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# Relative quantification of membrane-associated calcium in red spruce mesophyll cells

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**Abstract** We describe a method for localizing and comparing relative amounts of plasma membrane-associated calcium ions (mCa) in complex tissues and verify the procedure for mesophyll cells of red spruce (*Picea rubens* Sarg.) needles. This technique incorporates epifluorescence microscopy using the fluorescent probe chlorotetracycline (CTC) with computer image processing and analysis. Using an appropriate standardization for image brightness, the procedure allows relative quantitative comparison of CTC-fluorescence in the plasma membrane-cell wall region that corresponds to relative amounts of mCa. The technique effectively discerned mCa differences among red spruce needle sections exposed to treatments designed to alter mCa levels in vitro. Estimates of mCa for nine red spruce seedlings, were highly repeatable over a 6 week period in late summer. This repeatability verifies that the described methods produce reliable and reproducible estimates of foliar mCa in woody plant foliage. By incorporating image analysis, this technique allows for relative quantitative estimates of mCa specific to the physiologically-important and labile pool of Ca associated with the plasma membrane-wall complex. Such measurements have not previously been reported for woody plant tissues and thus may provide new insights into the relative roles and responsiveness of mCa vs total foliar Ca pools.

**Key words** Red spruce · Mesophyll cell · Membrane-associated calcium (mCa) · Chlorotetracycline (CTC) · Fluorescence microscopy

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## Introduction

Tree physiologists and forest ecologists frequently utilize measurements of total foliar concentrations of specific ions to examine the physiological status of trees or the responses of forests to environmental conditions. Such measurements, however, may not provide an estimate of physiologically important pools of ions, especially if the element in question is strictly compartmentalized. Ions can be unequally distributed through cells and tissues, with this partitioning playing a critical role in their physiological function (Marschner 1986). For instance, cytoplasmic concentrations of calcium (Ca) ions are assumed to be quite low (Hanson 1984; Hepler and Wayne 1985), although stores of Ca can be quite high elsewhere in plant cells, such as in vacuoles and other organelles (Hanson 1984). In addition, there may be spatial separation of Ca within complex tissues. High Ca concentrations may be precipitated, as calcium oxalate or pectate, along the outside walls of mesophyll cells and within walls of epidermal cells, sieve cells, and transfusion parenchyma (Fink 1991). In studies where total foliar Ca concentrations are measured, these large stored pools of Ca could mask any comparatively subtle differences among trees, or changes over time, in the compartmentalization within tissues or cells.

Calcium plays an integral role in cell membrane structure and function, stabilizing membranes by bridging phosphate and carboxylate groups of membrane phospholipids (Palta and Li 1978; Legge et al. 1982; Davies and Monk-Talbot 1990; Steponkus 1990). The association of Ca with plasma membranes strongly influences the response of cells to the environment, but meaningful estimates of Ca specific to the plasma membrane-cell wall region have been very difficult to obtain. Thus, the development of a method to measure membrane-associated Ca would greatly aid the study of plant-environment interactions.

The fluorescent probe chlorotetracycline (CTC) has been used extensively for visual localization of Ca associated with the plasma and organelle membranes. CTC is an appropriate marker for divalent cations associated with

biological membranes because the CTC dye molecule selectively binds to divalent cations, and has a moiety with an affinity for apolar environments. The chelation to divalent cations in close proximity to apolar environments (biological membranes) causes a conformational change in CTC, which results in an enhanced affinity for the cation, as well as a marked increase in the fluorescence of the molecule over that in entirely aqueous solutions (Caswell and Hutchison 1971). Despite the membrane specificity of the CTC probe, it is likely that CTC fluorescence specific to plasma membrane-associated Ca (mCa) reflects Ca ions in the membrane-wall complex, which are in dynamic equilibrium and distinctive from other dominant insoluble pools in conifer needles (Fink 1991).

CTC staining has been used to visually localize mCa within cells or in small groups of cells (e.g., Gray and House 1983; Tirlapur et al. 1993) and to visually monitor mCa development in isolated cells or small groups of cells (e.g., Gawlitta et al. 1980; Saunders and Hepler 1981). In conjunction with fluorescence spectrophotometry, fluorimetry, or microscope fluorimetry, CTC staining has also been used to compare mCa among cells of plant and animal tissues (e.g., Lynch et al. 1987; Jacob 1991). Most of these studies measured total cellular fluorescence, which reflects autofluorescence and CTC fluorescence from numerous organelles as well as CTC fluorescence from the plasma membrane-cell wall complex (eg., Cramer et al. 1985; Renard-Rooney et al. 1993). Tirlapur and Cresti (1992) used video image analysis in conjunction with CTC fluorescence to map the spatial distribution of mCa in growing tobacco pollen tubes, and Arora and Palta (1988) used similar procedures to examine the influence of supplemental Ca treatments on mCa of onion epidermal cells. Despite considerable use of CTC as a fluorescent probe for visualization of mCa, we are unaware of any studies examining mCa in leaf tissues of woody plants, or procedural descriptions for estimating mCa.

We present a detailed procedure for relative quantification of Ca associated with plasma membranes of woody plants. The procedure requires minimal chemical or physical alteration and as a result, membrane integrity and presumably mCa status are preserved. We also present estimates of mCa in mesophyll cells of red spruce seedlings grown under ambient environmental conditions in Burlington, Vt, USA.

## Materials and methods

### Procedures for relative quantification of mCa in spruce mesophyll cells

#### *Sectioning*

To minimize cellular disruption as a result of desiccation, osmotic shock, altered pH or extremes in temperature, and to prevent the leaching of ions into preparative solutions, red spruce needles were sectioned without chemical fixation, embedding, or freezing. Instead, fresh needles were supported in a small piece of carrot and 50 mm thick cross-sections were cut using a sliding microtome. Sections were immediately transferred, using a fine-tip paintbrush, to a buffer solution containing 10% sucrose and 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), adjusted to pH 6.5 with tetramethylammonium hydroxide.

#### *Staining*

After all sections were collected, buffer was removed and replaced with 90 µl of fresh buffer. To this we added 10 µl of 1.9 mM CTC solution for a final CTC concentration of 0.19 mM. This concentration was selected because lower CTC concentrations did not allow for the full development of fluorescence. Unstained sections from each needle were retained as a control to detect any autofluorescence in sections; we added 10 µl of buffer solution to control samples. All samples were stained overnight at 5°C in the dark. A preliminary study demonstrated that CTC fluorescence increased steadily at incubation times of 30 min to 7 h, and then remained stable until at least 20 h. Based on visual assessments using the cell viability stain fluorescein diacetate (FDA; Duncan and Widholm 1990), it was clear that cells consistently remained alive throughout the treatment and incubation period. Furthermore, examinations using the general membrane stain 3,3′ dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (Teraski 1989) indicated that cells appeared normal following the incubation period; organelles remained distributed throughout the cytoplasm with no visual evidence of appression. Because living plant cells regulate the distribution and compartmentalization of Ca (Hepler and Wayne 1985), we believe the cells we analyzed represented the naturally occurring distribution of Ca within red spruce mesophyll cells.

#### *Microscopy*

Microscopic analyses were performed using a microscope fitted with a tungsten-halogen incident epifluorescence system and a camera with an automatic exposure controller. The tungsten-halogen lamp was used because it produces little UV light, therefore minimizing photobleaching. Samples were illuminated through an FITC filter, which allowed wavelengths between 350 and 500 nm to excite the specimen. The optimal excitation wavelength for CTC-$Ca^{2+}$ fluorescence is 400 nm (Mathew and Balaram 1980). The dichroic beamsplitter had a cutoff wavelength of 500 nm, and the barrier filter was a narrow bandpass filter of $535 \pm 10$ nm. This narrow bandpass filter filtered out red chlorophyll autofluorescence and reduced the CTC-$Mg^{2+}$ fluorescence (peak, 520 nm) relative to that of CTC-$Ca^{2+}$ (peak, 530 nm; Caswell and Hutchison 1971). Photographs were taken with 400 ASA black and white professional film. All pictures were taken with the same exposure time (approximately 45 s), lamp voltage, and magnification, and with all other conditions and equipment settings as consistent as possible. The film was developed and all pictures were printed at a fixed density and contrast setting to maximize image uniformity within each roll of film. We chose to capture images on photographs and scan them into a computer for brightness analysis rather than using CCD image grabbing technology because of the potential for greater image resolution associated with the high resolution scanner.

#### *Image Processing and Analysis*

Photographs were scanned into a Macintosh computer via a flatbed image scanner at 498 dots per inch and 25% scaling. Scanning was done using NIH Image and the Microtek ScanMaker Plug-in from Adobe Photoshop (Adobe Systems, Mountain View, Calif.). All image processing and analyses were performed on a Macintosh Quadra 700 computer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Road., Springfield, VA 22161, part number PB93-504868). To determine the background pixel brightness level, all areas of high brightness (plasma membranes, chloroplasts, mitochondria, nuclei, and obvious artifacts) were shaded in black. Using this shaded picture, a mean pixel brightness value of all non-black (background) pixels in the picture was determined. All

**Table 1** Eleven mutually orthogonal contrasts used in statistical analyses to determine the influence of solution treatments, with and without chlorotetracycline (CTC) designed to potentially impact levels of membrane-associated calcium (mCa) in red spruce mesophyll cells. Means represent adjusted fluorescence brightness values that reflect relative levels of mCa, with higher numbers reflecting greater mCa

| Contrasted solution treatments | Contrasted mCa Means | Contrast Probability |
|---|---|---|
| CTC-stained buffer, $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ vs stained EGTA, EDTA, and all unstained treatments | 0.38 vs 0.07 | 0.00 ** |
| CTC-stained $CA^{2+}$ **vs** CTC-stained buffer, $Mg^{2+}$, $Na^{+}$ | 0.44 vs 0.36 | 0.01 ** |
| CTC-stained $Na^{+}$ and $Mg^{2+}$ **vs** CTC-stained buffer | 0.36 vs 0.38 | 0.70 |
| CTC-stained $Na^{+}$ **vs** CTC-stained $Mg^{2+}$ | 0.38 vs 0.33 | 0.17 |
| CTC-stained EDTA and EGTA **vs** all unstained treatments | 0.09 vs 0.13 | 0.59 |
| CTC-stained EGTA **vs** stained EDTA | 0.11 vs 0.07 | 0.52 |
| Unstained EDTA and EGTA **vs** unstained buffer, $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ | 0.05 vs 0.7 | 0.57 |
| Unstained EGTA **vs** unstained EDTA | 0.00 vs 0.10 | 0.21 |
| Unstained buffer **vs** unstained $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ | 0.04 vs 0.08 | 0.34 |
| Unstained $Na^{+}$ and $Mg^{2+}$ **vs** unstained $Ca^{2+}$ | 0.11 vs 0.04 | 0.17 |
| Unstained $Na^{+}$ **vs** unstained $Mg^{2+}$ | 0.05 vs 0.16 | 0.10 |

pictures were then normalized to a constant background level by adjusting pixel values for each entire picture. To determine the image brightness of plasma membrane-cell wall region, all areas other than the cell wall/membrane were shaded with black, and a mean non-black pixel value was determined for each picture. It would be substantially less time consuming to use thresholding to select the stained area, as was done by Takamatsu et al. (1986) using DAPI staining for DNA. Unfortunately, this was not possible because of the bright auto- and CTC-fluorescence from the cuticle and the membranes of some organelles.

Because of variation in film processing and/or autofluorescence, there was some variation in image fluorescence (usually less than 7%) among rolls of film that can be attributed to film processing or autofluorescence noise rather than mCa fluorescence. This artifact was adjusted out of mCa fluorescence estimates by creating a pixel brightness ratio, which was defined as the pixel brightness value of a stained sample divided by mean pixel brightness of unstained control plasma membranes for each roll of film. This ratio eliminated the background noise and allowed comparison among samples that were collected, photographed, and processed on different dates. Mean pixel brightness values were computer-generated and represent a numerical continuum of brightness from 255 (black) to 0 (white). We chose to reverse the scale so that increasing values correspond to increasing brightness and mCa. As such, mCa values are calculated as: mCa = 1 – pixel brightness ratio.

## Verification of procedures

### *Plant material*

Verification experiments were conducted on needles collected from a population of red spruce seedlings grown in a loamy sand nursery soil. At the time of sampling they were 7 years old and averaged about 50 cm in height. Seedlings were grown from a bulk red spruce seed collection from throughout Vermont and were expected to exhibit a range of mCa levels. For in vitro experiments, year-old shoots were collected from several different seedlings in June and August of 1994 and sectioned needles were subjected to various treatment solutions. For repeatability experiments that examined consistency of mCa measurements within individuals over time, current-year shoots were collected from lateral branches of nine seedlings in August and again in September of 1994.

### *Treatments*

To evaluate the effectiveness of the procedures and the specificity of CTC for Ca ions in red spruce leaf tissue, we exposed red spruce needle sections to one of an array of treatment solutions that would be expected to increase mCa, decrease mCa, or have no influence on mCa. Using the procedures described, we then quantified and compared relative mCa among mesophyll cells exposed to the various treatment solutions.

Standard buffer solutions used for all treatments were made with 10% sucrose, and 20 mM MES in deionized water, and were adjusted to pH 6.5 with tetramethylammonium hydroxide. Treatments included various additions, at concentrations of 50 mM, to this buffer. The twelve treatments consisted of additions of EGTA, EDTA, $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, and buffer with no additions, each with and without CTC staining. EGTA (ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetate) was added to selectively chelate $Ca^{2+}$ and remove it from membranes. We expected this to reduce CTC fluorescence. EDTA (ethylenediaminetetraacetic acid) was added to bind all divalent cations. EDTA has a stronger affinity for Ca than EGTA, but it also effectively chelates other divalent cations. Differences in CTC fluorescence of tissues treated with EGTA and EDTA should reflect the relative contributions of $Ca^{2+}$ and other divalent cations to the measured plasma membrane-associated cation pool. Calcium was added (as $CaCl_2 \cdot 2H_2O$) to increase mCa, and thus might be expected to increase fluorescence intensity. Magnesium ($Mg^{2+}$) was added (as $MgCl_2 \cdot 6H_2O$) to determine whether the emission filter was able to separate CTC-$Ca^{2+}$ fluorescence from that of CTC-$Mg^{2+}$. Sodium ($Na^{+}$) was added (as NaCl) to determine whether ionic additions alter either autofluorescence or CTC fluorescence.

On each treatment date, approximately 15 red spruce needle sections were placed in two test tubes for each of the six treatments. One tube was for the CTC stained treatment and the other served as an unstained control. Sectioned needles were initially placed into the buffer solution with no additions. After sectioning was complete, the buffer was removed and replaced with the appropriate treatment solutions. Samples were incubated overnight in the dark at 5°C. The treatment solutions were removed and the sections were rinsed three times with the basic buffer solution. Sections were then resuspended in 90 µl of the basic buffer and stained with CTC as described above. On each treatment date, membrane fluorescence was estimated and quantified in mesophyll cells from three to five needle sections from each of the stained and unstained treatments.

### *Data analysis*

For in vitro verification treatments, an analysis of variance was performed to determine the significance of variation between dates, among treatments, and in the treatment-by-date interaction. Eleven mutually orthogonal contrasts were selected to examine differences in mean pixel brightness ratios among specific treatment combinations, and therefore different levels of membrane-associated divalent cations (Table 1). For seedlings sampled repeatedly over time, Spearman′s Rank Order Correlations ($r_s$) were used to assess the consistency in ranking over time and repeatability of the procedure.

## Results

### In vitro verification experiments

Membrane fluorescence of red spruce mesophyll cells from needle sections soaked in the solution treatments were separable into three groups. Unstained cells (i.e., without CTC) and stained cells with EGTA or EDTA exhibited the lowest pixel brightness (mCa fluorescence value = 0.07), while CTC-stained cells from treatments with supplemental $Ca^{2+}$ consistently exhibited the greatest pixel brightness (mCa fluorescence value = 0.44; Table 1). CTC-stained cells from treatments with supplemental $Na^{+}$ or $Mg^{2+}$ or with no additional cations were intermediate between the extreme treatment groups (Table 1). No significant differences were found between sample dates, and the treatment-by-date interaction was not significant.

The consistently low pixel brightness of mesophyll cell membranes in all unstained solution treatments indicates that the treatments did not significantly alter autofluorescence. Although EDTA is expected to have a stronger affinity for $Ca^{2+}$ than EGTA, CTC-stained needle sections from EGTA and EDTA treatments exhibited pixel brightness values that were not significantly different from unstained controls (Table 1). As such, both components appeared to effectively remove all detectable $Ca^{2+}$ from the plasma membrane-cell wall region. Furthermore, it appears that these treatments effectively and equally removed the cations involved in CTC fluorescence indicating that cations other than $Ca^{2+}$ contributed minimally to fluorescence. The difference in fluorescence response to additional $Ca^{2+}$ in comparison with additional $Mg^{2+}$, $Na^{+}$, or buffer alone also indicate that the majority of the cations measured with this technique were $Ca^{2+}$. The narrow bandpass emission filter and tungsten-halogen lamp helped minimize the fluorescence of the Mg-CTC complex, which has an excitation wavelength of 377 nm and an emission wavelength of 520 nm. Caswell (1979) also noted, in studies with red blood cells, that CTC has a much greater affinity and fluorescence when complexed with $Ca^{2+}$ than with $Mg^{2+}$.

The lack of a difference between CTC-stained cells with supplemental $Na^{+}$ or $Mg^{2+}$ and no cation additions suggests that $Na^{+}$ and $Mg^{2+}$ do not affect mCa from mesophyll cells as measured by this procedure. In contrast, others (Lynch et al. 1987, with corn root protoplasts; and Cramer et al. 1985, with cotton root hairs) noted a reduction in CTC fluorescence in response to $Na^{+}$ treatments. Their studies, however, involved herbaceous plants, different cell and tissue types, and included total CTC fluorescence that was not restricted to the plasma membrane-cell wall complex.

### Repeatability of mCa estimates

Having verified the Ca-specificity and effectiveness of this procedure using in vitro manipulations, we then used the procedure to make relative quantitative in vivo comparisons of mCa in mesophyll cells of nine red spruce seedlings in August and September 1994. Seedlings differed considerably in relative mCa within each date, but relative mCa estimates among seedlings were consistent between the two sample dates ($r_s$ = 0.90, $P \leq 0.05$, $n$ = 9; Table 2). The strong individual tree repeatability of mCa measurements over the approximately 5 week time period is further validation of the effectiveness and utility of the procedures utilized. To the best of our knowledge, these relative quantitative estimates of mCa in mesophyll cells of red spruce are the first such estimates for any tree species.

**Table 2** Estimates and relative ranking of plasma membrane-associated Ca (mCa) in mesophyll cells from the same red spruce trees sampled in August and September 1994. Spearman's Rank Order Correlation ($r_s$) between sample dates was statistically significant ($r_s$ = 0.90, $P \leq 0.05$, $n$ = 9). Measurements of mCa represent adjusted fluorescence brightness values with higher numbers reflecting greater mCa

| | August 1994 | | September 1994 | |
|---|---|---|---|---|
| tree | mCa | rank | mCa | rank |
| A | 0.126 | 1 | 0.170 | 1 |
| B | 0.183 | 2 | 0.183 | 3 |
| C | 0.184 | 3 | 0.182 | 2 |
| D | 0.195 | 4 | 0.188 | 4 |
| E | 0.285 | 5 | 0.443 | 7 |
| F | 0.299 | 6 | 0.267 | 5 |
| G | 0.369 | 7 | 0.518 | 8 |
| H | 0.438 | 8 | 0.438 | 6 |
| I | 0.444 | 9 | 0.531 | 9 |

## Discussion

An important feature of this procedure for visualization and quantification of relative amounts of Ca in the plasma membrane-cell wall region of red spruce mesophyll cells is the use of image analysis in sample processing and assessment. By providing the capability to remove (through shading) selected tissue and cellular components, image analysis allowed us to generate brightness estimates of CTC fluorescence specific to the plasma membrane-cell wall region. This technique effectively eliminates from our mCa estimates autofluorescence and CTC fluorescence from the cuticle and vascular tissue, organelle membranes, and additional artifacts that were sometimes scattered throughout sections. These additional sources of non-mCa fluorescence in red spruce needle tissue were substantial and, without removal, would have precluded meaningful mCa estimates specific to the physiologically important membrane-wall pool of Ca ions. Masking of CTC fluorescence generated by cytoplasmic sources (e.g.., organelle membranes) also increased the precision of relative mCa estimates for individual plants. In a supplemental study designed to examine the efficacy of removing non-mCa fluorescence, the error variance in mCa estimates was 47–87% lower when shading was used to minimize extraneous sources of fluorescence. Finally, the ability to generate location-specific mCa estimates (i.e., specific to the plasma

membrane-cell wall region) through the use of image processing and analysis also made it possible to work with sectioned needle tissue as opposed to plasmolyzed cells or suspension cultures. Our view is that sectioning represented less severe alteration relative to Ca ion distribution than alternative sample preparation procedures.

Because of the relatively subtle nature of the endomembrane-plasma membrane transition in red spruce mesophyll cells, it is possible that there is a small amount of unmasked fluorescence from organelle membranes in our mCa estimates. However, visual assessments using $DiOC_6(3)$, which is specific to intracellular membranes (Terasaki 1989), revealed little or no evidence of endomembrane in the vicinity of the fluorescing plasma membrane-cell wall region of red spruce mesophyll cells.

This procedure provides accurate and repeatable relative estimates of mCa in mesophyll cells of both current-year and year-old needles of red spruce. It is important to emphasize that these estimates are relative. Individuals can be characterized as having more or less mCa than others from the same sampling time or the same individuals on different dates. The procedure has utility for studying seasonal or developmental variation in mCa or the mCa response to exogenous or endogenous treatments. We have not yet been able to calibrate these relative estimates for absolute quantification of mCa, nor have we experimented with other tissues or species.

We purposely refer to mCa as membrane-associated Ca rather than membrane-bound or membrane-specific Ca. There is likely a considerable amount of Ca that is cell wall-bound in spruce mesophyll cells (Marshner 1986). Although CTC molecules have a hydrophobic domain that inserts into the internal lipid region of membranes, the polar section of CTC binds both $Ca^{2+}$ on the plasma membrane and some free and displaced apoplastic Ca from the cell wall. Cramer et al. (1985) found that the contribution of $Ca^{2+}$ ions from the cell wall to CTC fluorescence was quite small. They plasmolyzed cotton (*Gossypium* sp.) root hair cells and found that cell wall Ca contributed less than 5% of the total CTC fluorescence. Based on their findings and the membrane specificity of the CTC probe, it is tempting to assume that the contribution of cell wall Ca to CTC fluorescence in the membrane-wall region of red spruce mesophyll cells is also likely to be small. We have not, however, ascertained the relative contributions of wall vs membrane Ca to total CTC fluorescence for red spruce needles and emphasize that our mCa estimates are based on $Ca^{2+}$ in the membrane-wall complex. Because Ca ions in the membrane-wall complex are expected to be in dynamic equilibrium, we believe this combined mCa pool represents a physiologically important pool in conifer needle tissue.

An additional complicating factor inherent in mCa estimates derived using this procedure may be the influence of variation in membrane density or volume throughout individual membranes or among cells or needle sections. It is conceivable, for example, that relatively high fluorescence may result from increased $Ca^{2+}$ per quantity of membrane or may simply be a result of greater membrane density (Saunders and Hepler 1981). If one is interested primarily in documenting changes in mCa, whether caused by changes in $Ca^{2+}$ per quantity of membrane or the availability of binding sites, this distinction may not be critical. If, in contrast, one is interested in understanding the basis for a CTC fluorescence gradient within an individual cell membrane, it would be difficult, using the procedure we describe, to distinguish between a $Ca^{2+}$ gradient and a gradient in membrane density. However, the clear and consistent fluorescence differences among our in vitro treatments and the highly repeatable relative mCa estimates within seedlings over time indicate that gradients in membrane fluorescence within cells or among cells within seedlings were relatively small. Saunders and Hepler (1981) have successfully employed a general membrane probe (N-phenyl-1-naphthylamine) to distinguish between gradients of $Ca^{2+}$ and membrane density.

The procedure we have described appears to be sufficiently precise to allow us to characterize natural variation in mCa among trees and sufficiently repeatable to accurately elucidate mCa patterns among individuals over time. The strong correspondence between pixel brightness ratios and the expected response to the in vitro treatments coupled with the consistency in response over two experimental dates indicates that the procedure described provides meaningful relative estimates of mCa in mesophyll cells of red spruce. This technique can be used to further examine the role of mCa in enabling woody plants to cope with environmental stress and to distinguish physiologically important mCa from the frequently measured total foliar Ca pools.

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