

Accretion, partitioning and sequestration of calcium and aluminum in red spruce foliage: implications for tree health

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Received September 19, 2003; accepted February 8, 2004; published online July 1, 2004

Summary Calcium (Ca) is an essential macronutrient in plants and is an important component of many cellular structures and physiological processes as well as overall forest function. Aluminum (Al) in soil solution can inhibit Ca uptake by plants and disrupt many Ca-dependent metabolic and physiological processes of plants. The ratio of Ca to Al in soil solution can be an important indicator of forest health, especially on acid soils. We used sequential chemical extractions (water, acetic acid and hydrochloric acid) to assess the chemical availability of Ca and Al in foliage from mature red spruce (*Picea rubens* Sarg.) trees growing under ambient environmental conditions. In plants deficient in Ca and with intermediate total foliar Ca concentration ([Ca]), Ca preferentially accrued in labile and physiologically available forms (water- and acetic acid-extractable). In plants with total foliar [Ca] above a “sufficiency” threshold, Ca also accrued in a chemically sequestered form with low solubility (HCl-extractable), suggesting that Ca sequestration is an inducible process in response to excess foliar Ca. Because it has low solubility, it is likely that sequestered Ca is unavailable for Ca-dependent physiological processes. Immobilization of Al in foliage was related to Ca sequestration, suggesting that Ca sequestration may provide a passive mechanism for Al tolerance in the foliage of these trees. Aluminum immobilization was evident based on the ratio of HCl-extractable Al to the more labile (water- and acetic acid-extractable) forms of Al. Sufficient labile Ca combined with Al sequestration was associated with plant health, including enhanced foliar accretion of Mg and Mn, greater tree growth, enhanced foliar cold hardiness and reduced winter injury. These findings demonstrate that not all chemical forms of foliar Ca and Al are of equal physiological significance and underscore the importance of assessing the biologically significant element forms in biogeochemical research.

Keywords: acid rain, Ca:Al, foliar extractions, membrane associated Ca (mCa), nutrient availability, *Picea rubens*.

Introduction

Long-term acidic deposition causes a reduction in soil avail-

ability of base cations. Acidifying processes inundate soils with hydrogen ions (Lynch et al. 1996, Butler et al. 2001, Driscoll et al. 2001), which replace base cations such as calcium (Ca) on soil exchange sites (Cronan 1991), leaving them susceptible to leaching (Likens et al. 1996, 1998). Soil acidification also increases the solubility of aluminum (Al), which competes with Ca for soil exchange sites (Lawrence et al. 1995). Further, high concentrations of available soil Al have been linked to reduced root uptake of mineral nutrients including Ca (Cronan 1991, Lawrence et al. 1995) as a result of impaired root growth (Schofield et al. 1998) or blockage of Ca channels (Huang et al. 1992, 1996).

Ratios of soil-solution or exchangeable Ca to Al (Ca:Al) have been widely used to assess site vulnerability to plant damage and decline as a result of acidifying processes and Al toxicity (e.g., Shortle and Smith 1988, Cronan and Grigal 1995, Heisey 1995). Cronan and Grigal (1995) reviewed many published articles and found that the most useful parameter for site comparisons was free Ca:Al in the soil solution, although they also found that foliar ratios of these elements are weakly related to plant growth and foliar nutrient concentrations. Cronan and Grigal (1995) estimated the threshold value of soil Ca:Al associated with reduced forest health primarily from the results of controlled experiments with young trees grown in hydroponic, sand or soil culture with varied inputs of Ca and Al. Few studies have evaluated the role and significance of Ca:Al in mature trees growing under natural conditions. In addition, the focus of most studies on Ca:Al has been soil solution chemistry and the resultant potential for impaired plant growth or nutrition. Details of the physiological processes affected and the mechanisms of impairment or tolerance in plants exposed to unfavorable soil Ca:Al remain undefined.

Calcium is an essential macronutrient that provides structural stability to cell membranes and walls, but is cytotoxic even at moderate concentrations because it precipitates with inorganic phosphate (e.g., Hepler and Wayne 1985, Bush 1995, Knight 2000). Sequestration of excess Ca outside the symplasm is therefore essential. Chemical precipitation with oxalate either within the cell or in the cell wall plays an impor-

tant role in foliar Ca sequestration (e.g., Franceschi and Horner 1980, Borchert 1986, Fink 1991); the relatively insoluble Ca oxalate crystals can represent a high proportion of total foliar Ca (Fink 1991). Thus, analyses of total foliar Ca concentrations indicate little about the true physiological status of Ca in plant foliage because they can be dominated by crystalline forms with low biological availability. In irrigated sand cultures, Swan (1971) defined thresholds for foliar calcium concentration ([Ca]) sufficiency ($> 1200 \text{ mg kg}^{-1}$) and deficiency ($< 800 \text{ mg kg}^{-1}$) for red spruce (*Picea rubens* Sarg.). Although the thresholds defined under these conditions may have little relevance for trees of other ages or growing under other conditions, DeHayes et al. (1999) reported a similar threshold for Ca deficiency associated with impaired midwinter cold hardiness in mature red spruce trees growing under ambient environmental conditions. DeHayes et al. (1999) found a significant linear relationship between cold hardiness and total foliar [Ca] only below this threshold.

Unlike Ca, Al plays no known beneficial physiological role. Research on Al toxicity and tolerance has centered primarily on the root apex, which is thought to be one of the primary sites of Al toxicity (e.g., Kochian 1995, Matsumoto 2000). Plant roots exhibit various species-specific mechanisms to reduce Al toxicity, including prevention of Al uptake (Taylor 1991, Kochian 1995) and cellular Al tolerance (Taylor 1991, Matsumoto 2000, Shen et al. 2002). Because of its charge density, Al may displace Ca on exchange sites in cell walls, including those comprising the xylem, thus influencing Ca mobility within plants (McLaughlin and Wimmer 1999). Although there is ample evidence that Al can accumulate in the foliage of many tree species (e.g., Young and Guinn 1966, David et al. 1988, Robarge et al. 1989, Heisey 1995), few studies have examined mechanisms of toxicity and tolerance once Al has accumulated. The broad spectrum of Al toxicity mechanisms (e.g., associating with chromatin, displacing Ca from the plasma membrane and cell walls, altering membrane permeability, altering phosphate metabolism (Kochian 1995, Matsumoto 2000)) indicates that Al toxicity should occur in a wide range of tissues and cell types unless Al is sequestered in an unavailable or nontoxic form or location. Without mechanisms for foliar detoxification, the potential exists for substantial Al toxicity, particularly in species that cannot avoid foliar Al accumulation. Research has recently begun to elucidate the role of organic acids in short-term mechanisms of foliar Al tolerance in Al hyperaccumulators (Ma et al. 1997, Shen and Ma 2001, Watanabe and Osaki 2002). However, little is known about foliar Al tolerance in other species, especially trees that accumulate only moderate amounts of foliar Al under ambient environmental conditions.

Although questions about the relationship of Ca and Al to the health and productivity of forest systems are pertinent to many tree species, red spruce decline in the northeastern United States provides a useful context in which to consider the likely physiological significance. The widespread and well-documented decline of red spruce (e.g., Johnson 1992) has been attributed to low Ca:Al (Shortle and Smith 1988, McLaughlin and Tjoelker 1992, Shortle et al. 1997). A Ca-spe-

cific mechanism to explain this decline was described by DeHayes et al. (1999). Reduced soil Ca availability, in conjunction with acid-induced foliar Ca leaching, can deplete the small, but physiologically important pool of foliar membrane-associated Ca (mCa). This, in turn, can impair foliar cold hardiness, leading to increased winter injury and species decline. Evidence indicates that this type of disruption is not unique to red spruce, but may occur in a variety of tree species (DeHayes et al. 1999).

Our study objective was to compare the biological significance of traditional measures of total foliar [Ca] and Al concentration ([Al]) with that of chemically separable foliar pools of these elements. We used sequential chemical extractions to determine the partitioning of Ca and Al into readily extractable versus relatively insoluble portions within red spruce foliage, and evaluated the relationship of these and total foliar [Ca] and [Al] to an array of measurements of tree growth and physiology. Earlier work (DeHayes et al. 1999) indicated that foliar Ca might have its greatest influence on physiology at concentrations below the threshold of foliar deficiency. Therefore, we selected trees to include a range of total foliar [Ca] from deficient to sufficient (Swan 1971).

Materials and methods

Plant material and site characteristics

Foliage samples were obtained from 41-year-old red spruce trees in a stand near Colebrook, Coos County, NH. The plantation is on level terrain at roughly 715 m elevation and the soil is predominantly Plaisted silt loam (Williams et al. 1943). Red spruce has a shallow rooting habit, with the majority of roots that are responsible for nutrient uptake growing in organic soil and in the top 2 cm of mineral soil (Burns and Honkala 1990). Samples were collected from the rooting zone with a soil corer. The forest floor and the relatively dark Oa soil horizon were identified based on color and texture (Fanning and Fanning 1989), and were clearly distinguishable from the yellow/orange horizon below. Mean depths of the forest floor and Oa layer were 1.3 and 2.3 cm, respectively. Exchangeable soil [Ca] and [Al] were determined by Modified Morgan extraction (McIntosh 1969) with ammonium acetate (pH 4.8) followed by analysis by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP/AES) with a Perkin Elmer Optima 3000DV ICP/AES (Perkin Elmer, Wellesley, MA). Mean soil exchangeable [Ca] and [Al] in the forest floor were 421 and 254 mg kg^{-1} , with 78 and 373 mg kg^{-1} in the Oa horizon, respectively. The exchangeable soil [Ca] was low compared with published data for spruce and spruce-fir forests (e.g., 1300–2360 mg kg^{-1} in the forest floor, McNulty et al. 1991; and 421–4409 mg kg^{-1} in the Oa layer, Lawrence et al. 1997). The soil Ca:Al (molar ratio) at the site was about 1.12 in the forest floor, comparable with the 1:1 ratio estimated by Cronan and Grigal (1995) to produce a 50% risk of adverse impacts on tree growth or nutrient uptake. Foliar [Ca] from red spruce at this site was also low compared with published foliar [Ca] for this species (e.g., Friedland et al. 1988, Robarge et al.

1989, Huntington et al. 1990). The 12 study trees were selected, based on previous work at this site, to include trees below the reported threshold of deficiency (800 mg kg^{-1}), and within the transition zone between deficient and sufficient foliar [Ca] ($800\text{--}1200 \text{ mg kg}^{-1}$) (Swan 1971). Sampling emphasized inclusion of trees with low foliar [Ca] to better assess the physiological consequences of Ca deficiency. Sun-exposed foliage from the upper third of the crown was collected in February 2002, and current-year and 1-year-old samples were processed separately.

Foliar cation analysis

Sequential extractions (Falkowski and Kukulka 1980, Fink 1991) were performed to assess element availability within the foliage. Five separate samples from each tree were flash-frozen in liquid nitrogen, freeze-dried, ground to produce a powder (2-mm mesh) and stored at -60°C for analysis. Samples (0.3 g) were sequentially extracted in 10 ml each of distilled deionized water, 2 N acetic acid and 2 N HCl. For each extraction step, samples were shaken overnight at room temperature, centrifuged at 2800 rpm for 8 min and the supernatant was collected with a pipet. To prevent carry-over or dilution, samples were rinsed with 40 ml of distilled deionized water and dried at 50°C overnight between extraction steps. Extraction was followed by cation analysis by ICP/AES. Blanks and duplicates were run for procedural verification, and were within acceptable limits. We report data only for cations that were consistently above instrument detection limits (Al, Ca, potassium (K), magnesium (Mg) and manganese (Mn)).

We calculated three additional parameters to help evaluate the potential physiological significance of Ca and Al partitioning and sequestration. First, we defined "available" [Ca] as the sum of water- and acetic acid-extractable [Ca]. Next, the ratio of sequestered (HCl-extractable) [Al] to available [Al] was calculated to represent the capacity of foliage to sequester Al in a form that is relatively unavailable and inert. We also calculated the Ca to Al molar ratio based on standard foliar digestions (rather than the sum of the extractions), allowing direct comparisons with published results (e.g., Cronan and Grigal 1995).

Membrane-associated calcium (mCa) was assessed in current-year foliage only, by standard fluorescence microscopy techniques (Borer et al. 1997) that have been used in various studies (DeHayes et al. 1997, Jiang and Jagels 1999, Schaberg et al. 2000, 2002) to determine the physiological Ca status of red spruce foliage. Four needles from each of five twigs per tree were sectioned and stained with chlorotetracycline (CTC), followed by evaluation by epifluorescence microscopy and digital image analysis. Chelation of CTC to divalent cations in close proximity to an apolar environment, such as a biological membrane, results in a conformational change in the CTC molecule, substantially increasing fluorescence intensity over that in an entirely polar environment (Caswell and Hutchinson 1971). Techniques and instrumentation have been evaluated to verify that Ca is the cation measured by these methods (Borer et al. 1997).

For total cation analysis, foliage samples from each tree

were oven-dried (65°C), ground to produce a powder (2-mm mesh), and digested by heating with nitric acid and hydrogen peroxide (adapted from Jones and Case 1990). Sample digestion was followed by ICP/AES to assess total foliar cation concentrations. We report data for elements that were consistently above detection limits for the sequential extractions as described above. Pine needles (SRM 1575) from the National Institute of Standards and Technology, sample duplicates and blanks were analyzed for procedural verification, and were within acceptable limits of variation.

Other response parameters

Because reductions in midwinter cold hardiness have been linked to perturbations in foliar [Ca] and to red spruce decline (e.g., DeHayes et al. 1999, Schaberg et al. 2000), cold tolerance was measured to further investigate the relationship between this important physiological response variable and foliar cation status. Cold tolerance was assessed by measuring relative electrolyte leakage of foliage (DeHayes and Williams 1989) at a series of test temperatures ranging from -15°C (expected to produce no cellular injury) to -90°C (expected to produce substantial cellular injury). Cold tolerance values (T_m) represent the temperature midway between uninjured and fully killed foliage, and were calculated by a nonlinear curve-fitting procedure applied to electrolyte leakage data for all test temperatures (Strimbeck and DeHayes 2000). Winter injury, which can result from insufficient development of cold tolerance and is also related to red spruce decline, is assessed annually at the site in 10% visible injury increments. Regionally, red spruce winter injury in the spring of 2003 was particularly widespread and severe, and data from 2003 allowed us to investigate the relationship between foliar Ca and Al partitions and vulnerability to subsequent winter injury events. Because tree growth can be closely related to soil Ca and Al availability (e.g., Cronan and Grigal 1995), we also measured tree diameter at breast height (DBH) at the time of foliage collection.

Statistical analysis

Regression and correlation analyses were performed to examine the relationship between foliar pools of Ca and Al (mCa, total foliar [Ca] and [Al], and sequentially extractable pools) and other parameters (cold tolerance, winter injury, DBH and foliar concentrations of other nutrients). The DBH data were transformed (arctan) to normalize the distribution. The relationship between acetic acid-extractable [Ca] and total foliar [Ca] was determined by a nonlinear curve-fitting procedure. Paired *t*-tests were performed to compare needle age classes. When data did not adhere to model assumptions, the non-parametric signed-rank test was used.

Results

Calcium accretion and partitioning

We present data for current-year foliage only; similar trends were observed in 1-year-old foliage. We found a significant linear relationship between total foliar [Ca], based on standard

foliar digestions and the sum of the sequential extractions ($r = 0.91$, $P < 0.001$; Figure 1A). The slope of the regression (0.87) was close to 1 and the intercept (57.0 mg kg^{-1}) was less than 10% of the [Ca] of the lowest sample. This close relationship demonstrates that the sum of sequential extractions is a good approximation of total foliar [Ca]. In the following analyses, the sum of the Ca contents from the three extractions is reported as total foliar [Ca] unless noted otherwise.

Relationships between [Ca] of the extractions and total foliar [Ca] are illustrated in Figure 1B, with greater proportions of acetic acid-extractable [Ca] and HCl-extractable [Ca] evident at higher total foliar [Ca]. In red spruce, 800 mg kg^{-1} is the total foliar [Ca] below which foliage is considered Ca-deficient (Swan 1971) and physiologically impaired (DeHayes et al. 1999). We found a significant increase in water-extractable [Ca] with increasing total foliar [Ca] only below this threshold of Ca deficiency (Table 1, Figure 1C) and no significant relationship above 800 mg kg^{-1} , indicating preferential accumulation to the most labile pool in Ca-deficient foliage. In contrast, there was a significant linear pattern of accretion of Ca into the HCl-extractable fraction only above a total foliar [Ca] of 800 mg kg^{-1} , with substantial accretion into this pool above 1200 mg kg^{-1} (Table 1, Figure 1C), a value described by Swan (1971) to be the minimum sufficient [Ca]. Below a total [Ca] of 800 mg kg^{-1} , there was no relationship between HCl-extractable and total foliar [Ca] (Table 1, Figure 1C), demonstrating that chemical Ca sequestration, presumably by crystallization with oxalate, occurs only after foliar Ca has accumulated to a sufficient concentration. The acetic acid-extractable [Ca] increased with total foliar [Ca] throughout the range studied ($r = 0.98$, $P < 0.001$; Figure 1C). The relationship be-

tween total foliar [Ca] and Ca accretion into the acetic acid-extractable fraction was best approximated by a nonlinear curve: acetic acid-extractable [Ca] = baseline + ((maximum - baseline)/(1 + $e^{k(\text{midpoint} - \text{sum Ca})}$)) with a baseline acetic acid-extractable [Ca] of 190 mg kg^{-1} , a maximum acetic acid-extractable [Ca] of 686 mg kg^{-1} , and a total foliar [Ca] midpoint of 1052 mg kg^{-1} for accretion of acetic acid-extractable Ca ($P \leq 0.05$; Figure 1D). Data from the sequential extractions illustrate different patterns of Ca accretion to these three pools as total foliar [Ca] increases from deficient through sufficient. In Ca-deficient foliage, Ca accrues first to the water-extractable pool. Acetic acid-extractable Ca accumulates as water-extractable [Ca] reaches a plateau. Finally, HCl-extractable Ca accumulates as acetic acid-extractable [Ca] plateaus.

Foliar Al partitioning

As verification of Al extraction efficiency, we found a significant linear relationship between total foliar [Al] based on standard procedures for foliar digestion and the sum of the three extractions ($r = 0.92$, $P < 0.001$; Figure 2A), demonstrating that the sum of the sequential extractions is a good approximation of total foliar [Al]. For the analyses described below, the sum of the [Al] from the three sequential foliar extractions is reported as total foliar [Al] unless noted otherwise. Although Ca partitioning was significantly related to total foliar [Ca] (Figure 1), the only chemical partition of Al that was significantly related to total [Al] was water-extractable [Al] ($r = 0.71$, $P = 0.010$; Figure 2B and 2C), perhaps indicating that this is the form of Al that is translocated in the foliage. Overall, Al partitioning to the three extractable pools was much less de-

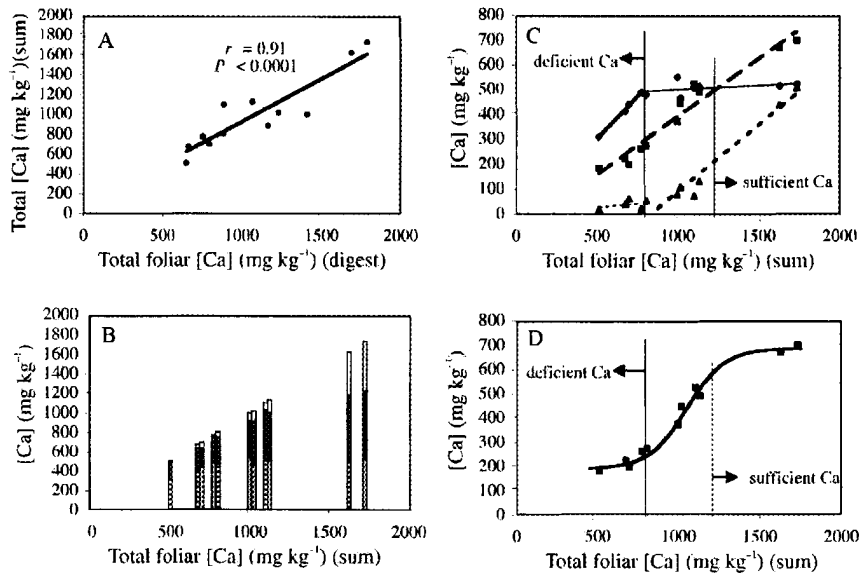


Figure 1. Foliar Ca concentrations ($[\text{Ca}]$; mg kg^{-1}) in sequential extractions of current-year red spruce needles. (A) Comparison of two methods to determine total foliar [Ca]. The sum of the three extractions is plotted against total foliar [Ca] determined by a standard foliar digestion procedure. (B) Stacked bar plot of water-extractable [Ca] (light grey), acetic acid-extractable [Ca] (dark grey) and HCl-extractable [Ca] (white) plotted against total foliar [Ca] (sum of the three extractions). (C) Water-extractable [Ca] (\blacklozenge , solid lines), acetic acid-extractable [Ca] (\blacksquare , dashed line) and HCl-extractable [Ca] (\blacktriangle , dotted lines) plotted against total foliar [Ca] (sum of the three extractions). (D) Acetic acid-extractable [Ca] versus total foliar [Ca] (sum of the three extractions) with a nonlinear curve fit. Vertical lines in (C) and (D) indicate Swan's (1971) ranges of total foliar deficiency and sufficiency.

Table 1. Correlations of Ca concentrations ([Ca]) (current-year foliage) in water- and HCl-extractions with total foliar [Ca] (sum of the three extractions), above and below Swan's (1971) foliar [Ca]-deficiency threshold of 800 mg kg⁻¹.

Extraction fraction	Total foliar [Ca] < 800 mg kg ⁻¹		Total foliar [Ca] > 800 mg kg ⁻¹	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Water	0.997	0.003	0.38	0.401
Hydrochloric acid	0.32	0.683	0.97	< 0.001

pendent on total Al accumulation than was the case for Ca partitioning.

Foliar Al partitioning, based on sequential extractions, was significantly related to foliar Ca partitioning. We found a weak positive relationship ($r = 0.51$, $P = 0.087$) between HCl-extractable [Ca] and [Al], and a significant inverse relationship ($r = -0.67$, $P = 0.017$) between acetic acid-extractable [Al] and HCl-extractable [Ca]. In contrast, there was no significant relationship between either the water-extractable [Al] or the total foliar [Al] and the HCl-extractable [Ca].

Calculated parameters

The results of sequential extractions were strongly related to other important measures of plant health. Although individual fractions of Ca and Al were significantly related to physiological response variables, the importance of foliar Ca and Al partitioning can be best understood by combining partitions (Tables 2 and 3). Available (sum of water- and acetic acid-extractable) [Ca] was significantly correlated with all response variables, with borderline significance only for its correlation with historical winter injury. These correlations suggest that this pool of foliar Ca has a unique physiological significance. In comparison, the correlations associated with the commonly reported total foliar Ca:Al were much weaker (Table 2). As ex-

pected, mCa was positively correlated with available [Ca] (Table 2). There was no relationship between foliar [Al] and mCa. Accretion of mCa thus appears to have been driven by Ca that was available or exchangeable in the apoplast, but was unaffected by Al toxicity or sequestration in the foliage. The ratio of sequestered (HCl-extractable) [Al] to available [Al] represents a tree's capacity to protect itself from Al toxicity by sequestering Al in a form that is relatively unavailable and inert. This ratio was significantly correlated with foliar Mg and Mn concentrations and tree growth, and was a significant predictor of subsequent winter injury, but was not significantly related to cold tolerance, historical winter injury, or mCa (Table 2). For comparisons with previously published studies, total foliar Ca:Al was calculated based on standard total foliar digestion procedures and found to be less closely related to response parameters (Table 2). Foliar Ca:Al was a significant predictor of subsequent winter injury, and was significantly correlated with tree growth and foliar Mn concentration, but was not related to cold tolerance, historical winter injury, mCa or foliar Mg concentration.

Foliar chemical partitioning of other elements

Foliar concentrations of all elements (Table 3) were within the previously reported ranges for mature red spruce growing un-

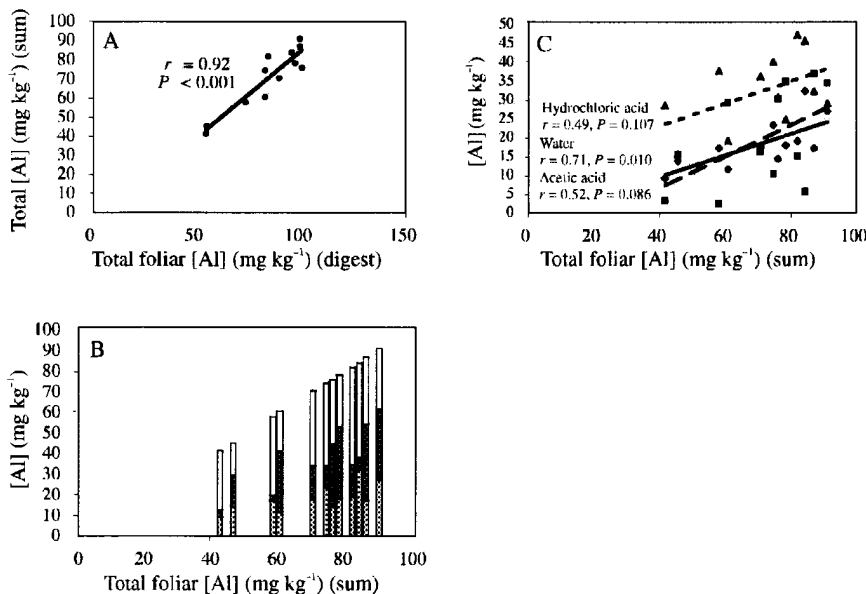


Figure 2. Foliar Al concentrations ([Al]; mg kg⁻¹) in sequential extractions of current-year red spruce needles. (A) Comparison of two methods to determine total foliar [Al]. The sum of the three extractions is plotted against total foliar [Al] that was determined via a standard foliar digestion procedure. (B) Stacked bar plot of water-extractable [Al] (light grey), acetic acid-extractable [Al] (dark grey) and HCl-extractable [Al] (white) plotted against total foliar [Al] (sum of the three extractions). (C) Water-extractable [Al] (◆, solid line), acetic acid-extractable [Al] (■, dashed line) and HCl-extractable [Al] (▲, dotted line) plotted against total foliar [Al] (sum of the three extractions).

Table 2. Correlations of physiological response variables with available foliar Ca concentration ([Ca]) (sum of the water and acetic acid extractions), with the ratio of HCl-extractable [Al] (HCl Al) to available [Al], and with the molar ratio of total foliar Ca:Al (based on standard foliar digestions). Abbreviations: DBH = tree diameter at breast height; and mCa = foliar membrane-associated Ca.

Response variable	Available foliar [Ca]		HCl Al:available [Al]		Ca:Al molar ratio	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Cold tolerance (T_m)	-0.63	0.028	-0.52	0.086	-0.37	0.231
Winter injury						
1988–1992	-0.56	0.057	-0.30	0.341	-0.36	0.246
2003	-0.73	0.010	-0.62	0.044	-0.66	0.019
DBH	0.65	0.036	0.89	0.001	0.73	0.014
mCa	0.67	0.018	0.41	0.191	0.48	0.114
Total foliar Mg	0.70	0.011	0.62	0.032	0.49	0.105
Total foliar Mn	0.59	0.043	0.73	0.008	0.76	0.004
HCl Al:available [Al]	0.80	0.002	–	–	0.83	0.001
Ca:Al molar ratio	0.84	0.001	–	–	–	–

der ambient environmental conditions (e.g., Safford et al. 1977, Friedland et al. 1988, Huntington et al. 1990). Data from the water-, acetic acid- and HCl-extractable fractions showed a wide range in total concentrations (sum of the three extractions) and differences in cation partitioning among the elements (Figure 3, Table 3). As expected, virtually all of the foliar K was extractable by water alone. Just over 80% of foliar Mg and Mn was extractable by water, with the rest of the Mg and Mn extractable by acetic acid. None of these nutrients (K, Mg or Mn) had any appreciable portion sequestered in a form extractable by HCl. There was no indication of differential foliar partitioning to the three extractable pools through the range of total foliar Mg, Mn and K. In contrast, both Ca and Al showed substantial partitioning to each of the three extraction pools (Table 3, Figure 3).

Comparison of foliar age classes

We found significantly more Ca and Mn in 1-year-old foliage than in current-year foliage and significantly more Mg and K in current-year foliage (Table 3). There were no significant differences between current-year and 1-year-old foliage in total [Al] or partitioning to the three extraction pools. In agreement with previous studies (Strimbeck and DeHayes 2000), we found cold hardiness to be significantly greater in older foliage than in current-year foliage (Table 4). We also found more available Ca and a larger Ca:Al in older foliage than in current-year foliage, presumably because of continued Ca accretion throughout the life of the foliage. We also found a greater ratio of HCl-extractable [Al] to available [Al] in older foliage (Table 4), although the individual parameters used to calculate this ratio did not differ significantly between foliar age classes (Table 3).

Discussion

Foliar element partitioning

Chemical and physical partitioning of elements in plant tissues is essential to mineral nutrition and physiology (Marschner

1995). Sequential chemical extractions of red spruce foliage demonstrated differences among elements in the relative proportion of foliar content that is readily available and physiologically significant (Figure 3). Because K is highly mobile and forms only weak complexes in plants (Marschner 1995), virtually all of the foliar K we measured was extractable by water. The acetic acid-extractable Mg likely represents the proportion that is bound in chlorophyll molecules and associated with pectate in the cell walls and was within the expected range for this pool (Marschner 1995). Because Mn complexes with numerous enzymes, it is not surprising that 20% of foliar Mn was not water extractable. As expected, based on the mineral nutrition of these elements, we found no appreciable K, Mg, or Mn sequestered in a form extractable by HCl alone. We also found no indication of differential foliar partitioning to the three extractable pools through the ranges of these elements. In contrast, substantial amounts of both Ca and Al were found in all three extractions (Table 3, Figure 3) and the relative proportions of these elements varied considerably throughout the range studied.

Calcium partitioning and physiological significance

Although it is an essential plant nutrient, Ca is toxic in the cytoplasm, even in moderate concentrations, because it precipitates inorganic phosphate. Cells have extensive systems of membrane channels and pumps that actively sequester Ca in organelles and in the apoplast, and thus maintain the essential cellular partitioning and localization of Ca. Cells also sequester excess Ca by chemical precipitation with oxalate in the cell wall region of conifers (Fink 1991) and in the vacuoles of many angiosperms (Franceschi and Horner 1980, Webb 1999). Fink (1991) noted that Ca oxalate can represent a high proportion of the total foliar Ca content. Cells depend on cytoplasmic Ca in signal transduction processes, which enable cells to respond to many environmental and hormonal cues and potential stresses (e.g., McAinsh and Hetherington 1998, Trewavas 1999, Berridge et al. 2000).

Our data showing differential foliar Ca availability are consistent with this understanding of Ca physiology and partition-

ing. We found that Ca-deficient red spruce trees preferentially partitioned foliar Ca to chemical forms that were extractable by water and acetic acid (Figure 1), which are likely to be the most physiologically important chemical forms of foliar Ca. Water-extractable Ca is immediately available for processes such as signal transduction, and was the first pool to accrue in Ca-deficient foliage. Acetic acid-extractable Ca is associated with pectin in the cell wall (Fink 1991), and accretion to this pool was best described by a nonlinear curve (Figure 1D). Acetic acid-extractable [Ca] remained low until a sufficient amount accrued to the water-extractable pool. We found minimal accumulation of acetic acid-extractable Ca below Swan's (1971) total foliar deficiency threshold (800 mg kg⁻¹) and no additional accumulation into this pool above Swan's sufficiency threshold (1200 mg kg⁻¹). Acetic acid-extractable [Ca] thus increases with total foliar [Ca] only for trees in an intermediate range of total [Ca] (between 800 and 1200 mg kg⁻¹). The water-extractable pool and most of the acetic acid-extractable pool are likely to be in dynamic equilibrium; Ca from each may be mobilized to fulfill the functions of the other. In contrast to water- and acetic acid-extractable Ca, the HCl-extractable Ca is chemically sequestered, presumably precipitated as Ca oxalate (Fink 1991). Marschner (1995) described Ca oxalate as only "sparingly soluble," and Franceschi (1989) reported that Ca oxalate can only be remobilized for a brief period after crystallization in idioblasts.

An appreciable quantity of this chemically sequestered Ca occurred only within the documented total foliar sufficiency range (Figure 1), above 1200 mg kg⁻¹ (Swan 1971). Chemical Ca sequestration, presumably by means of oxalate synthesis, thus appears to be an inducible response to excess available Ca, and not a constitutive process in red spruce foliage. The biochemical pathways by which oxalate is synthesized have not been elucidated, but it appears that different pathways may be important in different plant species (Webb 1999). It is plausible that production of enzymes responsible for oxalate biosynthesis is stimulated by foliar Ca in excess of a threshold concentration. Calcium crystallization with oxalate only occurs after the physiological Ca needs of the foliage are met, ensuring an adequate supply of available foliar Ca, especially when the Ca supply from the soil is limited.

Our data indicate that the Ca in these three extractable pools is not of equal physiological significance. Membrane-associated Ca was most closely related to the available (sum of water- and acetic acid-extractable) [Ca] ($r = 0.67, P = 0.018$), but was not significantly related to water-extractable [Ca] alone. Because mCa is electrostatically associated with negatively charged moieties at the cell membrane surface, it may require cation substitution for displacement and removal. This is more likely to occur during acetic acid extraction (pH 2) than with water extraction (pH 5.2). We have seen evidence for H⁺ displacement of mCa in studies with artificial acidic mist treatments (Schaberg et al. 2000). Although Fink (1991) interpreted the acetic acid-extractable Ca as specifically associated with pectin in the cell wall, our data indicate that mCa is also included in this fraction.

Recent data indicate that red spruce decline in the northeast-

Table 3. Mean foliar concentrations (mg kg⁻¹) of five elements in sequential extractions with water, acetic acid and hydrochloric acid and statistical comparisons of foliar age classes.

Element	P-value ¹											
	Current-year foliage					One-year-old foliage						
	Water	Acetic acid	HCl	Sum	Water	Acetic acid	HCl	Sum	Water	Acetic acid	HCl	Sum
Al	18.6	19.7	32.5	70.8	18.4	17.9	36.3	72.6	ns	ns	ns	ns
Ca	490.9	380.5	128.8	1000.1	486.2	527.7	240.9	1253.0	ns	<0.001	<0.001	0.002
K	5659.2	171.7	3.0	5837.8	4231.8	141.4	2.9	4378.6	<0.001	0.008	ns	<0.001
Mg	585.6	103.1	1.5	690.2	430.0	102.6	1.0	533.0	<0.001	ns	ns	<0.001
Mn	961.5	218.2	9.6	1189.2	1038.8	319.2	13.6	1370.6	0.003	<0.001	<0.001	<0.001

¹ Significance for one-tail paired t-tests between foliar age classes. The non-parametric signed-rank test was performed to assess the HCl-extractable Ca and HCl-extractable K because these parameters did not conform to model assumptions.

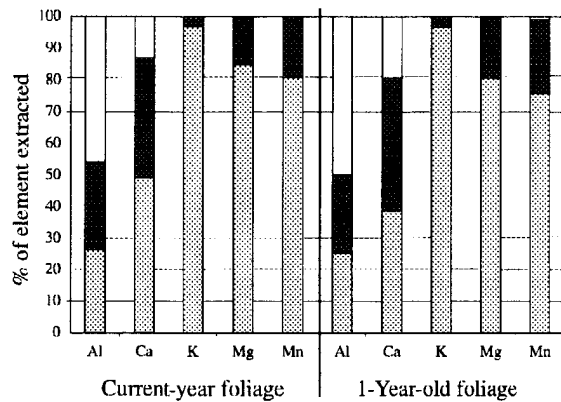


Figure 3. Foliar element partitioning (percent of the total for each element) in sequential extractions of current-year and 1-year-old red spruce foliage. Stacked percent content of water-extractable (light grey), acetic acid-extractable (dark grey) and HCl-extractable (white) portions of each element.

ern United States is caused by acidic deposition selectively depleting mCa, resulting in insufficient development of cold hardiness (DeHayes et al. 1999, Schaberg et al. 2000). This may occur through depletion of Ca that stabilizes cell membranes (DeHayes et al. 1999) or depletion of an essential pool of Ca for signal transduction, or both. Knight et al. (1996) reported that the process of cold acclimation in *Arabidopsis* includes Ca signaling, both from the apoplast and from the vacuole. Our data further demonstrate the differential physiological importance of the extractable foliar Ca pools in the context of this decline scenario. In addition to its relationship with mCa, available [Ca] was significantly correlated with foliar response variables (Table 2), including cold hardiness (T_m) and long-term winter injury, and was a significant predictor of subsequent winter injury.

Available [Ca] was also related to long-term plant health and net carbon accretion, as manifested here by DBH. This may result from increased energy demands for maintenance processes when Ca is limited (McLaughlin et al. 1993). In contrast, smaller trees may simply accrue less Ca because their smaller crowns transpire less. We found, however, no significant differences among crown classes or canopy exposure for any of the calculated parameters or any foliar partitions of Ca, so DBH differences likely reflect more than potential differences in transpiration. Available [Ca] was significantly corre-

lated with foliar concentrations of some other essential nutrients (Mg and Mn, but not K). These correlations may result from other physiological or ecological processes that influence nutrient accretion, including the influence of Al on overall soil cation availability and root uptake.

Our data suggest that Ca partitioning and sequestration play important roles in a variety of physiological processes. The close correlation we found between available [Ca] and physiological function, and the weaker association between total foliar [Ca] and physiological function are consistent with HCl-extractable Ca being chemically sequestered and biologically unavailable (Fink 1991). If, as predicted, chemically sequestered Ca cannot be remobilized, then reductions in labile Ca pools by acidic leaching (DeHayes et al. 1999, Schaberg et al. 2000) are likely to have long-term detrimental effects on the many processes that require available Ca. This is especially likely if leaching occurs when root uptake of Ca is limited by season (Schaberg et al. 2000), because of Ca-deficient soils or through Al inhibition of Ca uptake. In contrast, because Ca oxalate crystal-forming idioblasts are known to differentiate in some species even when the Ca supply is limited (Webb 1999), it is likely that constitutive Ca sequestration occurs in these species. Inducible Ca sequestration will likely be increasingly beneficial for forest species if anthropogenically mediated acidifying processes continue.

Aluminum partitioning and physiological significance

Aluminum is one of the most abundant elements in the lithosphere, but until recently was unavailable for uptake into biological systems (Martin 1988). As a result, organisms have not evolved mechanisms to utilize Al and may not have developed active mechanisms to detoxify it. Thus, Al sequestration may be a passive result of other metabolic processes. When soil Al is limited, foliar Al accumulation and toxicity are unlikely. However, Al uptake and foliar accumulation can occur in the foliage of red spruce and other tree species (e.g., Young and Guinn 1966, David et al. 1988, Robarge et al. 1989, Heisey 1995) where soil [Al] or soil acidity are high.

Mechanisms to exclude Al uptake and reduce its cytotoxicity in roots have been described for many plant species. These include Al precipitation with various organic acids and alteration of rhizosphere pH (Taylor 1991), as well as physical isolation in organelles such as the vacuole (Matsumoto 2000, Shen et al. 2002). Because of its charge density and dissociation constants, Al is thought to form similar chemical interactions to those formed by Ca in biological systems, but may

Table 4. Parameter comparisons between current-year and 1-year-old foliage.

Parameter	Current-year foliage	One-year-old foliage	P^1
Cold tolerance (T_m , °C)	-48.5	-56.4	0.001
Available [Ca] (mg kg ⁻¹)	871	1014	0.001
Total foliar Ca:Al molar ratio	10.2	12.1	0.060
HCl-extractable [Al]:available [Al]	0.763	0.926	0.016

¹ Significance for one-tail paired t -tests between foliar age-classes.

provide excessive and damaging rigidity to these structures (Kochian 1995, Matsumoto 2000). The similarity between Ca and Al binding at the cellular level raises the possibility that basic cellular mechanisms for preventing cytoplasmic Ca toxicity may also control Al toxicity. Despite the range of molar Ca:Al ratios in the foliage we studied (7.9 and above), and the potential for relatively low Al solubility at biological pH, our data demonstrate that impairment can result even from relatively low foliar [Al]. The relationships we present between Al forms and foliar physiology highlight the biological significance of differential Al sequestration and its relationship to Ca partitioning.

Unlike Ca, Al partitioning and sequestration had no statistically significant relationship with foliar [Al] within the range of foliar [Al] studied (Figure 2), suggesting that chemical partitioning of Al in red spruce foliage is not Al inducible. Although we found no relationship between total foliar [Al] and HCl-extractable [Ca], data from sequential chemical extractions demonstrated that Al partitioning was significantly correlated with Ca-induced foliar oxalate production. Foliage with high HCl-extractable [Ca] also had high HCl-extractable [Al] and low acetic acid-extractable [Al]. These results are consistent with Al-oxalate precipitation as a mechanism for mitigating foliar Al toxicity in red spruce foliage. Aluminum oxalate is reported to be stable and non-phytotoxic (Shen et al. 2002). Calcium oxalate crystals accumulate in the cell wall region in conifer foliage (Fink 1991), so Al oxalate may similarly precipitate outside of conifer cells. Calcium oxalate accumulates in angiosperm vacuoles (Franceschi and Horner 1980, Webb 1999), so Al oxalate may precipitate in vacuoles of those species.

Foliar partitioning of Al was significantly related to several important physiological parameters. The ratio of sequestered [Al] to available [Al] represents a plant's ability to protect itself from foliar Al toxicity (Table 2). There is a strong relationship between this ability and most response variables in Table 2. Greater Al sequestration was related to increased tree growth and uptake of Mg and Mn, and weakly related to enhanced foliar cold hardiness. Aluminum sequestration was more strongly correlated with growth than was Ca. This may be the result of the combined impacts of Al inhibition of Ca uptake as well as potential Al toxicity, even at total foliar [Al] below values reported to cause impairment (Raynal et al. 1990). Further, these data indicate that chemical sequestration of foliar Al could be an important mechanism of Al tolerance in red spruce, and that Al toxicity is strongly influenced by its chemical form rather than the total amount in foliage. Recent work has shown that foliar Al sequestration by precipitation with organic acids is a mechanism of short-term tolerance in some Al-accumulating species (Ma et al. 1997, 1998, Shen and Ma 2001, Watanabe and Osaki 2002). The data we present indicate that foliar sequestration of Al by precipitation with Ca-induced oxalate may also be a long-term mechanism of foliar Al detoxification in mature red spruce.

The strong relationship between available [Ca] and the ratio of sequestered to available [Al] (Table 2) likely reflects a combination of two concurrent processes. First, soil Al can inhibit

Ca uptake by reducing root growth (Schofield et al. 1998) and blocking Ca channels (Huang et al. 1992, 1996). Second, our data indicate that oxalate production, induced by excess foliar Ca, may passively reduce foliar Al toxicity by enhancing chemical Al sequestration. Chemical partitions of foliar Ca and Al showed much closer relationships with a range of physiological parameters than did standard foliar measures or the molar ratio of total foliar Ca:Al (Table 2). It is not surprising that all three calculated parameters (Table 2) were significantly correlated with winter injury in 2003, which was widespread and substantial. Winter injury during that year may reflect overall plant health, and may covary with other influences on health such as nutrient content. Available [Ca], in comparison, is a much more precise measurement and may allow specific resolution of the status of Ca-dependent physiological processes rather than generalized plant health as with foliar Ca:Al.

Calcium is an interesting mineral nutrient because of its wide-ranging roles in biological responses to environmental cues, its inherent cytotoxicity and need for specific cellular partitioning and localization, and its many important roles in ecosystem processes (e.g., McLaughlin and Wimmer 1999). Biogeochemical and environmental research of Ca processes is becoming increasingly important because of the many mechanisms by which humans are altering environmental Ca cycling. Our findings indicate that foliar partitioning and sequestration of both Ca and Al play important roles in plant health, and appear to be closely linked. Because foliar Ca and Al pools can include substantial portions that are chemically sequestered and biologically unavailable, frequently used methods of total foliar digestions do not specifically assess the physiological status of these elements. Targeting the physiologically available pools of Ca and Al could be particularly important in biogeochemical and physiological research examining the implications of anthropogenic cation disruptions and their influence on forest health.

Acknowledgments

We thank S. McLaughlin, C. Cronan, W. Shortle, P. Lintilhac, T. Hepner and T. Coe for their helpful suggestions on earlier drafts of this manuscript. We are grateful for excellent technical assistance from Tammy Coe, Brynne Lazarus, Heather Heitz, Michelle Hitchcock, Paula Murakami, Sam Nijensohn and James Tilley. This research was supported in part by a U.S. EPA Science to Achieve Results graduate fellowship to CHB. Additional funding support came from cooperative agreements with the U.S. EPA and the U.S. Forest Service, as well as the USDA Cooperative State Research, Education and Extension Services and McIntire-Stennis Forest Research Program.

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