Physiological Implications of Anthropogenic Environmental Calcium Depletion

Catherine H. Borer, Paul G. Schaberg, Donald H. DeHayes and Gary J. Hawley

Abstract

Recent evidence indicates that numerous anthropogenic factors can deplete calcium (Ca) from forested ecosystems. Although it is difficult to quantify the extent of this depletion, some reports indicate that the magnitude of Ca losses may be substantial.

The potential for Ca depletion raises important questions about tree health. Only a fraction of foliar Ca is physiologically important and labile, but this Ca exchanges membrane structure and function, and serves as a second messenger in the perception of environmental signals. Ca signaling may initiate plant response and defense systems that function to maintain plant health in the face of environmental change.

Acid deposition leaches Ca associated with mesophyll plasma membranes (mCa) in red spruce (*Picea rubens*) (DeHayes *et al.*, 1999). This loss destabilizes cell membranes and increases their susceptibility to the foliar freezing injury responsible for red spruce decline. Acid-induced perturbations of mCa and membrane stability also occur in other tree species, and soil-based treatments can limit mCa accrual and membrane integrity. These findings suggest that mCa disruptions may be more pervasive than the direct acid-induced foliar alterations noted for red spruce. We hypothesize that disruptions of biologically available Ca may impair tree stress response systems, and compromise tree responsiveness to otherwise inconsequential stresses.

Résumé

Des données récentes indiquent que de nombreux facteurs anthropogènes peuvent contribuer à l'épuisement du calcium (Ca) des écosystèmes forestiers. Bien qu'il soit difficile de quantifier cette diminution, certains rapports font état d'une perte substantielle du Ca.

Le potentiel d'épuisement du Ca soulève d'importantes questions relatives à la santé de l'arbre. Seule une fraction du Ca foliaire est physiologiquement important et labile, mais ce calcium améliore la structure et la fonction des membranes et sert de second messager dans la perception des signaux de l'environnement. Ces signes pourraient initier les réactions et les systèmes de défense qui ont pour rôle de maintenir la santé des plantes malgré les changements environnementaux.

Les dépôts d'acide lessivent le calcium associé aux membranes du plasma du mésophylle (mCa) de l'épinette rouge (*Picea rubens*) (DeHayes *et al.*, 1999). Cette perte déstabilise les membranes cellulaires et augmente leur susceptibilité aux lésions causées par le gel des aiguilles qui pourraient être responsable du déclin de l'épinette rouge. Des perturbations du mCa et de la stabilité membranaire induites par l'acidité s'observent aussi sur d'autres espèces et les traitements du sol peuvent limiter l'accroissement du mCa et l'intégrité de la membrane. Ces découvertes suggèrent que les perturbations du mCa pourraient être plus répandues que les altérations foliaires directes observées sur l'épinette rouge. Nous émettons l'hypothèse que des perturbations dans le calcium biologiquement disponible pourraient causer la défaillance

des systèmes de réaction au stress de l'arbre et compromettre la réaction de l'arbre à des stress autrement sans conséquence.

Calcium physiology

Calcium (Ca), an essential macronutrient, has a variety of important functions in plants. It helps enhance cell wall stability by bridging pectate molecules in cell walls. As a divalent cation of the appropriate atomic radius, Ca can also enhance the structural stability of cell membranes through electrostatic interactions with polar phosphate head groups of plasma membrane phospholipids and with membrane proteins (van Steveninck, 1965; Hanson, 1984). This structural stability may well impart an ability to withstand various physical assaults from the environment, including low temperature stress (Schaberg *et al.*, 2000), and may be an important factor that enables plants to withstand midwinter freezing events.

In addition to calcium's structural roles, cells use alterations in cytoplasmic Ca concentration as signals of a wide array of environmental and physiological cues, leading to a variety of cellular responses, including activation of gene transcription and enzymes. In response to various specific stimuli, receptors in the plasma membrane open Ca channels there, or stimulate the release of such compounds as inositol triphosphate, which diffuses through the cytoplasm, binds to and opens ligand-gated Ca channels in organelle membranes (Trewavas, 1999). Once released into the cytoplasm, Ca can bind to and activate such enzymes as calcium dependent protein kinases (CDPKs), stimulating a cascade of physiological events (Sheen, 1996). Many plant CDPKs differ from enzymes that are activated by Ca in animal cells in that they have a calmodulin-like domain rather than being dependent on Ca binding to and activating free calmodulin, which then binds to and activates the enzyme (Sheen, 1996), although calmodulin dependent pathways do exist in plants (Fromm and Poovaiah, 1996). Response specificity is obtained through the timing, magnitude, and localization of the cytoplasmic Ca response (Trewavas, 1999).

Because of its functions, Ca is likely to play a variety of important roles in plant environmental response systems, including responses to stress. Cell wall stability provides a physical barrier to external threats such as insect feeding and wounding. Plasma membrane stability can reduce membrane permeability, and influence solution movement across membranes and the ability to resist dehydration and freezing (Pomeroy and Andrews, 1985; Arora and Palta, 1988; Guy, 1990; Steponkus, 1990). Ca also plays a role in second messenger pathways, which can trigger physiological responses to a variety of stimuli, including drought, fungal elicitors, temperature changes, touch, and light fluctuations (Knight *et al.*, 1991; Bush, 1995; Sheen, 1996; Trewavas, 1999). Some physiological responses include phytoallexin production, formation of mycorrhizal symbioses, stimulation of genes that regulate cold acclimation, and a variety of stomatal responses.

Environmental Ca depletion

Within the context of Ca functions in plants, recent reports of reductions in environmental Ca availability (Johnson *et al.*, 1994; Lawrence *et al.*, 1995; Likens *et al.*, 1996; Likens *et al.*, 1998) take on an enhanced relevant. Although there is some disagreement over the magnitude of environmental Ca depletion, widespread reductions are not isolated or regional phenomena, but are likely to be affecting ecosystems throughout the United States (Johnson *et al.*, 1994; Hallett and Hornbeck, 1997; Lawrence *et al.*, 1997; NIST, 1998), and Europe (Huettl, 1989). Various factors can lead to this phenomenon. In recent years, overall particulate emissions and thus particulate deposition have been reduced (Likens *et al.*, 1996). This reduction has reduced Ca deposition to forested ecosystems both in the United States and Europe (Hedin *et al.*, 1994; Likens *et al.*, 1996; Lynch *et al.*, 1996).

In addition, despite some improvements, continued acidic deposition in many regions inundates the soil with hydrogen ions (Lynch *et al.*, 1996), replaces base cations on soil exchange sites, and leaves cations subject to leaching losses (Likens *et al.*, 1996; Likens *et al.*, 1998). Soil acidification also increases aluminum (Al) solubility, which may inhibit Ca uptake (Cronan, 1991; Lawrence *et al.*, 1995), or may compete for soil exchange sites with Ca, leaving Ca prone to leaching losses (Lawrence *et al.*, 1995). Al toxicity in plants may result in inhibition of signal-mediated root growth, even without significant Ca displacement on binding sites (Schofield *et al.*, 1998). Forest health declines have been noted in conjunction with alterations in ratios between soluble Ca and Al (Shortle and Smith, 1988; Heisey, 1995).

Cations, including Ca, are also removed from ecosystems via intensive timber harvest and removal from the site. Tritton *et al.* (1987) estimated that this removal could be an important mechanism of Ca loss from forests, and Federer (1989) estimated that whole tree harvest can remove 200-1100 kg/ha of Ca.

Nitrogen saturation may also reduce Ca availability. Ca in red spruce foliage has been found to be inversely proportional to annual nitrogen (N) deposition, and directly proportional to forest floor Ca in a range of sites in the northeastern United States (McNulty *et al.*, 1991). N saturation, a result of continued N deposition, may also enhance cation leaching losses through increased nitrate mobility and soil acidification (Aber *et al.*, 1998), and can lead to reductions in foliar Ca and Mg, as well as increased plant respiration (Schaberg *et al.*, 1997).

Calcium is an essential nutrient, yet numerous anthropogenic factors may be reducing its availability. Is there any evidence to document a real world basis for concern over plant health as a result of environmental Ca depletion?

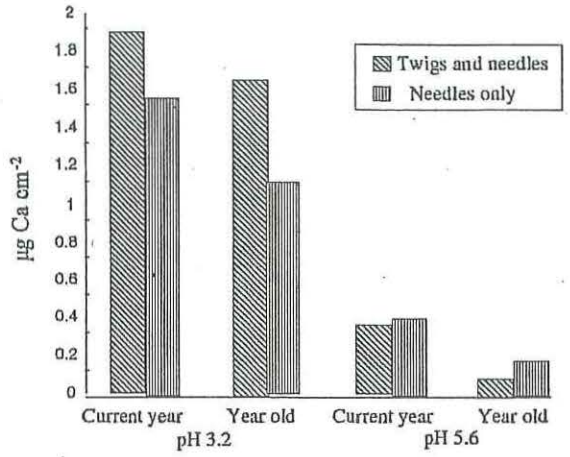

Figure 1. Partitioning throughfall calcium: in vitro *leaching of red spruce needles and twigs.*

Red spruce: a case study

As an illustration of the possible relevance of environmental Ca perturbation, we will summarize a series of investigations for one tree species (red spruce) and one environmental factor known to deplete Ca (acidic deposition). This is probably the best understood example of the potential physiological impacts of Ca depletion, and remains pertinent because acidic deposition continues to affect a variety of regions (Hedin *et al.*, 1994). Lessons from this example could be highly relevant to other species and other Ca-depleting factors.

In addition to its influence on soil Ca availability, acidic precipitation can leach Ca directly from tree foliage. A recent *in vitro* study with red spruce branches enabled us to partition and understand the primary sources of Ca leaching losses. Substantially more Ca is leached from branches treated with a pH 3.2 solution than those at pH 5.6 (Figure 1). At the lower pH, the majority of leached Ca comes from the needles, (with sealed ends) rather than from exchange sites in the bark or elsewhere on the twigs. Current-year needles are also more vulnerable to leaching losses than older needles, despite the typically greater total foliar Ca content of the older foliage (DeHayes *et al.*, 1999).

We found similar results in an autumn misting study, in which red spruce seedlings were subjected to artificial precipitation solutions (Schaberg *et al.*, 2000). Seedlings treated with artificial precipitation of pH 3 exhibited significantly greater Ca leaching losses than those subjected to a similar solution of pH 5. We also observed physiological perturbations in response to these treatments. Because red spruce typically shows sensitivity to extreme midwinter cold events, cold hardiness was assessed as the physiological response variable. Seedlings subjected to precipitation of pH 3 were consistently less cold tolerant than trees treated with pH 5 precipitation. This and other acid misting studies did not, however, result in any significant alterations in total foliar Ca, which is one of the most common methods of assessing a plant's physiological Ca status. However, considering Ca's partitioning in conifer foliage, total foliar Ca is probably not the best method of evaluating meaningful Ca perturbations.

Calcium tends to form an insoluble precipitate with inorganic phosphate; thus cytoplasmic Ca in high concentrations is toxic. Because of this, all cells have evolved mechanisms to maintain very low cytoplasmic Ca concentrations (0.1 µM, Hepler and Wayne, 1985; > 1 µM, Fink, 1991; about 10^{-7} M, or at least 1000X lower than in the apoplast, Evans *et al.*, 1991). These mechanisms of Ca partitioning include a variety of energy-utilizing Ca pumps and antiporters, which allow cells to pump Ca either out into the apoplast, or to sequester it inside of organelles (Hanson, 1984; Evans *et al.*, 1991; Bush, 1993; Bush, 1995). Plants have also developed mechanisms to maintain this sequestration, including forming calcium oxalate either inside the vacuole for many plant species (Hanson, 1984) or in the apoplast of some plants, including conifers (Fink, 1991). Fink (1991) estimated that greater than 90% of the total Ca in needles of Norway spruce is bound as sparingly soluble Ca oxalate. These large crystallized deposits are not readily available for plant use and are not likely to be readily lost via acidic leaching, although they dominate what is measured in total foliar Ca analyses. Thus, measurements of total foliar Ca are probably not a good indicator of the true physiological calcium status of a plant.

In order to address this apparent disparity between the Ca that is typically measured in plants, and Ca that may be physiologically important for plants, we developed techniques to assess relative amounts of membrane-associated calcium (mCa). This represents Ca ions in dynamic equilibrium between exchange sites on the cell walls and plasma membranes, as well as in solution in the apoplast (Borer *et al.*, 1997). This technique uses computer-based image processing in conjunction with fluorescence microscopy, using the fluorescent dye chlorotetracycline (CTC). By using filters that are specific to the emission wavelengths of the Ca-CTC complex, pixel brightness in the resultant images reflects relative mCa concentrations. The images are standardized and cell membrane

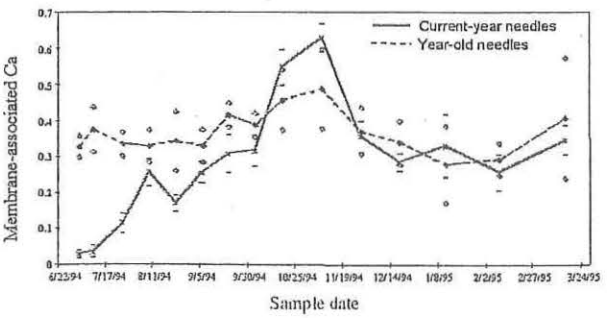

Figure 2. Membrane associated Ca in current-year and year-old foliage from red spruce seedlings.

brightnesses numerically compared using image analysis software.

After technique development and verification, we studied relative mCa over the course of a year in mesophyll cells of red spruce seedlings growing under ambient environmental conditions (Figure 2; DeHayes *et al.*, 1997). Current-year needles accrete mCa through the growing season and into the winter, but these developing needles are more sensitive to Ca leaching losses than year-old foliage (Figure 1). If accretion of a relatively large midwinter pool of mCa in current-year foliage is necessary for adequate environmental responsiveness and to develop the ability to withstand extreme midwinter temperatures, leaching losses during this important accretion period may leave plants vulnerable to further damage. Current-year foliage also tends to be much less cold tolerant than year-old foliage, and in red spruce, is often sensitive to temperatures that do occur within its ecological range (DeHayes *et al.*, 1999).

Seasonal patterns associated with environmental changes can also be detected in mCa from current-year foliage (DeHayes *et al.*, 1997). A substantial decline in mCa was associated with the first hard frost ($\leq$ 5 °C), and another was associated with an extreme midwinter thaw, which was followed by slow mCa recovery. These observations likely resulted from temperature-induced alterations in the membrane structure, but may also reflect signal transduction processes. In either case, these changes may result in altered membrane stability. A similar midwinter pattern (a sudden and dramatic decline followed by a gradual recovery) was discernible for the cold tolerance of red spruce trees measured during the same midwinter thaw event (Strimbeck *et al.*, 1995). Temperature-induced changes in mCa may reflect Ca signal transduction and are likely associated with altered physiology (DeHayes *et al.*, 1999).

Despite the apparent physiological importance of the pool of Ca that we measure as mCa, we have observed no significant relationship between measurements of mCa and total foliar Ca (DeHayes *et al.*, 1997). This suggests that total foliar Ca measurements are not the most appropriate assessments of physiologically meaningful Ca.

Further research, using controlled seedling studies, has confirmed the importance of mCa assessments (Schaberg *et al.*, 2000). We attempted to manipulate Ca physiology of red spruce seedlings through Al and Ca soil treatments, in conjunction with acidic mist treatments. Both soil treatments significantly affected total foliar Ca, but acidic mist did not significantly impact total foliar Ca. Gas exchange measurements were affected by soil treatments only during the growing season. As with previous work, acidic mist resulted in a significant amount of Ca leaching, and acidic mist was the only factor that significantly altered both mCa and cold tolerance. Acidic mist was also the only factor that produced significant reductions in membrane stability (measured as foliar electrolyte leakage). Interestingly, attempts to manipulate mCa through soil Ca availability failed in this study. Although plants were given controlled soil nutrient solutions, they were not protected from ambient precipitation, which may have been a source of Ca for these trees.

A plausible explanation of the processes and mechanisms by which acidic deposition can lead to physiological perturbation in red spruce, and ultimately may contribute to species decline, can be derived from these data (Figure 3; DeHayes *et al.*, 1999). Acidic deposition inundates plant foliage with substantial quantities of hydrogen ions. This large influx of hydrogen ions selectively displaces Ca from the labile pool of mCa, which is vulnerable to leaching losses directly from plant foliage. Reduced mCa may impair signaling processes, or it may destabilize cell membranes, or both. Reductions in cold tolerance result either from diminished environmental signaling or from membrane destabilization, or from both processes. If this model is correct, one would expect to find substantial freezing injury during years when substantial leaching of mCa coincides with extreme winter cold events or other factors that can freeze-injure foliage.

Broader implications of this work

To the best of our knowledge, the only factor in this process that is unique to red spruce is its sensitivity to extreme winter cold events. We have observed aspects of this model in a variety of tree species, including physiological and mCa perturbations as a result of acidic mist treatments. Foliar Ca leaching has been documented for a variety of tree species (Lovett *et al.*, 1985; Lovett *et al.*, 1991). We have also found membrane destabilization in balsam fir, a reduction in cold tolerance of eastern white pine, and reductions in mCa in eastern hemlock as a result of acid treatments (DeHayes *et al.*, 1999). The general physiological functions of Ca, including mCa, are virtually ubiquitous among cell types and species. Ca

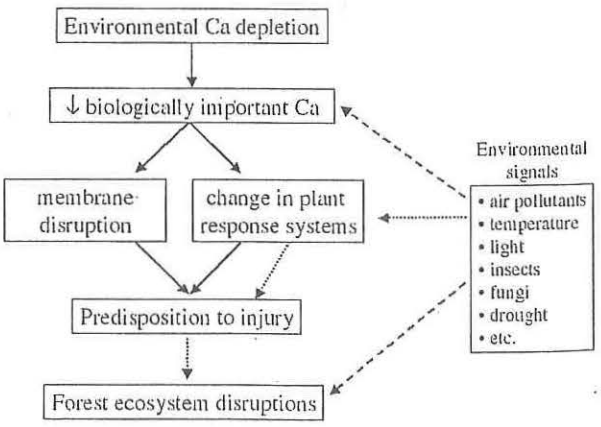

Figure 3. Possible physiological consequences of environmental calcium depletion. Solid lines indicate known relationships, dashed lines indicate hypothesized connections, and dotted lines indicate hypothesized connections that are currently being investigated.

signal transduction enables plants to perceive and respond to their environment, and it may play an important role in overall plant response systems, including plants' abilities to respond to stressors.

Acidic deposition can result in both soil Ca depletion and foliar mCa depletion. Inadequate mCa can result in both membrane disruption (decreased membrane stability) and altered plant responsiveness to environmental signals or secondary stresses. These, in turn, would likely predispose plants to injury and may lead to alterations in ecosystem health.

Current research

We are currently working on a variety of studies designed to address some of the gaps in our knowledge and understanding concerning the broader applicability of the model of mCa perturbation that we have proposed (Figure 3). The first of these studies is designed to investigate the role of mCa in responsiveness to changing environmental conditions. We are using soil Ca treatments in conjunction with acidic leaching to perturb foliar mCa in red spruce seedlings grown outdoors but protected from ambient Ca inputs. Through soil manipulations alone, we have produced seedlings with no apparent signs of decline, but which do exhibit altered mCa, total foliar Ca, and height and diameter growth. Because Ca is thought to be an important mediator of environmentally-induced changes in physiology, we will soon assess differences in physiological responses to short-term environmental cues (e.g. changing light and temperature) among plants in Ca perturbation treatments.

A second study is designed to determine whether ambient conditions at a site with low soil Ca availability can influence mCa enough to affect plant physiology. At this site, we have observed a direct correlation between total foliar Ca and physiology (measured as cold tolerance) only in the documented Ca "deficiency" range for the species (Swan, 1971). We are investigating whether a similar relationship is found between mCa and cold tolerance levels.

We are also assessing mCa and cold tolerance physiology at a site that has been manipulated to simulate nitrogen saturating conditions, and we have initiated work to examine the possible role for mCa in sugar maple decline. We are also exploring the relationship between mCa changes and signal transduction at the cellular level.

Conclusion

We are currently working to bridge the gaps in our knowledge and understanding of the potential ramifications to the future health and sustainability of forested ecosystems of widespread declines in environmental Ca availability. Although much of our work has been with red spruce and its responsiveness to cold events, it is likely that similar Ca perturbations are occurring in other plant species. These perturbations may leave plants vulnerable or inadequately responsive to environmental stresses that might otherwise pose no significant threat. The potential ramifications of this could be substantial. If environmental Ca depletion does impair plant stress response systems, the combination of this deficiency and the growing onslaught of anthropogenic stresses (e.g. pollution, exotic pests, pathogens, climate change, etc.) could have serious ramifications on forest health.

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