistently reduced the cold tolerance of current-year foliage (Table 4). Mean reductions in cold tolerance (3.5, 5.6, and 4.6 °C in November, January, and February, respectively) were comparable to decreases documented for red spruce saplings exposed to ambient acid cloud water in late autumn (3 °C) and winter (5 °C) (DeHayes et al. 1991). An even larger reduction in cold tolerance (8 °C) was detected for acid-treated saplings in the supplemental experiment that received equal concentrations of SO_4^{2-} across mist treatments (Table 4). No effects of soil solution Ca or Al treatments on cold tolerance were detected and no interactions among treatments were found.

Table 2. Effects of acid mist and soil solution Ca and Al treatments on leaching of cations from current-year foliage of red spruce seedlings in August 1994. Similar results were found for analyses conducted in July and September 1994.

Factor	Treatment	Mean element concentration in leachate ($\mu g\ l^{-1}$)								
		Al	B	Ca	Fe	K	Mg	Mn	P	Zn
Mist pH	3	68***[1]	35	233***	38***	221	38**	6***	17**	104***
	5	8	15	64	0	153	22	1	6	23
Soil Ca	0	32	23	172	14	214	35	3	37	61
	25	27	17	101	4	45	26	2	33	54
	75	38	26	165	30	275	27	4	59	74
	225	51	33	150	24	216	30	4	29	63
Soil Al	0	45	32	162	22	317	37***	4	51	69
	200	30	19	133	14	71	23	3	29	58

[1] Significance values: ** and *** denote $P \leq 0.05$ and 0.01, respectively (ANOVA).

Table 3. Effects of acid mist and soil solution Ca and Al treatments on membrane-associated Ca (mCa) in the current-year foliage of red spruce seedlings.

Factor	Treatment	Mean relative mCa concentration[1]			
		September 94	November 94	January 95	February 98
Mist pH	3	0.213	0.161**[2]	0.118**	0.204*
	5	0.221	0.190	0.177	0.246
Soil Ca	0	0.208	0.178	0.148	–
	225	0.226	0.173	0.146	–
Soil Al	0	0.209	0.175	0.140	–
	200	0.225	0.175	0.154	–

[1] Values presented are adjusted pixel brightness ratios that range from 0 (no Ca) to 1 (high Ca) (Borer et al. 1997).
[2] Significance values: * and ** denote $P \leq 0.10$ and 0.05, respectively (ANOVA).

Table 4. Effects of acid mist and soil solution Ca and Al treatments on cold tolerance of current-year foliage of red spruce seedlings.

Factor	Treatment	Mean critical temperature[1] (°C)			
		November 94	January 95	February 95	February 98
Mist pH	3	–44.5***[2]	–51.5**	–44.9**	–44.5**
	5	–48.0	–57.0	–49.4	–53.8
Soil Ca	0	–45.4	–53.8	–45.6	–
	25	–47.0	–56.2	–49.9	–
	75	–45.2	–51.5	–46.5	–
	225	–47.2	–55.8	–46.7	–
Soil Al	0	–46.5	–54.0	–47.8	–
	200	–45.9	–54.6	–46.6	–

[1] Temperature indicated is the highest at which significant electrolyte leakage occurred.
[2] Significance values: ** and *** denote $P \leq 0.05$ and 0.01, respectively (ANOVA).

Table 5. Effects of acid mist and soil solution Ca and Al treatments on diameter, height and shoot lengths of red spruce seedlings measured August 1994.

Factor	Treatment	Mean growth parameters (mm)			
		Stem diameter	Total height	Lateral shoot length	Leader length
Mist pH	3	10.2	442.5	35.4*[1]	49.2*
	5	10.4	440.8	31.9	39.8
Soil Ca	0	10.3	443.2	32.3	40.0
	25	10.2	431.5	32.4	45.7
	75	10.6	450.9	36.0	47.2
	225	10.3	441.2	33.8	44.3
Soil Al	0	10.5***	450.2***	37.4***	51.7***
	200	10.1	433.1	29.9	37.1

[1] Significance values: * and *** denote $P \leq 0.10$ and 0.01, respectively (ANOVA).

Table 6. Effects of acid mist and soil solution Ca and Al treatments on mean photosynthesis (P, µmol m^{-2} s^{-1}), respiration (R, µmol m^{-2} s^{-1}), and photosynthesis to respiration ratios (P:R) for red spruce seedlings.

Factor	Treatment	July 1994			September 1994			November 1994		
		P	R	P:R	P	R	P:R	P	R	P:R
Mist pH	3	8.92	2.21	4.62	9.65	2.09	5.09	4.22	0.97	4.74
	5	9.44	2.28	4.75	10.15	2.03	5.54	4.42	0.97	4.86
Soil Ca	0	9.77	1.90 a**[1,2]	5.66 a**	10.04	2.04	5.32	4.48	1.04	4.68
	25	9.02	2.37 b	4.34 b	10.26	2.25	4.92	4.39	0.92	4.99
	75	9.39	2.22 b	4.72 b	9.55	1.94	5.36	3.99	0.94	4.63
	225	8.55	2.48 b	4.03 b	9.73	2.00	5.62	4.44	0.98	4.90
Soil Al	0	8.82*	2.47**	4.08**	10.29**	2.10	5.43	4.40	0.95	4.92
	200	9.56	2.01	5.32	9.50	2.03	5.18	4.24	0.99	4.68

[1] Calcium treatment means with the same letter are not different ($P \leq 0.10$) based on the Tukey-Kramer HSD test.

[2] Significance values: * and ** denote $P \leq 0.10$ and 0.05, respectively (ANOVA).

Membrane integrity

In the supplemental study in February 1998, there was greater electrolyte leakage from current-year needles of pH 3.0-treated saplings than from current-year needles from pH 5.0-treated saplings (mean relative electrolyte leakage of 0.38 and 0.25, respectively, $P = 0.06$, $n = 16$), indicating that acid mist destabilized plasma membranes. Enhanced electrolyte leakage from pH 3.0-treated foliage significantly exceeded conductivity differences expected for treatment-associated H^+ residues and the enhancement was evident even when differential H^+ loading was accounted for by assessing relative conductivity. Acid-induced membrane destabilization occurred even though saplings only received the mist treatments from September to November 1997.

Growth

Saplings treated with pH 3.0 mist had longer leader and lateral shoots than saplings treated with pH 5.0 mist (Table 5). Soil Al additions resulted in significant reductions in all measurements of stem growth (Table 5). A mist pH × Al treatment interaction for leader length was also evident and indicated that increases in leader length associated with the pH 3.0 treatment were offset by the inhibitory influence of added soil Al (Figure 1). The Ca treatment had no effect on stem growth.

Gas exchange

Photosynthetic rates were high through September (Table 6), but by November, photosynthetic rates had decreased to less than half of maximal rates. For all dates, rates of respiration were approximately 20% of photosynthetic rates. Stomatal conductances in July and September were 165 ± 3 and 172 ± 3 mmol l^{-1} s^{-1}, respectively. These values are similar to those reported for red spruce during the growing season (Eamus and Fowler 1990, Amundson et al. 1992), whereas the November values (52 ± 2 mmol l^{-1} s^{-1}) indicated some stomatal restriction. No treatment differences in stomatal conductance were found (data not shown), and no differences in net photosynthesis or respiration attributable to acid mist treatment were detected (Table 6). However, several alterations in gas exchange associated with soil solution treatments were found (Table 6). Soil treatment effects were most evident in July when soil Al addition resulted in lower respiration rates and higher net photosynthesis and photosynthesis to respiration ratios (*P*:*R*). Because periodic measurements indicated that shoots were still elongating in July (data not shown), it seems probable that the Al treatment reduced not only growth (Table 5) but also growth-associated respiration (Table 6), thereby increasing net photosynthesis (Table 6). Calcium additions resulted in higher rates of respiration and lower *P*:*R* ratios in July. Calcium-induced increases in respiration were not the result of differential stem growth (Table 5), but may have been associated with alterations in some unmeasured parameter (e.g., root growth, and rate or timing of shoot development) that influenced respiration. In September, the only effect of treatment on gas exchange was an Al-induced reduction in net photosynthesis. No treatment-associated differences in carbon exchange were evident in November.

Discussion

Calcium additions had comparatively little influence on foliar chemistry and physiology, whereas Al additions reduced foliar concentrations of Ca, Mg, Mn, P and Zn, disrupted gas exchange during July and September, and resulted in smaller stem diameters, plant heights, and shoot lengths. Reductions in aboveground growth in response to approximately 200 μM Al have previously been reported for young (≥ 1year old) red spruce (Thornton et al. 1987, Joslin and Wolfe 1988, Cumming and Brown 1994, Schier 1996); however, declines in root growth are generally more pronounced (Thornton et al. 1987, Joslin and Wolfe 1988, Schier 1996). Our findings indicate that Al-induced reductions in stem growth can also occur in older (7-year-old) saplings grown outdoors.

While shoots were elongating in July, Al addition decreased respiration and increased net photosynthesis (Table 6), probably as a result of Al-induced reductions in growth (Table 5). Presumably because of the timing of measurements and the prominence of growth respiration, our results contrast with reports of Al-induced reductions in net photosynthesis (McCanny et al. 1995) and increases in respiration (McLaughlin et al. 1990, 1991) for mature shoots. However, measurements made in September following growth cessation were more consistent with the literature, and showed reductions in net photosynthesis in response to the Al treatment (Table 6).

Reductions in net photosynthesis have been reported for red spruce seedlings grown in sand culture in the presence of 250 μM Al (McCanny et al. 1995). Increases in respiration and reductions in *P*:*R* ratios have also been documented for montane red spruce with high foliar Al concentrations (McLaughlin et al. 1990, 1991). Aluminum may influence gas exchange and growth through an indirect effect on plant mineral nutrition, particularly Ca nutrition (McLaughlin et al. 1990, 1991). The concomitant effects of Al on plant nutrition and physiology that we observed could be interpreted as evidence of this indirect influence. However, other evidence indicates that Al can be directly phytotoxic (Haug 1984, Kochian 1995) as a result of binding with orthophosphate groups of critical biomolecules such as nucleotides, nucleic acids, ATP, calmodulin, membrane phospholipids, ATPases, and other enzymes.

Although Al treatment reduced the concentrations of six elements in foliage (Table 1), the resulting concentrations were within the range of sufficiency established for red spruce seedlings (Swan 1971). The finding that Al treatment reduced sapling growth even though foliar concentrations of Ca and other elements were well above reported deficiency values is suggestive of a toxicity mechanism that is separate from the influence of Al on nutrition. Aluminum-induced reductions in growth were also found by Joslin and Wolfe (1988) for red spruce seedlings with sufficient foliar Ca and Mg concentrations. Similar findings were recently reported for red spruce cells grown in suspension, where Al-induced reductions in cell growth occurred without consistent effects on cellular Ca, Mg or Mn concentration (Minocha et al. 1996). In our study, the absence of significant Al × Ca interactions on growth suggests that the effect of Al was not the result of Ca deficiency. Furthermore, Al-induced reductions in growth were not ameliorated by soil solution Ca additions. The independence of Al toxicity development and Ca nutrition is also supported by the lack of an Al treatment effect on mCa concentrations (Table 3). Aluminum addition reduced growth and altered gas exchange but had no influence on this biologically important Ca pool.

Whatever the mechanism of Al toxicity, our data indicate that Al concentrations within the range reported for soil solutions in red spruce forests (Miller et al. 1992) can disrupt the mineral nutrition, carbon relations and growth of red spruce saplings. However, Al treatment had no effect on foliar cold tolerance. Because freezing injury is a fundamental component of red spruce decline in the northeastern USA, the lack of a response in cold tolerance to Al treatment raises questions about the relevance of Al-induced changes in physiology to red spruce decline in northern forests.

Although soil Ca and Al treatments both influenced total foliar Ca concentrations, neither treatment altered mCa concentrations. Total foliar Ca and mCa concentrations are not well correlated at total Ca concentrations above deficiency values (DeHayes et al. 1999). This lack of correspondence probably results from the accretion of Ca in the apoplast of needles when Ca is abundant, where it accumulates as insoluble Ca oxalate crystals within cell walls (Fink 1991). These crystals typically comprise the bulk of total Ca within spruce needles (Fink 1991). Despite their growth in low Ca soils, even plants that received 0 μM supplemental Ca had total foliar Ca concentrations (Table 1) above the 800 mg kg^{-1} deficiency value determined for red spruce seedlings (Swan 1971). Although Ca and Al treatments altered the accumulation of total foliar Ca (which is dominated by Ca oxalate crystals), these treatments had no influence on the physiologically important pool of Ca associated with the plasma membranes of mesophyll cells (mCa).

Acid mist treatment resulted in increased foliar Fe and K concentrations (Table 1), possibly as a result of the increased availability of these cations in acidified soils (Bohn et al.

1985). Acid additions also increased leaching of Ca, Mg, Mn, Zn, Fe, and Al from foliage (Table 2). Numerous studies have shown that acid deposition leaches an array of cations, including Ca, Mg, K, Mn, Zn, and Fe, from foliage (Scherbatskoy and Klein 1983, Mengel et al. 1987, Joslin et al. 1988, Klemm et al. 1989, Schier and Jensen 1992). It has been proposed that acid deposition-induced cation leaching could result from inputs of H^+, or SO_4^{2-}, or both (Edwards et al. 1995). However, reductions in H^+ concentrations in throughfall following foliar contact without similar changes in SO_4^{2-} concentrations (Joslin et al. 1988, McLaughlin et al. 1996) indicate that leaching losses are not driven by SO_4^{2-} inputs, but are more likely the result of H^+-induced cation exchange. The relative importance of H^+ versus SO_4^{2-} additions to cation leaching is also highlighted by additional data from our laboratory. In a separate study, we found significantly greater cation leaching (P = 0.05) from saplings treated with pH 3.0 mist than from saplings treated with pH 5.0 mist, even when mists contained no SO_4^{2-} (DeHayes et al. 1999).

Similar to results from both field (Joslin et al. 1988) and simulation (Edwards et al. 1995) studies, leaching losses of Ca were considerably greater than losses of other cations. Preferential leaching of Ca was probably associated, in part, with the relative abundance of Ca compared with many other foliar elements (Table 1). The location of elemental pools may have also influenced leaching. Although cations such as K have a greater permeability to transport through the cuticle, Ca can exhibit a greater rate of leaching, presumably because of its high concentration in cell walls (Klemm et al. 1989). Leached cations are derived from the surface or intercellular free space of foliage (Schier and Jensen 1992), and Ca is unique among cations in that a dominant and physiologically important pool exists external to the cytoplasm (Marschner 1986). However, not all extracellular Ca is equally vulnerable to leaching. The major fraction of Ca in red spruce needles exists as insoluble Ca-oxalate crystals in the apoplast (Fink 1991). In contrast to this large, but stable pool, Ca associated with the plasma membrane (mCa) is more readily available for acid-induced cation exchange and loss through the cuticle. This differential susceptibility of extracellular Ca pools is reflected in our data. Calcium leaching resulted in significant reductions in mCa, whereas no changes in total foliar Ca were detected. Furthermore, because the supplemental study had similar SO_4^{2-} concentrations in pH 3.0 and 5.0 mists, and because mist application occurred only in autumn, it is evident that the leaching-induced mCa reduction is independent of SO_4^{2-} exposure and can be induced after growth has ceased.

In addition to the high vulnerability of Ca to leaching loss, Ca depletion may be harder to overcome than the loss of other foliar cations because Ca is not mobile in the phloem (Marschner 1986). Leaching-induced reductions in other cations can be compensated for by phloem transport, whereas the resupply of depleted Ca is dependent on recharge through the xylem. Reliance on Ca input by the transpiration stream might particularly limit the re-supply of Ca leached during autumn, when transpiration rates are typically low (Havranek and Tranquillini 1995). The finding that acid-mist-induced reductions in mCa were not detected until November may reflect the influence of seasonal declines in Ca supply on the development of mCa deficits.

Acid mist treatment significantly enhanced shoot growth (Table 5), probably as a result of H^+-induced increases in cell wall loosening and growth (Salisbury and Ross 1985). Although these results are corroborated by other reports of acid-induced increases in red spruce growth (Taylor et al. 1986, Lee et al. 1990), numerous reports of negative (McLaughlin and Kohut 1992, McLaughlin et al. 1993) or neutral (Seiler and Paganelli 1987, Laurence et al. 1989, Kohut et al. 1990) growth effects also exist.

No acid-induced alterations in net photosynthesis or respiration were detected (Table 6). Reports of neutral or positive effects of acid deposition on photosynthesis predominate in the literature (Taylor et al. 1986, Seiler and Paganelli 1987, Kohut et al. 1988, Laurence et al. 1989, Kohut et al. 1990, Eamus and Fowler 1990, Thornton et al. 1990, Thornton et al. 1994). However, the lack of a respiration response in our study contrasts with the documented acid-induced increases in respiration reported by McLaughlin and Tjoelker (1992) and McLaughlin et al. (1993). This discrepancy may reflect differences in aggregate H^+ loads applied. Although total water additions were somewhat greater in our study, acid mist inputs were approximately four times greater in the McLaughlin studies (McLaughlin and Tjoelker 1992, McLaughlin et al. 1993). Although acid inputs did not alter respiration rates in our study, they caused other effects on cation relations (leaching and mCa concentrations), growth, and physiology (cold tolerance).

Acid mist exposure significantly reduced the extent of fall and winter cold tolerance (Table 4) (cf. Fowler et al. 1989, DeHayes et al. 1991, Jacobson et al. 1992, Vann et al. 1992, Waite et al. 1994). Although the chemical composition of cloud water in the eastern United States is dominated by NO_3^-, SO_4^{2-}, NH_4^+, and H^+ (Mohnen 1992), evidence indicates that H^+ ions are responsible for reducing the foliar cold tolerance of red spruce during winter. For example, although anionic additions, especially SO_4^{2-} (Cape et al. 1991), can decrease foliar cold tolerance in autumn (Cape et al. 1991, Jacobson et al. 1992), only H^+ additions reduce cold tolerance in both autumn and winter (Jacobson et al. 1992). Data from our 1997–98 supplemental study indicated that the pH 3.0 mist treatment resulted in an 8 °C reduction in cold tolerance relative to the pH 5.0 treatment (Table 4) even though mist solutions contained similar concentrations of SO_4^{2-}. Significant reductions in winter cold tolerance have also been observed in saplings treated with acid mists containing no NO_3^-, SO_4^{2-} or NH_4^+ (solutions were acidified with HCl) (DeHayes et al. 1999).

Despite evidence for the specific influence of H^+ on cold tolerance, the mechanism by which H^+ inputs alter cold tolerance has not been explored. We propose that acid-induced leaching of mCa from mesophyll cells is the mechanism for H^+-induced reductions in cold tolerance. Calcium in the plasma membrane cell wall region of plant cells functions in the protection of plants from low temperature injury. Calcium is an integral component of cell membrane structure and function, stabilizing membranes by bridging phosphate and carboxylate groups of membrane phospholipids and proteins (Palta and Li 1978, Legge et al. 1982, Davies and Monk-Talbot 1990). The plasma membrane plays a central role in cold acclimation and low temperature injury in plants (Pomeroy and Andrews 1985, Davies and Monk-Talbot 1990, Steponkus 1990). By influencing membrane architecture, Ca influences solution movement across membranes, the ability of cells to resist dehydration, extracellular ice damage, and, at least in some cases, intracellular freezing during cold acclimation. Extracellular Ca, including membrane-associated Ca (Atkinson et al. 1990), also serves as an important second messenger and plays a crucial role in the perception and transduction of low temperature signals during cold acclimation (Dhindsa et al. 1993). Studies with crop plants have demonstrated that the loss or restriction of Ca enhances susceptibility to freezing injury (Pomeroy and Andrews 1985, Arora and Palta 1986, Monroy et al. 1993, Crotty and Poole 1995), whereas the addition of Ca to the extracellular solution can prevent freezing injury (Arora and Palta 1988). If H^+ of acid mist displaced and leached Ca from the plasma membrane, resulting losses of mCa could destabilize membranes and enhance their susceptibility to freezing injury.

The effect of the acid mist treatments on mCa pools that we document supports a mechanism by which acidic inputs increase the susceptibility of red spruce to freezing injury. Acid deposition can increase the solubility of soil Al and interfere with the availability and uptake of Ca (Clarkson and Sanderson 1971, Haynes 1980, Godbold et al. 1988, Cronan 1991). Our manipulations of soil solution Al and Ca concentrations within the range reported for red spruce forests altered total foliar Ca and Al concentrations as well as other foliar cation concentrations. Treatment-induced reductions in total foliar Ca reflect a reduction in extracellular Ca accumulation as a result of low soil availability or Al-induced reductions in Ca uptake and assimilation. Importantly, soil-based Ca and Al treatments had measurable effects on sapling physiology: they altered sapling growth (Al only) and gas exchange (Ca and Al). However, perhaps because Ca and Al manipulations did not alter critical mCa concentrations, these treatments did not influence cold tolerance. In contrast, acid mist leached Ca from foliage, reduced mCa concentrations in mesophyll cells, and consistently decreased foliar cold tolerance. In addition, acid-induced mCa reductions were accompanied by significant declines in membrane stability that were evident before freezing stress, but were exacerbated by subfreezing temperatures (i.e., reduced cold tolerance). Combined with extensive documentation of the important role that Ca plays in plant cold tolerance, these results support the hypothesis that acid-mist-induced reductions in cold tolerance are mediated through a depletion of mCa. Foliar leaching of mCa resulted in cold tolerance reductions even among plants given Ca abundant soil treatments and containing total foliar Ca concentrations that seemed sufficient (Swan 1971). Although direct acid-induced disruption of mCa appeared to override soil Ca influences, greater correspondence between soil Ca and mCa pools would be expected in more severely limited soil Ca environments because soil Ca is the ultimate source of foliar mCa.

Acid-deposition-induced Ca deficiency has been largely acknowledged as the explanation for the reduced growth of red spruce in the southern Appalachians, whereas freezing injury has been largely accepted as the explanation for the mortality and reduced growth of red spruce in the northern Appalachians (Johnson et al. 1992). The proposed mCa–cold tolerance relationship (acid-deposition-induced Ca depletion) provides a unifying and viable explanation for red spruce decline throughout much of its range.

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