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Physiological implications of seasonal variation in membrane-associated calcium in red spruce mesophyll cells

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Summary We examined the pattern of seasonal variation in total foliar calcium (Ca) pools and plasma membrane-associated Ca (mCa) in mesophyll cells of current-year and 1-year-old needles of red spruce (*Picea rubens* Sarg.) and the relationship between mCa and total foliar Ca on an individual plant and seasonal basis. Foliar samples were collected from seedlings and analyzed on 16 dates at 2- to 3-week intervals between June 1994 and March 1995. Concentrations of mCa in current-year needles were more seasonally dynamic and responsive to temporal environmental changes than either mCa concentrations of 1-year-old needles, which were largely stable, or total foliar Ca concentrations in both tissues. In current-year needles, mCa was barely evident in early summer, increased steadily through summer, and then increased dramatically in early fall and surpassed the concentrations in 1-year-old needles. Coincident with the first severe frost, mCa concentrations in current-year needles declined significantly and subsequently maintained concentrations comparable to those of 1-year-old needles. Following an extended January thaw, which included 5 days of minimum temperatures > 5 °C, mCa concentrations of current-year needles temporarily, but significantly, declined. However, there was no change in mCa concentrations of 1-year-old needles or total Ca concentrations of either tissue. Total Ca concentrations were stable through midsummer in both tissues, doubled in late summer, and then were stable in both tissues throughout fall and winter. Total Ca concentrations were consistently higher in 1-year-old than in current-year needles. Correlations between concentrations of mCa and total foliar Ca were consistently low and mostly nonsignificant. Thus, the dominant, but insoluble, extracellular Ca pool reflected in commonly measured total foliar Ca concentrations is not a meaningful surrogate for the physiologically important and labile pool associated with the plasma membrane–cell wall compartment of red spruce mesophyll cells. It is likely that shifts in the critical mCa compartment would not be detected by analysis of total foliar Ca pools. Seasonal changes in mCa concentration seemed to parallel seasonal changes in membrane structure, and possibly the important role of extracellular Ca in transducing messages associated with environmental signals.

Keywords: cold acclimation, compartmentation, extracellular calcium, frost, Picea rubens, plasma membrane, total foliar calcium, winter thaw.

Introduction

Concern about the influences of atmospheric pollution on forest health has enhanced both interest in and the frequency of measurement of nutrient chemistry in forest trees and ecosystems. Such measurements most frequently include quantitative estimates of total foliar ion pools and biogeochemical assessments of soil, soil solution, and surface waters. Interpretation of foliar chemistry data in trees can be complicated because of foliar age class differences, dynamic seasonal patterns of uptake and distribution, and unequal allocation and physiological importance of individual ions within and between cells and tissues.

Calcium (Ca), which is an abundant element in trees and a major cation in soil and surface waters, has been a particular focus of recent attention because of potential losses resulting from acid deposition, diminished base cation deposition, foliar leaching, and competitive interactions with soil aluminum (Joslin et al. 1988, Shortle and Smith 1988, Lawrence et al. 1995, Likens et al. 1996). Foliar pools of Ca have been shown to vary substantially with site, foliage age class, and season (Schomaker 1973, Czapowskyj et al. 1980, MacLean and Robertson 1981, Friedland et al. 1988, Oren et al. 1988, Robarge et al. 1989, Fernandez et al. 1990, Huntington et al. 1990, McLaughlin et al. 1990, 1991). More importantly, however, Ca is also compartmentalized within cells and tissues and this partitioning is critical to its physiological function in plants. For example, Ca is of vital importance for cell wall formation and plays an important role in 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, Steponkus 1990). In contrast, cytosolic Ca can be phy-

totoxic and concentrations are extremely low (Hanson 1984), although intracellular Ca acts as a metabolic regulator for many processes and as a second messenger in binding to proteins such as calmodulin (Hepler and Wayne 1985).

Despite these physiologically critical roles for Ca in plant cells, the major fraction of Ca in conifer needles is insoluble Ca oxalate crystals that occur on the outside walls of mesophyll cells and to a lesser extent within cell walls of epidermal cells (Fink 1991*a*). In addition, Ca pectate occurs in high concentration in the cell walls of sieve cells and transfusion parenchyma (Fink 1991*a*). Measures of the total foliar concentration of Ca ions in conifer foliage may primarily reflect these large stored pools rather than the smaller, but physiologically critical and labile, pool of Ca ions associated with the plasma membrane–cell wall complex. In particular, membrane-associated Ca (mCa), although a relatively small fraction of total foliar Ca ion pools, strongly influences the response of cells to changing environmental conditions and stress (Hanson 1984, Hepler and Wayne 1985, Dhindsa et al. 1993, McLaughlin et al. 1993). Until recently (Borer et al. 1997), it has not been possible to produce meaningful relative quantitative estimates of this particular Ca fraction in the foliage of woody plants, or to assess whether estimates of total foliar Ca pools provide insight into physiologically important pools of Ca.

In this paper, we describe the pattern of seasonal variation in total foliar Ca pools and plasma membrane-associated Ca (mCa) in mesophyll cells of current-year and 1-year-old needles of red spruce (*Picea rubens* Sarg.). We also examine the relationship between mCa and total foliar Ca on both an individual plant and seasonal basis. Although we refer to mCa as plasma membrane-associated Ca, we fully recognize mCa estimates may include both plasma membrane and some free and displaced apoplastic Ca from the cell wall. However, because Ca ions in the membrane–wall complex are expected to be in dynamic equilibrium and are distinctive from other dominant pools of Ca in conifer needles (Fink 1991*a*), we believe this combined labile mCa pool represents a physiologically important pool of Ca in conifer needle tissue.

Materials and methods

Plant material

Foliar Ca assessments were made on needles from a population of approximately 150 red spruce seedlings growing in a loamy sand nursery soil in Burlington, VT. Seedlings were grown from a bulk seed collection from throughout Vermont and were 8 years old and averaged about 63 cm in height at the end of the 1994 growing season. Seedlings received ambient precipitation periodically supplemented by watering during dry summer periods. Analyses of foliage from these seedlings revealed nutrient concentrations within the normal range reported for both red spruce seedlings (Swan 1971) and mature trees (MacLean and Robertson 1981, Friedland et al. 1988, Robarge et al. 1989, Fernandez et al. 1990).

Beginning in late June 1994 (when current-year shoots were nearly fully extended) and continuing until late March 1995, current-year and 1-year-old needles were collected for mCa and total foliar Ca analyses. At 2- or 3-week intervals, six seedlings were chosen with replacement from the 150 seedlings described above. On each date, current- and 1-year-old needles were collected from the upper two whorls of each tree and were kept separate by individual seedling and age class. Needles were prepared for mCa and total foliar Ca analysis within a few hours of collection.

On November 21, 1994 and February 28, 1995, current-year needles were collected from the uppermost whorls of 24 seedlings, providing a sufficient sample size to examine the relationship between concentrations of mCa and total foliar Ca for individual plants on specific dates. To determine the extent of the mCa and total foliar Ca responses to an extended midwinter thaw, we sampled a group of six seedlings immediately before and again 5 days into a 9-day midwinter thaw (January 12–21, 1995), which set several climatic records throughout northern New England and adjacent Canada (Strimbeck et al. 1995). During the first 4 days of the thaw (January 12–15), maximum and minimum temperatures in Burlington remained above 0 °C and on January 15 the maximum temperature reached 18.8 °C. After January 21, maximum and minimum temperatures were again consistently below 0 °C for most of the next 2 months.

Membrane-associated calcium analyses

The procedure used to estimate relative amounts of mCa in red spruce mesophyll cells incorporates 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 of divalent cations in close proximity to apolar environments (biological membranes) causes a conformational change in CTC that results in 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 demonstrated that mCa is the cation associated with CTC fluorescence in samples prepared and analyzed following the same protocol (Borer et al. 1997).

For seasonal assessments, mCa analyses were conducted on current-year needles from six trees per sample date and on 1-year-old needles from three trees per sample date. For samples collected from 24 trees on November 21, 1994 and February 28, 1995, only current-year foliage was analyzed for relative concentrations of mCa. Needle sections were used in all mCa analyses and individual trees were generally represented by several sections per date. To minimize cellular disruption and prevent the leaching of ions into preparative solutions, red spruce needles were sectioned without chemical fixation, embedding, or freezing. Fresh needles were supported in a small piece of carrot and 50-µm-thick cross sections were cut with a sliding microtome. Sections were immediately placed in a buffer solution containing 10% sucrose and 20 mM 2-(*N*-morpholine) ethanesulfonic acid (MES), adjusted to pH 6.5 with tetramethylammonium hydroxide. After all sections were collected, buffer was replaced

with 90 µl of fresh buffer and 10 µl of 1.9 mM CTC solution for a final CTC concentration of 0.19 mM. To detect autofluorescence, unstained sections from each needle were soaked in 10 µl of buffer solution to be used as controls. Samples were incubated overnight at 5 °C in the dark. Examinations based on cell viability stains indicated that cells consistently remained alive throughout the incubation and treatment period (Borer et al. 1997). Therefore, we believe the cells maintained their naturally occurring distribution of Ca.

Microscopy included a Balplan microscope with a tungsten-halogen incident epifluorescence system and a 35-mm camera with an AX-1 automatic exposure controller (Bausch and Lomb, Rochester, NY). The tungsten-halogen lamp produces little UV light thereby minimizing photobleaching. Samples were illuminated through a Fluorescein-5-isothiocyanate filter (FITC filter, Bausch and Lomb), which allowed wavelengths between 350 and 500 nm to excite the specimen. The optimal excitation wavelength for CTC-Ca^{2+} is 400 nm (Mathew and Balaram 1980). The dichroic beamsplitter had a cutoff wavelength of 500 nm, and the barrier filter had a narrow bandpass of 535 ± 10 nm that excluded red chlorophyll autofluorescence and that of CTC-Mg^{2+} (peak 520 nm) relative to that of CTC-Ca^{2+} (peak 530 nm) (Caswell and Hutchison 1971). Preliminary studies demonstrated that Mg ions did not contribute to CTC fluorescence in red spruce mesophyll cells (Borer et al. 1997). For brightness analysis, we captured images with photographs and digitized them by means of a flatbed scanner at 498 dots per inch and 25% scaling rather than using a CCD image grabbing system because of the potential for greater image resolution with the high resolution scanner. All pictures were taken with 400-ASA black and white professional film using an exposure time of 45 s. Lamp voltage, magnification, and all other conditions and settings were kept as consistent as possible. Pictures were printed at a fixed density and contrast to maximize image uniformity.

Image processing and analyses were performed with the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institute of Health, Washington, DC). By providing the capability to remove (through shading) selected tissue, cellular components, and artifacts as well as produce brightness estimates on a continuous brightness scale, image processing and analysis allowed us to generate CTC-Ca^{2+} fluorescence brightness estimates specific to the plasma membrane–cell wall region. Brightness estimates of CTC fluorescence from the plasma membrane–cell wall region were generated by determining pixel brightness of only the plasma membrane–cell wall region (by shading all other areas) and normalizing each picture to its background pixel brightness (Borer et al. 1997).

Because there is some variation in image fluorescence attributable to film processing and autofluorescence noise rather than to mCa fluorescence, we adjusted for this background variation by creating a pixel brightness ratio consisting of the pixel brightness value of CTC stained samples divided by the mean pixel brightness of unstained control plasma membranes for each roll of film. This ratio eliminated background noise and allowed comparison among samples collected, photographed, and processed on different dates. Because pixel brightness values were generated on a scale from 255 (black) to 0 (white), we reversed the scale by calculating mCa values as mCa = 1 − pixel brightness ratio so that increasing values would correspond to increasing brightness and mCa concentrations.

Total foliar Ca analyses

Analyses were conducted on foliar samples representing six individual seedlings for current-year foliage, whereas 1-year-old needles were bulked to form two samples, each representing three different seedlings. Although this bulking precluded individual seedling analysis for 1-year-old foliage, it was cost-efficient and still allowed calculation of a sample date mean and error estimate. Following each collection, oven-dried current-year and 1-year-old red spruce needle samples were 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 Westco Scientific AD-4020 block digestor (Westco Inc., Danbury, CT). Foliar samples were predigested overnight at room temperature and then digested for 20 min at 250 °C and 1.5–2.0 h at 450 °C. Digests were diluted to 75 ml with distilled deionized water and analyzed for Ca by inductively coupled plasma atomic emission spectroscopy (ICP.AES, PlasmaSpec 2.5, Leeman Labs, Lowell, MA). The reference for all analyses was white pine needles from the National Bureau of Standards and Technology (SRM 1575, Gaithersburg, MD).

Data analysis

Analyses of variance were used to determine the significance of differences among sampling dates for both mCa and total foliar Ca of current-year and 1-year-old foliage. The significance of differences between specific date means were examined by a Tukey-Kramer Test. *t*-Tests were used to contrast amounts of mCa and total foliar Ca before and after 5 days of the January 1995 thaw.

Correlation analyses were conducted to examine relationships between concentrations of mCa and total foliar Ca. Because 1-year-old needles were bulked by date for total foliar Ca analyses, only seasonal (across dates) relationships between these parameters could be assessed. For current-year foliage, relationships between concentrations of mCa and total foliar Ca were examined in three different ways. First, to assess seasonal relationships, correlations were calculated between mCa and total foliar Ca means across all sample dates. Second, to minimize the influence of seasonal trends and achieve better resolution of the overall relationship between mCa and total foliar Ca, this correlation was also evaluated after data for individual trees were expressed as a percentage of the mean for each sampling date, thus removing sample date and seasonal differences. Third, to better assess the relationship of mCa and total foliar Ca of current-year foliage on specific dates, correlations were made with an expanded sample of seedlings on November 21, 1994 and February 28, 1995. Except where indicated, all tests were considered significant if $P \leq 0.05$.

Results and discussion

Seasonal variation in mCa and total foliar Ca

In contrast with total foliar Ca pools, mCa in current-year needles of red spruce was much more seasonally dynamic and appeared to be responsive to temporal changes in the environment (Figures 1A and 1B). In 1-year-old needles, mCa was largely stable throughout the year except for a slight increase to a peak in late autumn. In late June 1994, when sampling was initiated, there was little or no mCa fluorescence in the region of the plasma membrane–cell wall complex of current-year red spruce needle mesophyll cells. There was, however, considerable fluorescence in the resin ducts and vascular bundles of current-year needles and the plasma membrane–cell wall compartment of 1-year-old needles. Throughout summer and early autumn, mCa concentration increased steadily in mesophyll cells of current-year needles presumably as a result of the synthesis of new ion exchange sites or continuing accretion of Ca on existing exchange sites in the plasma membrane–cell wall area as needles mature (Figure 1A). In October, mCa concentration in mesophyll cells of current-year needles abruptly and significantly increased, and surpassed that of 1-year-old needles. The increase in mCa from early October to early November represented a doubling of mCa concentration in current-year needles (Figure 1A), resulting in a sixfold increase over values in late June and early July. Equally abruptly, and coincident with the first severe frost (< −5 °C) of the season, mCa in mesophyll cells of current-year needles declined significantly in mid-November and subsequently reached and maintained a concentration comparable to that of mCa in mesophyll cells of 1-year-old needles.

Unlike mCa, total foliar Ca pools in both current-year and 1-year-old red spruce needles were stable from June through August, although the total foliar concentration of Ca in 1-year-old needles was about 60% greater than that of current-year needles during summer (Figure 1B). In early September, Ca concentrations in 1-year-old needles more than doubled, whereas the seasonal increase was more gradual and somewhat less pronounced in current-year needles (Figure 1B). Although total Ca in the two tissues differed substantially and consis-

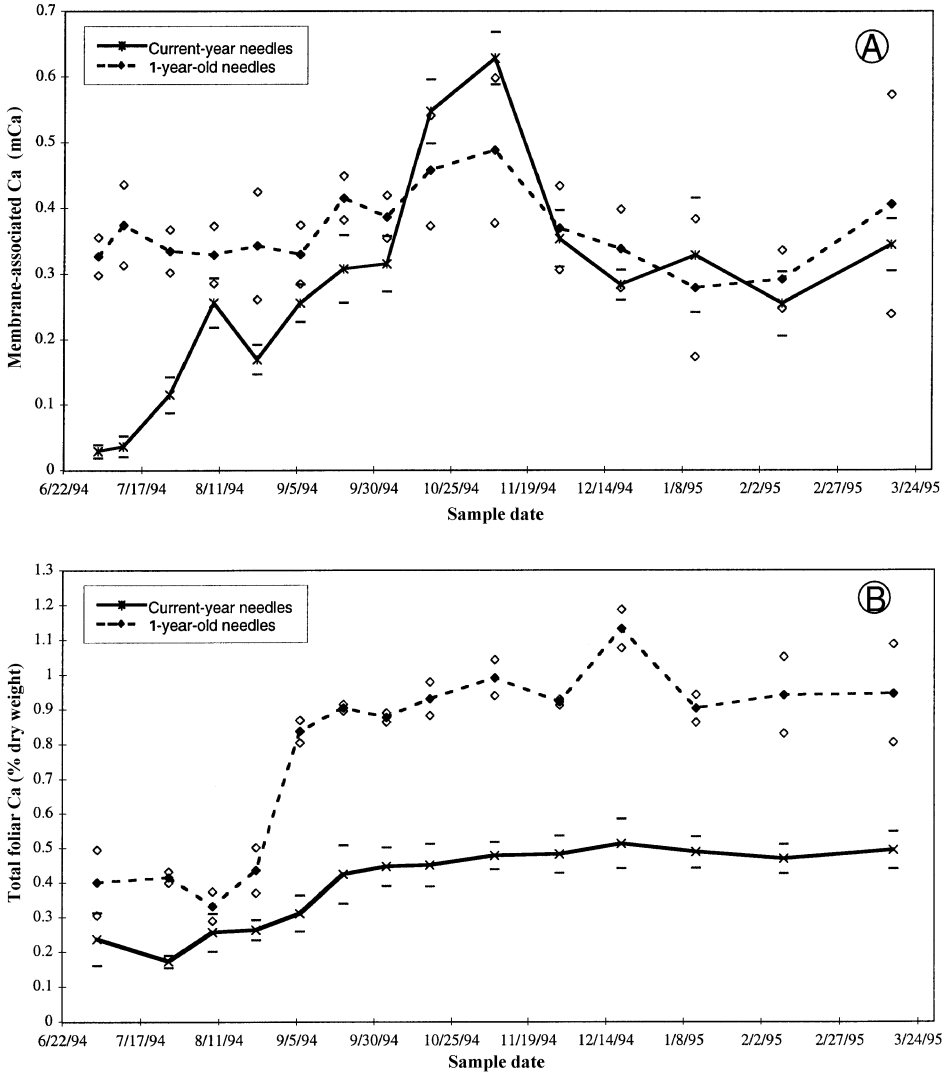

Figure 1. Seasonal patterns of membrane-associated Ca (mCa) (A) and total foliar Ca (B) of current-year (×) and 1-year-old (◆) needles of red spruce seedlings. Values are means ± standard errors for each date.

tently, Ca concentrations in both tissues were approximately double their respective midsummer concentrations by late September. The dramatic late summer increase in total foliar Ca of 1-year-old needles preceded the increases in mCa of both current- and 1-year-old needles by about 1 month. Total foliar Ca concentrations were subsequently stable in both tissue groups and about double in 1-year-old compared to current-year needles from early autumn through early spring when sampling ceased.

The difference in total Ca concentration associated with needle age class is largely a reflection of the means of Ca transport and its lack of mobility in the phloem (Marschner 1986). Although Ca transport is largely an ion exchange process (Fink 1991*a*), Ca continues to enter 1-year-old needles as part of the mass flow of water associated with transpiration. Because Ca is immobile in phloem, it accumulates with age of tissue even though the need for Ca in older needles is thought to be low. Several other studies have reported similar needle age class increases in foliar Ca concentration of red spruce and other conifers (MacLean and Robertson 1981, Friedland et al. 1988, Oren et al. 1988, Robarge et al. 1989, Fernandez et al. 1990, McLaughlin et al. 1990). The late summer increase in total foliar Ca is consistent with most other findings for north temperate conifers, including red spruce (MacLean and Robertson 1981, Oren et al. 1988, Fernandez et al. 1990). Probably, the approximate doubling in total foliar Ca concentration in current- and 1-year-old needles in late summer–early fall was driven by changes in Ca sinks. For example, it is expected that the cessation of stem growth during this time would reduce Ca sinks more proximal to roots and thereby allow a greater fraction of Ca in the transpiration stream to reach the foliage. Presumably there is also an increase in Ca ion binding sites associated with the primary bound Ca pools in spruce needle tissues—extracellular Ca oxalate crystals, Ca pectate in middle lamellae, sieve cells, and parenchyma, and cell walls of epidermal cells (Fink 1991*a*). Regardless of the mechanism, the apparent abrupt seasonal increase in total foliar Ca pools would probably complicate interpretation of foliar Ca comparisons among trees on different sites or even between soil and foliar pools at the same site.

Influence of a January thaw on mCa and total foliar Ca

On January 12, 1995, current-year and 1-year-old needles were collected as part of our ongoing mCa and total Ca sampling protocol. However, that day also marked the beginning of an extended January thaw that included 5 consecutive days of minimum temperatures above 0 °C and maximum temperatures exceeding 12 °C (National Oceanic and Atmospheric Administration, National Weather Service, S. Burlington, VT). On January 17, we resampled the same trees examined 5 days earlier and noted a dramatic and significant reduction in mCa concentrations in mesophyll cells of current-year needles, but no significant changes in mCa concentrations of 1-year-old needles or total Ca pools in either current-year or 1-year-old needles (Table 1). Following exposure to 5 days of above-freezing temperatures in January, concentrations of mCa dropped uniformly in all seedlings from what appeared to be a mid- to late winter equilibrium value (about 0.33) to the approximate value observed in mid- to late August. On return of subfreezing temperatures in late January, mCa concentrations in mesophyll cells of current-year needles returned to prethaw values, which were similar to and not significantly different from mCa values in 1-year-old needles throughout most of the year.

Relationship between mCa and total foliar Ca

We examined the relationship between concentrations of mCa and total foliar Ca on a seasonal mean basis, a seedling basis across all sample dates, and on an individual seedling basis in November 1994 and February 1995. Because total foliar Ca measures for 1-year-old needles were represented by a bulk collection on each sample date, correlations involving 1-year-old needles could only be examined on a seasonal basis. Correlations between concentrations of mCa and total foliar Ca were consistently low and mostly nonsignificant (Table 2). The strongest association between mCa and total foliar Ca was on a seasonal basis. The seasonal increases in mCa and total Ca pools were sufficiently synchronized to result in modest and significant correlations (Table 2) even though the specific seasonal changes in the concentrations of mCa and total Ca did not appear to coincide on a weekly basis.

The relationship between mCa in current-year and 1-year-old needles was modest and significant on a seasonal basis (Table 2), despite disparate patterns in mCa concentrations between both tissues during summer. The correlation was driven largely by the consistent early autumn rise, mid-autumn peak, and late autumn decline in mCa that was evident in both tissues. The relationship between total Ca concentrations in current-year and 1-year-old needles was strong ($r = 0.884$, $P \leq 0.001$), despite the substantial abrupt increase in total Ca in late summer that was unique to 1-year-old needles.

Table 1. Mean total foliar calcium (Ca) and mesophyll cell membrane-associated Ca (mCa) concentrations in current-year and 1-year-old needles of red spruce seedlings before (January 12, 1995) and 5 days after (January 17, 1995) a thaw.

Date and condition	Current-year needles		1-Year-old needles	
	mCa	Total Ca (% dry wt.)	mCa	Total Ca (% dry wt.)
January 12/Prethaw	0.328	0.488	0.278	0.902
January 17/Thaw	0.209	0.502	0.435	0.878
P-value	0.065	0.843	0.317	0.625

Table 2. Correlations (r) between mesophyll cell membrane-associated calcium (mCa) and total foliar calcium (Ca) concentrations of red spruce seedlings on a seasonal basis (across 15 dates from June through March), on a seedling basis over all dates and adjusted for date means (n = 92), and on an individual seedling basis in November 1994 and February 1995 (n = 24 seedlings).

	Correlation coefficients (r)			
	mCa of current-year needles	P-value	mCa of 1-year-old needles	P-value
Seasonal basis				
mCa of 1-year-old needles	0.502	0.057	1.000	< 0.001
Total Ca of current-year needles	0.513	0.050	0.392	0.149
Total Ca of 1-year-old needles	0.577	0.021	0.351	0.200
Seedling basis (adjusted by date)				
Total Ca of current-year needles	−0.058	0.582	–[1]	
Individual seedling basis				
Total Ca of current-year needles				
November 1994	−0.238	0.262	–[2]	
February 1995	−0.089	0.708	–[2]	

[1] Correlations were not possible because the 1-year-old needles were bulked for total foliar Ca analysis.
[2] Only current-year needles were collected on these dates.

Correlations between mCa and total foliar Ca concentrations on a seedling basis were weak for both current-year and 1-year-old foliage even though mCa measurements made on the same seedlings over time were highly repeatable (Borer et al. 1997). When the influence of sample date (i.e., seasonal variation) was adjusted out of the data, the cumulative correlation between mCa and total foliar Ca across 92 samples for current-year needles approached 0 (Table 2). When the correlation was examined for 24 seedlings on specific dates in November and then again in February, the correlations were also quite low and nonsignificant (Table 2). These weak correlations indicate that the commonly measured total foliar Ca ion pool is not a meaningful surrogate measure for the physiologically important pool of Ca associated with the plasma membrane–cell wall compartment in red spruce mesophyll cells. Although total foliar Ca may be a good indicator of the ecological availability of Ca within soils, mCa may be a better measure of the physiological availability of Ca within foliage. Because the major pool of Ca in red spruce needles is in the form of insoluble Ca oxalate crystals located in extracellular spaces within the mesophyll cells and within walls of epidermal cells (Fink 1991*a*), the physiological significance of estimates of total foliar Ca is questionable. Calcium ions in the plasma membrane–cell wall compartment are critical to cell wall structure, membrane stability and permeability, and the perception and transduction of environmental signals (Hanson 1984, Hepler and Wayne 1985). It is possible that shifts in this critical Ca compartment, whether pollution-induced or attributed to natural seasonal or developmental variation, would not be detected in an analysis of total foliar Ca content.

Physiological implications of seasonal changes in mCa

Seasonal changes in mCa concentration reflect the dynamic and environmentally responsive nature of Ca ion exchange sites in the plasma membrane–cell wall compartment of red spruce mesophyll cells, especially in current-year needles. Despite the membrane and Ca specificity of the CTC probe and evidence that the contribution of Ca ions from the cell wall to CTC fluorescence may be quite small (Cramer et al. 1985), we have not ascertained the relative contributions of wall versus membrane Ca to total CTC fluorescence in this critical compartment for red spruce needles. It is probable, however, that the increase in mCa from June through October is a reflection of increased Ca binding sites in both the developing cell wall and plasma membrane. The latter is associated with seasonal increases in polar lipids of plasma membranes in late summer and early autumn (Senser and Beck 1982). In contrast, changes in mCa that occur throughout the winter and appear to coincide with changing environmental conditions (e.g., fall frost) are probably a reflection of seasonal augmentation of membrane phospholipids and a concomitant incorporation of polyunsaturated fatty acids into membrane lipids (Senser and Beck 1982). Seasonal fluxes in mCa may also reflect the important second messenger role of extracellular Ca. There is increasing evidence that mCa plays a critical role in the transduction of low temperature signals during cold acclimation (Dhindsa et al. 1993) and possibly the dehardening response associated with climatic warming (Woods et al. 1984). Calcium ions in the membrane–wall complex are a likely source of such messenger Ca (Atkinson et al. 1990).

We propose a hypothetical model that depicts seasonal variation in mCa of red spruce mesophyll cells within a context of seasonal alterations to membrane structure and functional interrelationships with red spruce freezing tolerance (Figure 2). Although hypothetical, empirical support for many elements of the model is abundant. It is well established that the lipid composition of membranes in leaf tissue of north temperate conifers and other woody plant species changes throughout the fall during the onset of cold acclimation, and that the development of freezing tolerance of conifer needles is characterized by increased proportions of cell-membrane lipids containing

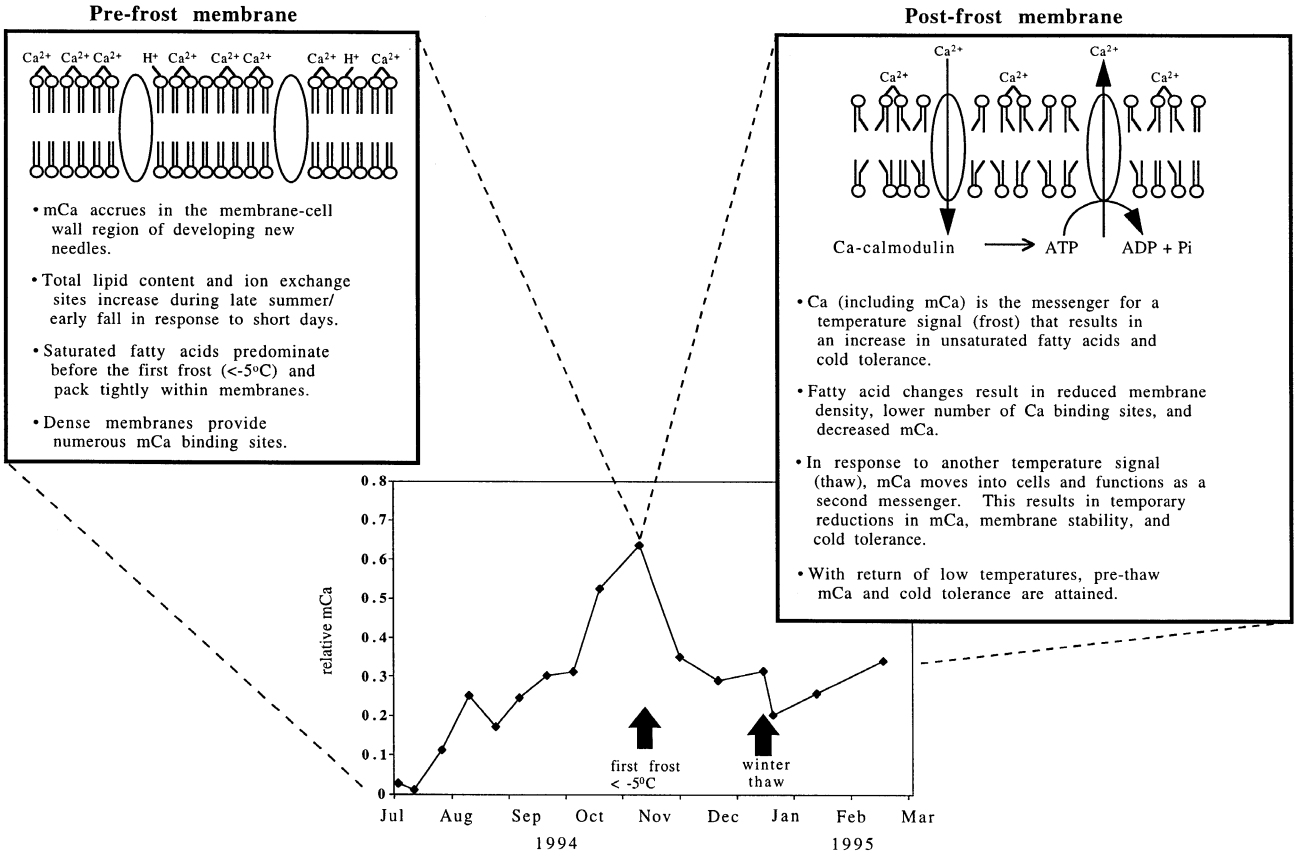

Figure 2. Seasonal variation in membrane-associated Ca (mCa) of mesophyll cells in current-year red spruce needles and a hypothetical model depicting interrelated alterations in mCa, membrane structure and cold tolerance.

unsaturated fatty acids (DeYoe and Brown 1979, Senser and Beck 1982 and 1984, Sakai and Larcher 1987, Lag et al. 1991, Latsague et al. 1992). In fact, Senser and Beck (1982) have demonstrated augmentation of phospholipids in Norway spruce (*Picea abies* (L.) Karst.) needles in response to shortening days, and a preferential incorporation of polyunsaturated fatty acids into membrane lipids induced by subfreezing temperatures.

It is likely that the abrupt increase in mCa concentration observed in late September–early October (Figure 2) reflects an increase in Ca binding sites associated with short-day-induced increases in membrane lipids, especially phospholipids (Senser and Beck 1982). The subsequent significant reduction in mCa of red spruce mesophyll cells that coincided with the first severe frost in autumn (Figure 2) may be a function of frost-induced changes in the fatty acid composition of cell membrane lipids. Saturated fatty acids that predominate before subfreezing temperatures pack tightly within membranes (Cullis et al. 1983, Gennis 1989), resulting in dense membranes that are expected to provide numerous Ca binding sites. Lipid changes in response to subfreezing temperatures (Senser and Beck 1982), which include the preferential incorporation of polyunsaturated fatty acids into membrane lipids, are accompanied by a reduction in membrane density, which is expected to lead to a lower density of Ca binding sites and to reduced mCa as observed in red spruce. The apparent frost-induced reduction in mCa in autumn may also reflect the role of Ca in the perception and transduction of the freezing temperature signal that leads to the increase in unsaturated fatty acids associated with developmental frost tolerance as has been suggested for some agronomic crops (Dhindsa et al. 1993, Monroy et al. 1993, Crotty and Poole 1995).

Although an explanation for the abrupt, but temporary, mCa reduction in current-year needles in response to the January thaw (Table 1, Figure 2) is less certain, it may reflect the role of mCa as a messenger in transducing a temperature signal or an mCa response to thaw-related alterations in physiology. For example, with respect to the latter, it is likely that there is a reorganization of organelle membranes associated with documented changes in photosynthesis (Schaberg et al. 1995, 1996), respiration (Schaberg et al. 1996), and cold tolerance (Strimbeck et al. 1995, Schaberg et al. 1996) during winter thaws. Such organelle membrane restructuring could create sinks for labile Ca, including mCa. With regard to the messenger role, the January thaw represents a warm temperature signal. A possible role of mCa in transducing a warm temperature signal is consistent with the increased concentration of intracellular Ca following warming observed by Woods et al. (1984). Interestingly, the January thaw and associated reduction in mCa was also accompanied by a significant deharden-

ing response of red spruce during the first 3 days of the thaw (Strimbeck et al. 1995). After the thaw, both mCa and cold tolerance of red spruce returned to prethaw values.

We believe that the documented seasonal changes in mCa of red spruce are consistent with expected and well established changes in membrane lipids that influence the number of Ca binding sites, and the expected dynamic physiological role of Ca as a second messenger. In contrast, the relative stability of total foliar Ca pools, especially in current-year needles, appears consistent with the observations of Fink (1991*a*, 1991*b*) that the majority of Ca in north temperate conifer needles is insoluble and immobile. Given the tissue specification and cellular compartmentation of Ca in conifer needles, it is quite probable that, although a useful ecological indicator, total foliar Ca pools are physiologically inconsequential. Although measurements of mCa are time-consuming, we advocate a much greater emphasis on the physiologically active and labile plasma membrane–cell wall pool of Ca ions in plant tissues, especially in studies examining plant responses to stressors, such as pollution or temperature change.

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