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Seasonal Alteration of the Cytosolic and Nuclear Ca^{2+} Concentrations in Overwintering Woody and Herbaceous Perennials in Relation to the Development of Dormancy and Cold Hardiness

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Abstract. Seasonal alteration of the cytosolic and nuclear Ca^{2+} concentrations of spruce (*Picea engelmannii* Parry) and brome grass (*Bromus inermis* Leyss) was investigated by the antimonate precipitation cytochemical technique. Electron microscopic (EM) observations revealed that electron-dense Ca^{2+} antimonate deposits, an indication of Ca^{2+} localization, were seen mainly in the vacuole, the cell wall and the intercellular space in samples of both species, collected on 14 July 1997. Few deposits were found in the cytosol and nuclei, showing a low resting level during summer months. On 8 Aug. 1997 following a decrease in daylength of 1 hour and 12 minutes, Ca^{2+} accumulation was initiated in spruce with increased cytosolic and nuclear Ca^{2+} deposits, but not in brome grass. On 8 Sept. 1997, Ca^{2+} accumulation occurred in the cytosol of brome grass. This followed a drop in ambient temperature to 12 °C. Cytosolic and nuclear Ca^{2+} deposits continued to increase in spruce. Controlled experiments confirmed that it was the low temperature, not shortening daylength, that triggered Ca^{2+} accumulation in brome grass. High cytosolic and nuclear Ca^{2+} concentrations lasted about three months in spruce from early August to early November. However, the high cytosolic and nuclear Ca^{2+} concentrations in brome grass lasted only about 20 days from early September to the end of the month. During winter and spring, both species had low resting cytosolic and nuclear Ca^{2+} concentrations. The relationship between the duration of the high cytosolic and nuclear Ca^{2+} concentrations and the status of the developed dormancy/cold hardiness is discussed in light of current findings.

Temperate woody perennials undergo an annual cycle of rhythmic changes, which are induced by the seasonal signals of shortening daylength and/or lowering temperature (Nooden and Weber, 1978; Weiser, 1970). In summer, plants are actively growing with little cold hardiness. As the daylength shortens and the ambient temperature decreases plant growth rate decreases and, ultimately, ceases at which time plants enter the stage of physiological dormancy (Lang et al., 1985, 1987). Concomitantly, the cold hardiness of plants also becomes well-developed (Sakai and Larcher, 1987; Weiser, 1970). In spring, as the daylength becomes longer and the ambient temperature increases, plants deacclimate and resume growth (van Huystee et al., 1967). Contrary to woody perennials, overwintering herbaceous plants do not develop the stage of so-called physiological dormancy (Lang et al., 1985, 1987) under a similar environment. Overwintering herbaceous plants maintain their capability to grow throughout the winter whenever subjected to favorable growing conditions. It is also known that low temperatures are the primary factor in cold acclimation of overwintering herbaceous plants like winter rape (Kacperska-Palacz, 1978), and winter wheat (Gusta et al., 1982).

Calcium, as a second messenger, plays an important role in plant growth and its adaptation to the current environment (Bush, 1995; Hepler and Wayne, 1985; Knight, 2000; Poovaiah and Reddy, 1993). Change in cytosolic Ca^{2+} concentration provides the basis for Ca^{2+} to serve as a second messenger. Jian et al. (1997) reported that 8-h short days at room temperature induced Ca^{2+} accumulation in poplar apical bud cells, which was closely associated with growth cessation and the development of dormancy/cold hardiness. Shortening daylength in nature also plays a primary role in signaling Ca^{2+} accumulation in the cytosol of mulberry apical bud cells (Jian et al., 2000). Monroy et al. (1993) and Monroy and Dhindsa (1995) reported that low temperatures induced Ca^{2+} influx in alfalfa, which was necessary for the development of cold hardiness. We wondered if there was a difference in the signal for Ca^{2+} accumulation in the cytosol and nuclei between overwintering *Picea,* a woody perennial and *Bromus,* a herbaceous perennial in nature. We attempted to answer this question using spruce and brome grass.

Materials and Methods

Plant materials. One-year-old twigs of spruce (*Picea engelmannii* Parry) from a 30-year-old tree and brome grass (*Bromus inermis* Leyss) were used. Both species were grown at the location of 45°N latitude in St. Paul, Minn. Samples were collected on 14 July, 8 Aug., 8 and 29 Sept., 8 Oct., 1 Nov., and 13 Dec. 1997 and 23 Jan. and 10 May 1998.

Controlled experiments for brome grass. Controlled experiments were carried out in growth chambers. For Experiment I, with a 14-h photoperiod, the chamber had only changes in diurnal temperatures. Temperatures were programmed in the chamber to closely resemble field conditions that occurred on the night prior to the 8 Sept. 1997 collection date. It was 12 °C from 2100 to 0600 hr, 14 °C from 0600 to 0800 hr, 16 °C from 0800 to 1000 hr, 18 °C from 1000 to 1200 hr, 22 °C from 1200 to 1800 hr, 18 °C from 1800 to 1900 hr, 16 °C from 1900 to 2000 hr, and 14

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°C from 2000 to 2100 HR. The lowest temperature was set at 12 °C for 9 h from 2100 to 0600 HR. For Experiment II, with temperatures at 25/20 °C (day/night), the chamber was set up with a 10-h photoperiod, an average daylength in late September in Minnesota.

Field grown plants were planted in 15-cm-diameter (5.6-L) pots in early September and then transferred to a greenhouse with a regime of 14 h light and 25/20 °C (day/night) temperature. After a 10-d growth period, they were transferred to growth chambers. Samples were collected from Experiment I after 11 h and 2, 5, and 8-d growth. Samples were collected from Experiment II after 1, 15, and 31 d growth. Plants grown in the greenhouse served as the controls for the comparison of cold hardiness. Cold hardiness of the plants after 21 d in Experiment I and 31 d in Experiment II was determined (Jian et al., 1997)

SAMPLE PREPARATION FOR CALCIUM LOCALIZATION. Sample preparation for Ca^{2+} localization was carried out by the method as previously described (Jian et al., 1997). Briefly, after removing the scales and young leaves, apical buds were cut into $0.5 \times 0.5 \times 0.5$-mm^3 slices. Slices were immediately immersed in a fixative solution containing 4% glutaraldehyde and 2% potassium antimonate ($K_2H_2Sb_2O_7 \cdot 4H_2O$) in 0.1 M potassium phosphate buffer (pH 7.6) at 4 °C for 6 h. After fixation, samples were washed three times, each for 20 min, with the same buffer containing 2% potassium antimonate. The washed samples were post-fixed at 4 °C overnight in phosphate buffer (pH 7.6) containing 1% osmium tetroxide (OsO_4) and 2% potassium antimonate. After post-fixation, samples were washed twice with the same buffer containing 2% potassium antimonate, followed by two washes with pH 7.6 distilled water. Thereafter, samples were dehydrated in an ethanol series and then embedded in Embed 812 (EMS, Fort Washington, Pa.). The embedded samples were sectioned with a diamond knife and an RMC (Tucson, Ariz.) MT 4000 ultramicrotome. The sections were stained with uranyl acetate, and then observed and photographed with a TEM (Philips CM12; F.E.I. Co., Tacoma, Wash.) operated at 60 kV.

VERIFICATION OF CALCIUM DEPOSITS. Two methods were used for verifying Ca^{2+} deposits. 1) Chelating of Ca^{2+} deposits with EGTA: grids that had been mounted with tissue sections and examined by EM were immersed in a 100 mM EGTA solution (pH 8.0) for 1 h at 60 °C. After the treatment, the grids were rinsed briefly with distilled water, stained with uranyl acetate again, and examined with an EM. 2) Energy-dispersive X-ray microanalysis of Ca^{2+} antimonate precipitates: tissue sections were cut at ≈0.25 μm, mounted onto copper grids, and then stabilized with carbon film using a KSE-2A-M vacuum evaporator (Kinney Vacuum Co., Boston, Mass.). X-ray microanalysis of the precipitates in these sections was performed by a TEM (Philips CM-12), operated at 60 kV using EDAX power system (MXEDS model) with an ultrathin window detector, which was interfaced with a Power Mac computer. Complete spectra from 0 to 16 KeV were obtained from the precipitates observed in the cytosol and the nucleus. Supporting film and tissue-free Embed 812 embedding material were also analyzed as controls. Spectra were recorded and counts were made over 80 s. Calcium (Ca^{2+}) and antimony (Sb) emission spectra were separated by deconvolution with the aid of the computer.

Results

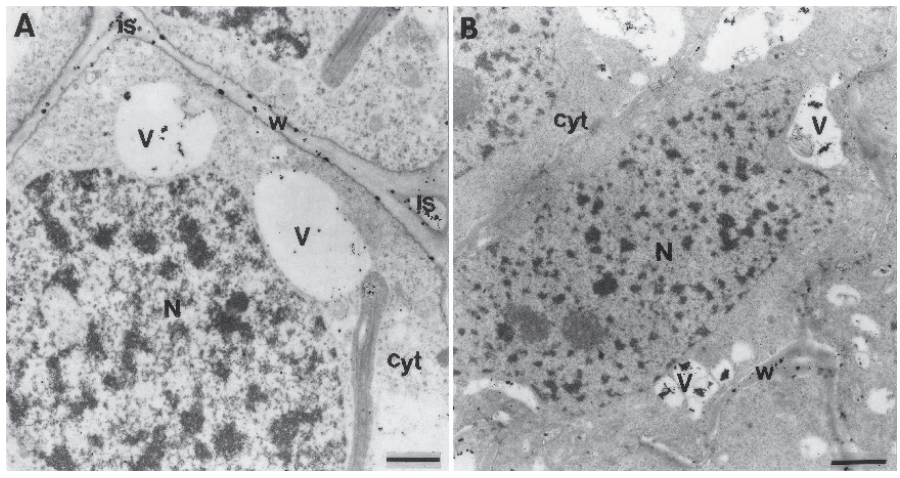
SUBCELLULAR Ca^{2+} LOCALIZATION IN SUMMER. Electron microscopy observations revealed that electron-dense Ca^{2+} antimonate deposits in the apical bud cells of spruce and brome grass from samples collected on 14 July were mainly localized in the vacuole, the cell wall and the intercellular space, which appeared to be the major calcium stores. Few deposits were found in the cytosol and nuclei. The subcellular Ca^{2+} localization pattern showed no difference in the summer months between spruce (Fig. 1A) and brome grass (Fig. 1B).

CALCIUM SUBCELLULAR LOCALIZATION DURING SHORTENING DAYLENGTH AND DECREASING AMBIENT TEMPERATURE IN AUGUST to OCTOBER. On 8 Aug. following a daylength decrease of 1 h 12 min but while ambient temperatures remained high, the apical bud cells of spruce exhibited an accumulation of Ca^{2+} deposits present in the cytosol and nuclei (Fig. 2A). However, brome grass collected on the same date the Ca^{2+} deposits remained in the intercellular space and the vacuole (Fig. 2B). As the daylength continuously shortened and the ambient temperatures decreased in early September, Ca^{2+} deposits in spruce (collected on 8 September) were further increased in the cytosol and, particularly, in the nucleus (Fig. 2C). Brome grass collected at the same date also showed an accumulation of Ca^{2+} in the cytosol and nucleus (Fig. 2D). Many cytosolic and nuclear Ca^{2+} deposits were still observed in the brome grass collected 29 Sept. (Fig. 2E).

When weather data recorded in the St. Paul area during the early September 1997 were examined closely, we found that there was one night when the temperature dropped to 12 °C before the sample collection date on 8 Sept. We wondered if the one night of 12 °C triggered the Ca^{2+} accumulation in brome grass. As shown in Fig. 3A and B from the controlled experiments, when brome grass was exposed to 12 °C with a 14-h photoperiod, Ca^{2+} accumulation occurred. A 10-h photoperiod, which is 5 h and 37 min shorter than the longest daylength in the St. Paul area, was not able to trigger Ca^{2+} accumulation in brome grass even after a 31-d exposure (Fig. 3C and D).

After an 8-d exposure at 12 °C, cold hardiness of brome grass leaves was about 2 °C hardier than the greenhouse grown control (killing temperature was about –4/–5 °C). No hardiness improve-

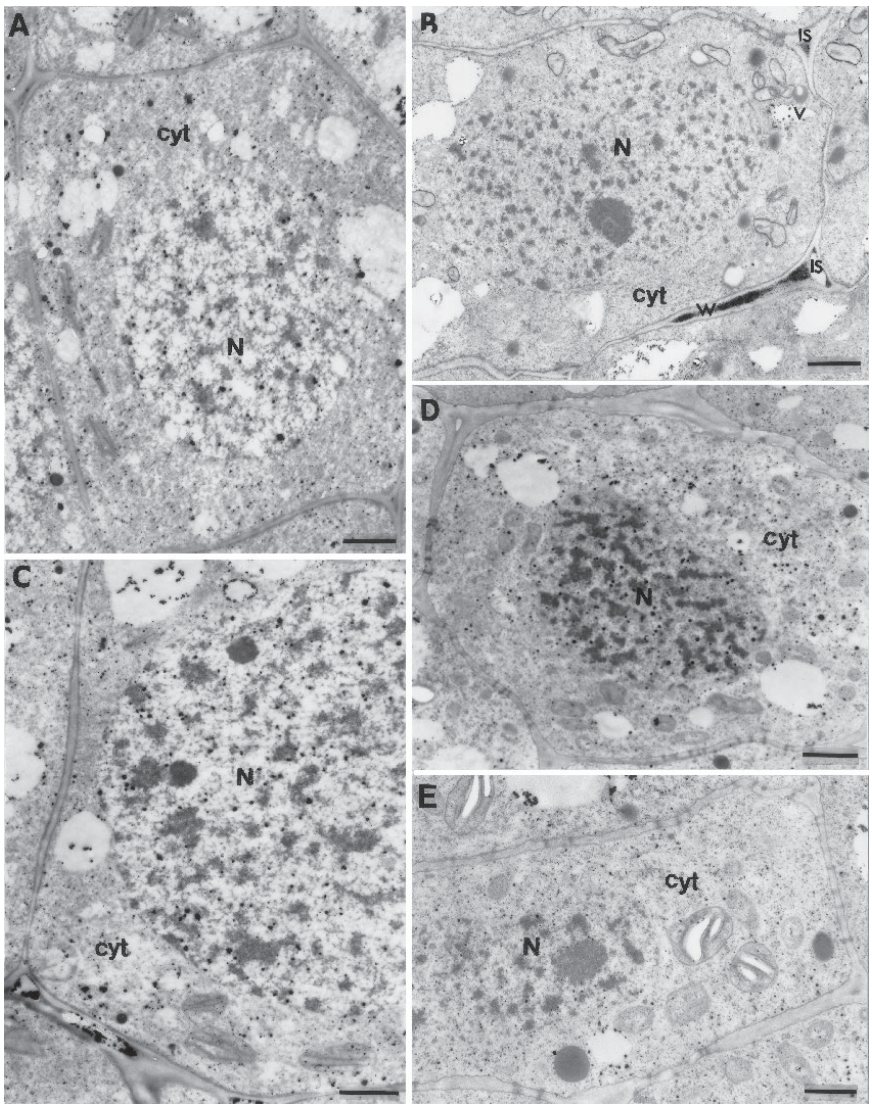
Fig. 1. Subcellular Ca^{2+} localization in spruce (**A**) and brome grass (**B**) collected on 14 July . Ca^{2+} antimonate deposits were localized mainly in the vacuole (V), the cell wall (W) and the intercellular space (IS). Few deposits were seen in the cytosol (Cyt) and nucleus (N). Bar = 1 μm.



ment was found in the short-day-treated plants, even after a 31-d exposure. Plants resumed growth shortly after being transferred to the greenhouse in Experiment I and II (data not shown). We thus concluded that low temperature, not shortening daylength initiated the Ca^{2+} accumulation in brome grass.

From 8 Oct. to the end of the month, spruce apical bud cells still had a large number of Ca^{2+} deposits in the cytosol and nuclei. There were also many deposits present in the vacuole (Fig. 4A and B). During this same period, brome grass showed few Ca^{2+} deposits in the cytosol and nuclei (Fig. 4C and D). Deposits were localized again in the cell wall, the vacuole and the intercellular space (Fig. 4C). Brome grass, collected on 1 November, also showed a peculiar phenomenon in that some Ca^{2+} deposits tended to be localized in certain regions of the cytoplasm (Fig. 4D, arrows), whereas spruce cells had many plastids with starch granules, which were surrounded by Ca^{2+} deposits (Fig. 4B, arrows).

Fig. 2. Intracellular Ca^{2+} deposits in spruce and brome grass from August to September. Cytosolic and nuclear Ca^{2+} concentrations increased in spruce (**A**), but not in brome grass (**B**) from samples collected on 8 August. Ca^{2+} deposits further increased in the cytosol and nuclei of spruce (**C**), whereas Ca^{2+} accumulation was initiated in brome grass (**D**) from samples collected on 8 September. Increased deposits in the cytosol and nuclei were still observed in brome grass on 29 September (E). Bar = 1 μm.



The high cytosolic and nuclear Ca^{2+} concentration in brome grass lasted about 20 d from early September to the end of the month. The high cytosolic and nuclear Ca^{2+} concentration in spruce, however, lasted about three months, from early August to early November.

Calcium subcellular localization in winter and spring. Spruce collected on 13 Dec. and 23 Jan. had protoplasm contraction. Deposits of Ca^{2+} appeared in the space between the plasmalemma and the cell wall (Fig. 5A and C, arrows). Few Ca^{2+} deposits were found in the cytosol and nuclei (Fig. 5A–C). Some Ca^{2+} deposits were observed in plastids, mitochondria and vacuoles (Fig. 5C), and some even appeared in the perinuclear space of the nuclear envelope (Fig. 5B, arrows).

In brome grass collected during this same period, a very low level of Ca^{2+} deposits was observed in the cytosol and nuclei. Most of the Ca^{2+} deposits were seen in the vacuole, cell wall and intercellular space (Fig. 5D and E). Some deposits were also seen in the plastids (Fig. 5E), which were swollen.

When spruce and brome grass were de-acclimated and resumed active growth in late spring, i.e., 10 May, their patterns of Ca^{2+} subcellular localization showed no difference from the samples collected on 14 July (Fig. 1A and B). Both species had low resting cytosolic and nuclear Ca^{2+} concentrations. Again, Ca^{2+} deposits were found mainly in the vacuole, the cell wall and the intercellular space (Fig. 6A and B).

Discussion

Calcium antimonate deposits. Wick and Hepler (1982) and Slocum and Roux (1982) first demonstrated the usefulness of the antimonate precipitation cytochemical technique in localizing Ca^{2+} in plant cells. Since then, many researchers have successfully used the technique to determine the cellular localization of Ca^{2+} in plants (Dauwalder et al., 1985; Hilaire et al., 1995; Lazzaro and Thomson, 1992; Tretyn et al., 1992; Wang and Jian, 1994). We have used it to investigate Ca^{2+} localization as affected by short days and/or low temperatures in poplar, mulberry, maize and winter wheat (Jian et al., 1997, 1999, 2000). However, the technique has shortcomings. For example, it cannot distinguish free from bound Ca^{2+} and also can only detect Ca^{2+} above sub-micromolar concentrations (Slocum and Roux, 1982). In addition, the experimental design of the present study did not allow us to examine the Ca^{2+} oscillations with periods of days, hours, or minutes. Due to many factors, which may cause the formation of electron-dense mass with antimonate, verification of Ca^{2+} deposits is necessary (Slocum and Roux, 1982). In the present study, we used the EGTA Ca^{2+} chelating and energy dispersive X-ray microanalysis to verify the electron-dense masses, which are truly Ca^{2+} deposits. As shown in Fig. 6C, there were no electron-dense deposits present, but many transparent holes (arrows), where Ca^{2+} deposits were chelated by EGTA. Energy dispersive X-Ray microanalysis revealed that overlapping Ca/Sb peaks were

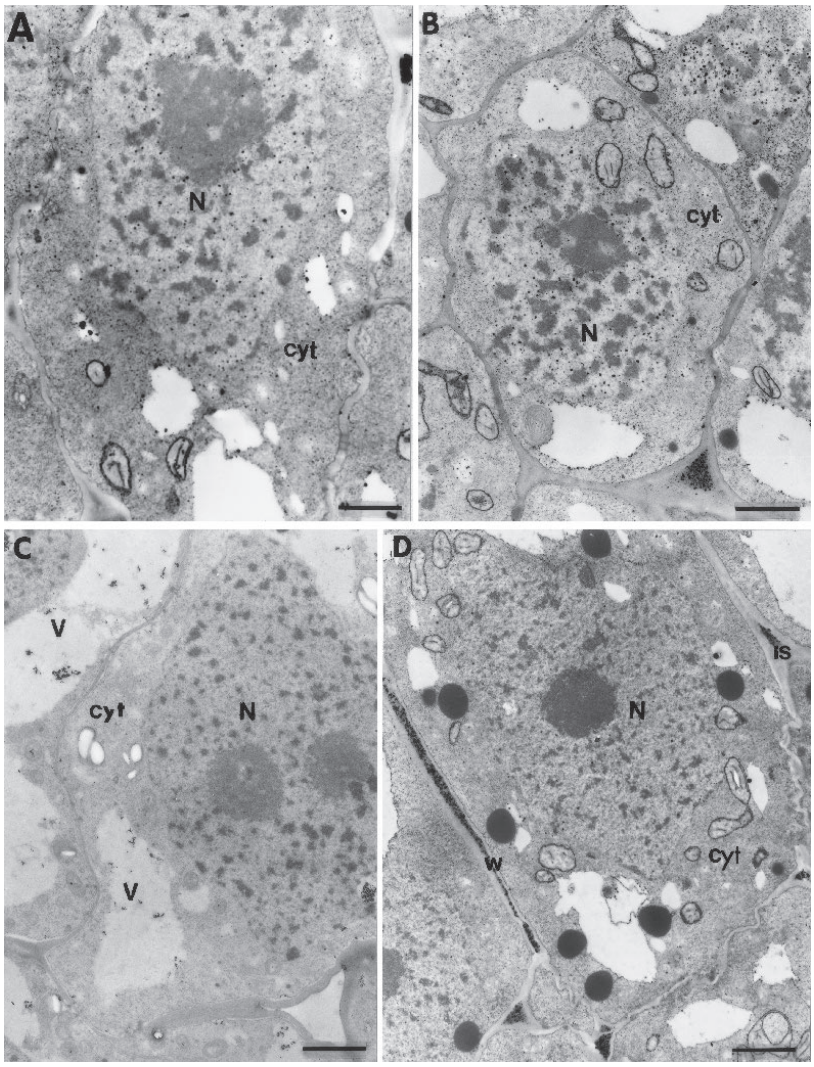


Fig. 3. Localization of Ca^{2+} in brome grass grown in controlled environments. Accumulation of Ca^{2+} in the cytosol and nuclei was observed either after an 11-h exposure to12 °C (**A**) or after 8 d at 12 °C (**B**). Under 10-h photoperiod with 25/20 °C, day/night, temperatures, few Ca^{2+} deposits were observed after 1 d (**C**) or after 31 d (**D**) growth. Bar = 1 μm.

generated at 3.5 to 4.2 KeV (Fig. 7A-a and B-a). After separation of Ca and Sb peaks by deconvolution, calcium peak locations were identified at Ca-Kα = 3.690 KeV and Ca-Kß = 4.012 KeV (Fig. 7A-b and B-b). The results are consistent with reports by Jian et al. (1999), Slocum and Roux (1982), and Tretyn et al. (1992). We realize the interpretation of the results obtained from this technique might be subjective, but we believe that the information should be published. For example, this study may provide a reasonable answer for the re-growth behavior of dormant red-osier dogwood reported earlier (van Huystee et al., 1967) and the often observed rapid de-acclimation of perennials in winter months (last paragraph below).

Shortening daylength and decreasing ambient temperature trigger Ca^{2+} accumulation in spruce and brome grass, respectively, in nature. We observed that Ca^{2+} accumulation in spruce had occurred on 8 Aug. (Fig. 2A) when the daylength was reduced by 1 h 12 min from the daylength of 14 July at the 45°N latitude of St. Paul while days/nights were still very warm. It is consistent with a previous report on mulberry, also grown in St. Paul (Jian et al., 2000), that the Ca^{2+} accumulation occurred in early August. Surprisingly, we did not observe Ca^{2+} accumulation in brome grass at this time of the year. We did, however, observe Ca^{2+} accumulation in brome grass (Fig. 2D), but it occurred a month later on 8 September when one night of the ambient temperature had previously reached 12 °C in 1997. It has been shown that the threshold of low temperatures for the cold acclimation of herbaceous plants falls around 12 °C (Chen and Li, 1980). On 8 September, the daylength was reduced by 2 h and 47 min compared to the 14 July daylength. Controlled experiments confirmed our suspicion that brome grass Ca^{2+} accumulation was triggered by low temperatures (Fig. 3A and B), not by shortening of the daylength (Fig. 3C and D). Reducing the temperature from 25 °C to 17-18 °C can cause an elevation of cytosolic Ca^{2+} level in tobacco roots and cotyledons (Campbell et al., 1996). We do not know, however, the minimum temperature required in nature to trigger Ca^{2+} accumulation in brome grass. We also do not know if Ca^{2+} accumulation in the cytosol and nuclei is due to Ca^{2+} influx or Ca^{2+} release from internal stores, or from both.

Our findings also confirm our belief that woody perennials are sensitive to a shortening of daylength in nature which triggers Ca^{2+} accumulation. At 45°N, 93°W location (St. Paul), about 1 h in daylength reduction in late summer was able to trigger a Ca^{2+} accumulation. It has been suggested that Ca^{2+} accumulation signals the sensory process for the development of dormancy and cold hardiness in plants (Jian, et al., 1997; Knight, 2000; Monroy et al., 1993; Monroy and Dhindsa, 1995; Sheen, 1996). It is also known that short days at moderate temperatures initiate growth cessation and the development of dormancy and cold hardiness in woody perennials (Jian et al., 1997; van Huystee et al., 1967). From our studies, we suggest that the sensory process for cold hardiness development in *Pieca engelmannii* is initiated in the middle of August at 45°N in St. Paul. No increase in cold hardiness from the summer level could be detected at that time. For *Bromus inermis*, our findings indicate that short days neither trigger Ca^{2+} accumulation nor induce cold hardiness. Initiation of the sensory process in *Bromus inermi* seems to be only sensitive to low ambient temperatures. Consequently, the date for the initiation of cold hardiness development will vary depending on when a minimum low ambient temperature occurs in nature. In 1997, for example, the sensory process seemed to occur in early September following a drop in ambient temperature to 12 °C.

Duration of high cytosoloic and nuclear Ca^{2+} concentrations in relation to developed dormancy and cold hardiness. We found that low ambient temperatures induced high cytosolic and nuclear Ca^{2+} concentration in brome grass, which lasted about 20 d from early September to the end of the month in 1997 in St. Paul. It has been reported that low temperature, light, wind, salt, drought, etc., can trigger an influx of Ca^{2+} with an increase in cytosolic Ca^{2+} concentration (Bush, 1995; Gehring et al., 1990; Gilroy and Jones, 1992; Hilaire et al., 1995; Knight et al., 1991, 1992; Knight, 2000; Shacklock et al., 1992). In many cases, increased cytosolic Ca^{2+} concentrations are transient. The duration of high cytosolic Ca^{2+} level varies from several seconds to several hours. For example, 4 °C exposure induced an increase in

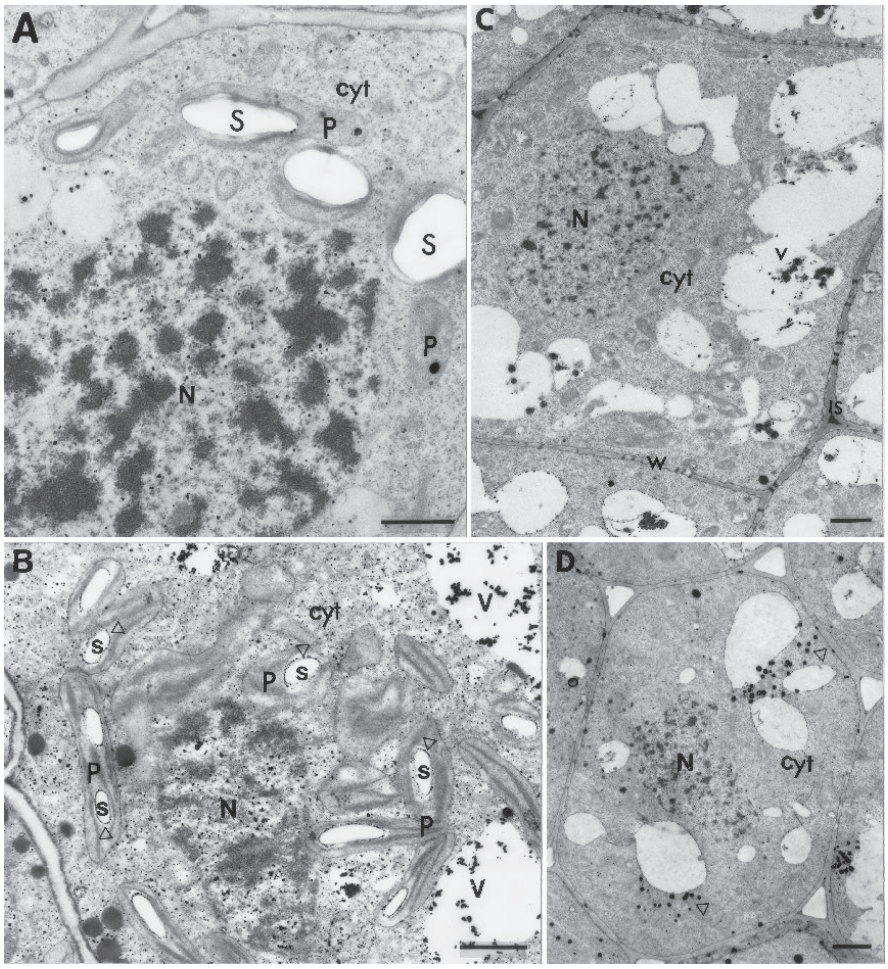


Fig. 4. Distribution of Ca^{2+} deposits in spruce samples collected on 8 October (**A**) and 1 November (B). High Ca^{2+} levels were still observed in the cytosol and nuclei. Many plastids (P) surrounded the nucleus (**B**). These plastids contained starch granules (S) surrounded by a large number of Ca^{2+} deposits (arrows). Distribution of Ca^{2+} deposits in brome grass samples collected on 8 October (**C**) and 1 November (**D**). Few Ca^{2+} deposits were localized in the cytosol. and nuclei. Some were gathered together in certain cytoplasmic regions (D. arrows). Bar = 1 μm.

cytosolic Ca^{2+} levels that lasted ≈30 min in tobacco and *Arabidopsis* seedlings (Knight et al., 1996). The rather long period of high cytosolic Ca^{2+} concentration in brome grass observed in nature may be necessary for an accumulative development of cold hardiness. Knight et al. (1996) reported that exposure of *Arabidopsis* seedlings to 4 °C for 3 h daily for 3 consecutive days resulted in a prolonged elevation of cytosolic Ca^{2+}. Previous cold stimulation could be retained and might have some bearing on the ability for an accumulated cold hardiness (Knight et al., 1996).

We also observed a 3-month period, from early August to early November, of high cytosolic and nuclear Ca^{2+} concentrations in spruce. A similar long period of high cytosolic and nuclear Ca^{2+} concentrations has been reported for mulberry grown in St. Paul (Jian et al., 2000). When mulberry twigs collected during the period from early September to early November when cells had high cytosolic Ca^{2+} concentrations (Jian et al., 2000) were subjected to a regime of 14-h photoperiod and 25/20 °C, day/night temperatures, their buds did not show any sign of budbreak after many weeks. However, mulberry samples collected in mid-December, when apical bud cells had low level of cytosolic Ca^{2+} (Jian et al., 2000), had buds break within a week after subjecting to the same regime (unpublished data). In fact in 1967, van Huystee et al. (1967) reported that plants of red-osier dogwood (*Cornus stolonifer* Michx), collected in mid-November or before from the field and grown in a greenhouse with long days and high temperatures, did not show any sign of growth until the following May. Red-osier dogwood resumed growth within 1 month after being placed in warm temperatures on 23 Nov., and within 2 weeks when exposed to high temperatures on 1 Jan. Our Ca^{2+} findings seem to suggest that the long period of high cytosolic and nuclear Ca^{2+} concentrations observed in spruce (Figs. 2 and 4) and mulberry (Jian et al., 2000) may play a critical role not only in the development of the physiological dormancy but also possibly in maintaining the status of the developed dormancy. Foresters and horticulturists have often noticed that diurnal high temperatures in midwinter can rapidly decrease the cold hardiness of woody plants such as spruce. Would the low levels of cytosolic and nuclear Ca^{2+}, as observed in midwinter in spruce (Fig. 5), have an influence on the plant's response to warm temperatures in winter months for the rapid deacclimation?

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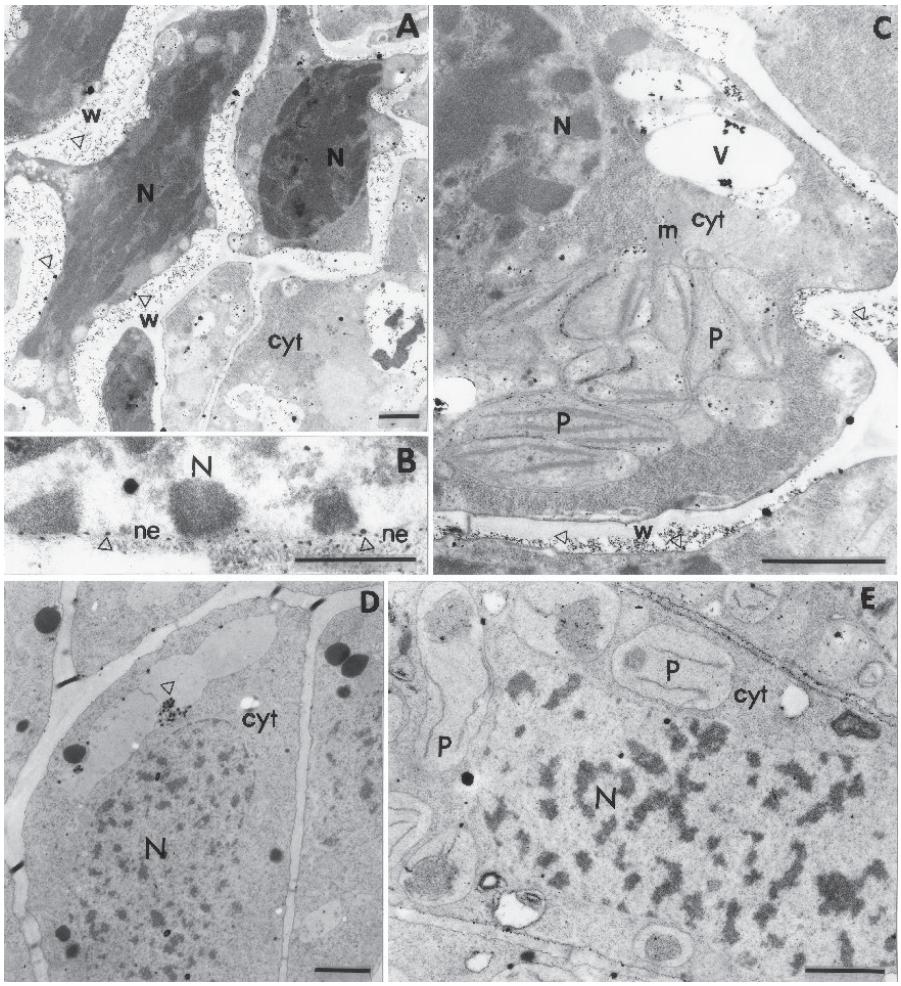

Fig. 5 . Distribution of Ca^{2+} in spruce and brome grass during midwinter. (**A** and **B**) Spruce samples collected on 13 December. Protoplasm was dehydrated and contracted. Many Ca^{2+} deposits appeared in the spaces between the plasmalemma and the cell wall (A, arrows) and some in the nuclear envelope (ne) (**B, arrows**). Few Ca^{2+} deposits were found in the cytosol and nuclei. (**C**) Spruce sample collected on 23 January. Again, few Ca^{2+} deposits were distributed in the cytosol and nuclei. Some were localized in the vacuole, plastid, mitochondrion (m) and the space between the plasmalemma and the cell wall. (**D** and **E**) Brome grass samples collected on 13 December and 23 January, respectively. Cytosol and nuclei contained low level of Ca^{2+} deposits. Some still appeared as a domain distribution in the cytoplasm (**D, arrow**). Some were localized in the plastids which showed swelling (**E**). Bar = 1 μm.

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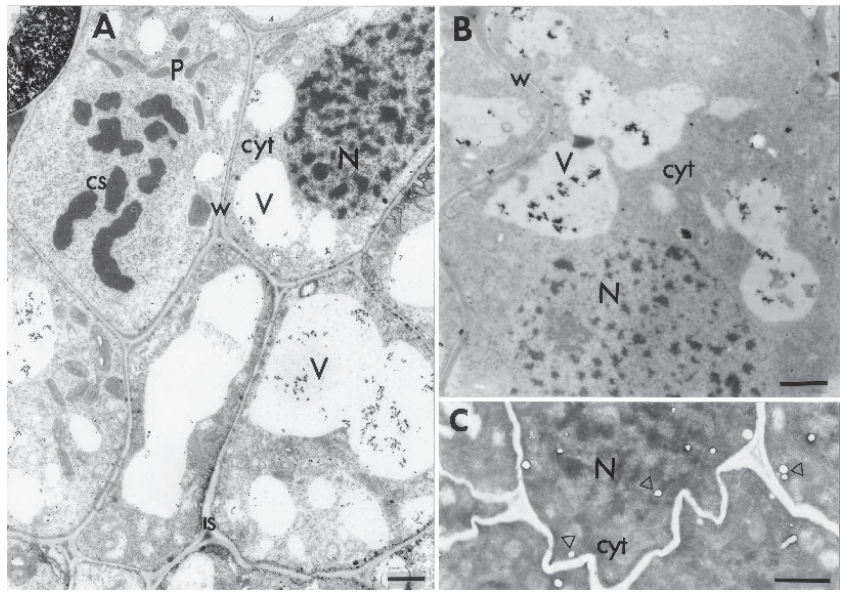

Fig. 6. Localization of Ca^{2+} in spruce (**A**) and brome grass (**B**) from samples collected on 10 May. Ca^{2+} deposits were distributed mainly in the vacuole, the cell wall and the intercellular space. Ca^{2+} deposits from spruce sample collected on 1 November were chelated by EGTA (**C**) showing the transparent holes (arrows), where Ca^{2+} deposits were localized prior to EGTA treatment; cs = chromosome. Bar = 1 μm.

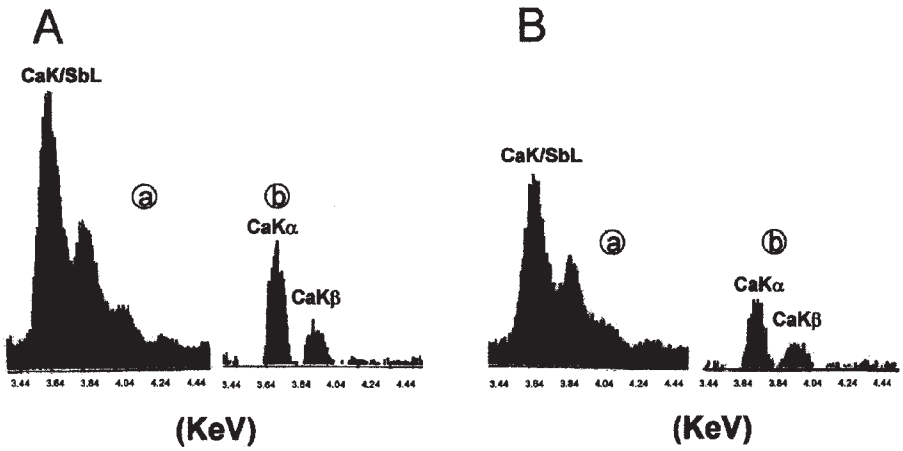

Fig. 7. Energy dispersive X-ray microanalysis of calcium antimonate deposits from spruce sample collected on 1 November. (**A**) The Ca^{2+} deposits were localized in the nucleus. (**B**) The Ca^{2+} deposits were localized in the cytosol. (**a**) Strong signals at 3.4 to 4.4 KeV correspond to the combination of antimony (Sb) and calcium (Ca). (**b**) Energy spectrum for calcium revealed by the deconvolution of the Sb peak from the total spectra shown in **a**.

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