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Cold in the common garden: comparative low-temperature tolerance of boreal and temperate conifer foliage

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Abstract Because they maintain green foliage throughout the winter season, evergreen conifers may face special physiological challenges in a warming world. We assessed the midwinter low-temperature (LT) tolerance of foliage from eight temperate and boreal species in each of the genera *Abies*, *Picea*, and *Pinus* growing in an arboretum in Trondheim, Norway, using relative electrolyte leakage (REL) as an index of cell injury. Relatively LT sensitive species came from temperate coastal and Mediterranean environments and displayed a well-defined sigmoidal response to LT stress, with LT_{50} ranging from –27 to –38°C. Species originating from boreal regions were not lethally stressed by slow freezing to temperatures as low as –80°C, while species from temperate mountains and continental interiors displayed intermediate responses, with LT50s ranging from –33 to –44°C. Further evaluation of one sensitive and one insensitive species in each genus showed that boreal species can survive quenching in liquid nitrogen at –196°C provided they are first slowly cooled to –30°C or lower. Quantitative image analysis of color changes resulting from LT stress followed by exposure to light showed that foliage from nonlethally stressed boreal species developed mild to moderate chlorosis while more sensitive species developed a mixture of chlorosis and necrosis, with significant necrosis occurring mainly at temperatures resulting in REL of 50% or more. Sensitive and insensitive trees differed significantly in total raffinose, sucrose, and total sugar concentrations, and raffinose and sucrose correlated significantly with LT_{50} within the sensitive group.

Keywords Frost tolerance · Winter physiology · Sugars · Biogeography

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Introduction

In a warming world, trees growing in cold-seasonal environments may encounter new winter stresses associated with delayed acclimation, midwinter deacclimation, winter respiration, and light stress, even if they are well-adapted to surviving low-temperature stress in existing climatic regimes. Boreal and temperate conifers that maintain foliage through the winter months may be especially vulnerable. Therefore, an understanding of the degree and mechanisms of low temperature (LT) tolerance will be an important element in predicting the effects of global warming on tree and forest health and productivity.

Conifers in northern boreal and cold temperate regions face contrasting winter climate conditions and accordingly employ different strategies for surviving the winter months. In continental boreal regions such as central Siberia and Canada, midwinter temperatures may fall below –60°C and remain below –40°C for weeks at a time (Trewartha and Horn 1971). The foliage of evergreen conifers in these regions generally survives these extreme temperatures in a

dormant state and resumes photosynthetic activity during the growing season. The foliage and other aerial parts of many boreal species can survive immersion in liquid nitrogen (LN_2) at –196°C (Sakai 1960, 1983), so for all practical purposes they have achieved absolute low temperature tolerance. Conifers of temperate continental interiors are rarely if ever exposed to temperatures below –40°C and their wood and bud tissues may employ alternate mechanisms of LT survival such as deep supercooling (Becwar et al. 1981; Pukacki 1987; Sakai 1978). LT_{50}s for these species are often in the –40 to –60°C range, and some will partially deacclimate and fix carbon during extended periods of mild winter weather (Schaberg et al. 1998, 1996; Strimbeck et al. 1995). A few temperate conifer species are known to suffer winter injury in their natural range (DeHayes et al. 2001; Hiratsuka and Zalasky 1993). In temperate coastal regions with an oceanic climate trees experience only episodic, mild frost, and may maintain some level of photosynthetic activity during the winter months (Hawkins et al. 1995).

In a review of LT tolerance and geographic distribution, Bannister and Neuner (2001) used the natural distributions of nearly 500 conifer species from tropical to subarctic regions worldwide to list them in USDA plant hardiness zones, with supporting LT tolerance data for about 150 species gleaned from the literature. The overwhelming majority of this data comes from the exhaustive work of Akira Sakai and coworkers (e.g., Sakai 1983; Sakai and Okada 1971; Sakai and Weiser 1973), who used laboratory freezing and the subsequent development of visual injury symptoms to arrive at an approximate maximum survival temperature for various tissues of each species investigated, with a resolution of 10°C, the increment used in the freezing tests. Although the basic method was standardized, many of these studies involved plant material collected at different locations and transported over long distances, and in some cases relied on an artificial hardening protocol to attain maximum hardiness. This approach confounds the potential influences of site-specific environmental cues that drive deep hardiness, and possible influences of transport-associated dehardening and/or artificial re-hardening, with inherent species capacities to achieve maximum cold tolerance. This leaves some uncertainty when comparing the results of various published works. Indeed, in some cases the results of early studies do not agree with those of more recent studies of the same species (e.g., *Picea rubens*; DeHayes 1992; Sakai and Okada 1971).

Sakai and coworkers (Sakai 1960; Sakai and Weiser 1973) also reported that winter-acclimated foliage, bark or buds of various boreal gymnosperm and angiosperm species, such as *Larix laricina*, *Pinus banksiana*, *Betula papyrifera*, and various *Salix* species, could survive quenching in LN_2 at –196°C, provided they were precooled to around –30°C before immersion. These results were also based on assessment of tissue browning as the main symptom of injury.

Accumulation of sucrose and oligosscaharides such as raffinose is closely associated with LT acclimation in a wide variety plants (Sakai and Larcher 1987). They are thought to function as cryoprotectants and may also serve as regulators of various processes, including photosynthesis (Schaberg et al. 1999). Sugar reserves may be depleted by respiration during warm periods in winter, with effects on overall carbon balance and LT tolerance (Ögren et al. 1997).

In this study, we remove the confounding influences of differing sites and sample protocols by evaluating trees in a common garden on a single day using uniform sample collection and analysis procedures. In particular, we compare the LT tolerance of foliage from 24 boreal and north-temperate coniferous species in the genera *Abies*, *Picea*, and *Pinus* growing in a common environment, and quantitatively assess the visual injury symptoms and the liquid nitrogen (LN_2) quenching tolerance of two species in each genus. We also examine the relationship between the concentrations of mono-, di-, and oligosaccharides and LT tolerance in these species. Our results offer an updated and more complete and quantitative analysis of the comparative responses of boreal and temperate conifer foliage to LT stress.

Materials and methods

Site description and sample collection

The Ringve Botanical Garden in Trondheim, Norway (63° 25′ N, 10° 25′ E) supports an arboretum that includes 53 conifer species as well as numerous angiosperm trees and shrubs, all from temperate and boreal regions around the northern hemisphere. The garden is only a few meters above sea level and less than a kilometer away from Trondheimsfjord, which remains unfrozen during the winter. The local climate is classified as temperate oceanic in global maps (Trewartha and Horn 1971) or southern boreal and slightly oceanic in the higher resolution Norwegian classification (Moen 1999), with winter temperatures occasionally falling below –20°C, but also with periods of thaw weather during the winter months, especially in recent years. The area where the trees are planted is nearly level, with uniform soil originating from marine clays.

Samples were collected on 13 February and 13 March 2006. On the first date, samples were collected from 24 species, eight species each in the genera *Abies*, *Picea*, and *Pinus*, originating from boreal, temperate continental, and

temperate oceanic climates around the northern hemisphere (Table 1). Tolerance to slow freezing to temperatures as low as –80°C was estimated using controlled freezing and electrolyte leakage to measure stress response. The results of the first sampling were used to select one boreal and one temperate species in each genus for sampling one month later. These were tested for both slow freezing and LN_2 quenching tolerance, using both electrolyte leakage and quantitative image analysis of visual injury as responses.

Three individuals of each species were sampled by excising current-year shoots from the tips of several branches within reach of the ground. Different trees were sampled on the two dates. Sample material was stored in plastic bags, transported in an insulated box, and stored overnight at 0°C before preparation for freezing.

Freezing and electrolyte leakage

For LT tolerance testing using electrolyte leakage, the foliage and twigs of several current-year shoots from each tree were cut into 5 mm sections and mixed to produce a uniform sample of needle and twig pieces for each tree. Approximately 1–2 ml subsamples were measured into 200 × 20 mm glass test tubes, and 1 ml of 0.1% Triton-X solution containing a biological ice nucleator (Snomax, York Snow, Victor, NY, USA) was added to each sample to prevent supercooling.

Samples were cooled in a custom, computer-controlled freezing chamber based on a Julabo FW95-SL circulator and Julabo EasyTemp control software (Julabo Labortechnik GmbH, Seelbach, Germany). Temperature in an empty test tube inserted among the samples was monitored using a Pt100 sensor, which also provided the signal for the control unit. Samples were cooled at a rate of 0.6°C h^{-1} to a series of 10–12 temperatures ranging from –5 to –65°C, with a 40–50 min hold at each temperature to allow equilibration. One set of samples was transferred to a cold room and held at +4°C without freezing treatment, and an additional set was transferred from the controlled freezer at the lowest temperature to an ultralow freezer at –80°C and allowed to equilibrate for 1 h. At the end of each equilibration period, a rack containing one sample from each tree was removed from the freezer in a precooled box made of 5 cm thick polystyrene foam and transferred to a cold room at +4°C. The racks were left to warm slowly overnight in the insulated boxes.

For liquid nitrogen quenching, at each test temperature a sample rack was placed in an insulated box inside the freezing chamber, and liquid nitrogen was poured into the box until the test tubes were immersed to a depth of 20–30 mm, well above the level of the sample in the tube. For whole shoot samples used for visual injury assessment, liquid nitrogen was poured directly into the open test tubes in the rack. The box was transferred to the cold room, where the nitrogen evaporated and the samples warmed passively overnight.

Ten milliliter of 0.1% Triton-X solution was added to each sample and the samples were held at 4°C for an additional 24 ± 1 h to allow electrolyte leakage to equilibrate. Initial conductivity was measured with an automated system (Radiometer SAC80 sample changer and CDM conductivity meter; Radiometer Analytical, Villeurbanne Cedex, France). Samples were autoclaved for 20 min at 120°C, allowed to cool overnight, and the final conductivity measured. Conductivities were corrected by subtracting the mean conductivity of blanks containing the Snomax and Triton-X solutions inserted in each sample rack, for a total of 20–24 blanks in all. Relative electrolyte leakage (REL) was expressed as the ratio of initial to final conductivity.

Visual injury

For visual injury assessment of the samples collected on 13 March, individual current-year shoots were placed in 200 × 20 mm glass test tubes. For the *Pinus* species needles projecting from the tube were cut off so the sample could be covered. These samples were placed in the freezer with the electrolyte leakage samples and followed the same freezing and thawing protocol. For development of visual injury symptoms, the base of the stem was inserted in a water-saturated floral foam block (Smithers-Oasis Denmark A/S, Frederikssund, Denmark) and the samples were kept at 15°C under approximately 250 μmol m^{-2} s^{-1} PAR for 2 weeks. The samples were then sliced into 5 mm twig and needle sections and the twig sections were removed. The needle sections were placed in 12-well clear plastic culture dishes and scanned at 300 d.p.i. using a flatbed scanner. Images were stored as high-quality JPEG files.

ImageJ public domain software (U.S. National Institutes of Health, Bethesda, MD, USA) was used to measure the needle area in each sample with green, yellow, and orange coloration (Details in figure S4). Green coloration was interpreted as uninjured tissue, yellow coloration was interpreted as chlorosis, and orange coloration was interpreted as necrosis resulting from cell death.

Sugars

Sugar contents were measured in samples drawn from the bulk samples prepared for LT tolerance testing for the 24 species collection. Samples from the three trees in each species were mixed to produce a single sample for each species. The samples of needle and stem pieces were freeze-dried, and the stem pieces were removed, leaving a sample of needle pieces only. Cuticular waxes were

Table 1 Species information and low-temperature tolerance results

Species	Continent	Main range	Elevation	Trewartha climate type	T_m Mean ± SD	Y_{max}
Abies balsamea	North America	Eastern Canada	0–1,700	E, Dc	(–36.7) ± 3.0	0.34
A. sibirica	Eurasia	Siberia	?–2,400	E	(–30.6) ± 2.7	0.45
A. homolepis	Eurasia	Mountains, southern Japan	700–2,200	Dc, Cf	(–33.6) ± 1.4	0.47
A. lasiocarpa s.l.[a]	North America	Rocky Mountains and northwest Coast Ranges	600–3,700	BS, Do	(–37.4) ± 1.8	0.48
A. veitchii	Eurasia	Mountains, central Japan	1,600–1,900	Dc	–32.6 ± 5.4	0.55
A. amabilis	North America	Pacific northwest coast	1,000–2,300	Do	–37.7 ± 7.3	0.63
A. procera	North America	Pacific northwest mountains	60–2,700	Do	–32.4 ± 4.3	0.68
A. alba	Eurasia	Alps & Pyrenees	300–1,950	Do, Dc, Cs	–28.8 ± 2.7	0.73
Picea obovata	Eurasia	Siberia		E	(–37.0) ± 7.3	0.26
P. glauca	North America	Canada	0–2,100	E, Dc	(–41.0) ± 7.7	0.31
P. abies	Eurasia	Northern Europe	0–2,200	E, Dc	(–47.9) ± 6.6	0.43
P. omorika	Eurasia	Balkans	400–1,700	Dc	–43.8 ± 2.2	0.53
P. engelmanni	North America	Rocky Mountains	1,000–3,000	BS	–44.0 ± 2.6	0.54
P. sitchensis	North America	Pacific northwest coast	0–1,000	Do	–35.7 ± 0.6	0.68
P. rubens	North America	Northeastern US, Southeastern Canada, Appalachian Mountains	0–2,000	Dc	–38.3 ± 7.2	0.69
P. jezoensis	Eurasia	Japan and adjacent mainland	40–1,000	Dc, E	–32.7 ± 1.6	0.70
Pinus sylvestris	Eurasia	Northern Europe to Siberia	0–1,000	E	(–38.1) ± 5.3	0.35
P. cembra	Eurasia	Alps and Carpathians	1,300–2,400	Dc	(–42.9) ± 2.3	0.37
P. koraiensis	Eurasia	Korea and Japan	600–1,800	Dc	(–35.2) ± 3.7	0.46
P. strobus	North America	Northeast US and southeast Canada	0–1,500	Dc	(–36.6) ± 6.0	0.49
P. contorta	North America	Rocky Mountains, Coast Ranges, Sierra Nevada, northwest coast	0–3,500	BS, Do	–39.5 ± 1.2	0.52
P. banksiana	North America	Eastern Canada	0–800	E, Dc	–27.3 ± 6.3	0.55
P. nigra	Eurasia	Mountains in Mediterranean region	200–2,000	Cs (H), Dc	–26.8 ± 1.4	0.60
P. jeffreyi	North America	Sierra Nevada	2,000–3,100	Cs	–30.4 ± 7.5	0.68

Nomenclature and information on species distribution and elevation comes primarily from Earle (2006). Species within each genus are ranked by the parameter Y_{max}, an estimate of the maximum stress or injury produced by slow freezing. Parentheses indicate T_m values for trees that were probably not completely injured at the lowest test temperature. Climate types follow the classification in Trewartha and Horn (1971), summarized as follows: *E* boreal; *Dc* temperate continental; *Do* temperate oceanic; *Cf* subtropical humid; *Cs* Subtropical dry summer (Mediterranean); *BS* steppe or semiarid. Climate type was determined by comparison of range maps or descriptions (Earle 2006) to Trewartha's map. The climate zone in the main part of the species range is given first, followed by other climate zones where the species is found. For montane species, the climate of the surrounding region is given

[a] *Abies lasiocarpa* s.s. includes only populations in coastal mountain ranges; Rocky Mountain populations have been segregated as *A. bifolia* (Earle 2006). The provenance of the trees in the arboretum is unknown, so they might have come from either region

removed using hexane, and then soluble sugars were extracted using 80% ethanol (Hinesley et al. 1992). The supernatant was filtered using a Waters C18 Sep-Pac Plus Cartridge (Waters Corporation, Milford, MA, USA) to remove chlorophyll. A subsample was dried at 37°C, reconstituted in 0.1 mM Ca EDTA, and filtered again. Samples were analyzed for glucose, fructose, sucrose, stachyose, raffinose and xylose using a Waters Alliance 2690 HPLC system with a Waters Sugar-Pak column at 90°C with 0.1 mM Ca EDTA as the mobile phase at a flow rate of 0.6 ml/min. Soluble sugar standards encompassing low, medium and high anticipated sugar concentrations were run prior to each sample set.

Data analysis

The LT_{50} for each tree was estimated by non-linear curve fitting (JMP, SAS Institute, Cry, NC, USA) using the formula:

$$Y_T = Y\min + \frac{Y\max - Y\min}{1 + e^{k(Tm-T)}}$$

(Anderson et al. 1988). Y_T is the value of the response variable (REL or visual injury) at temperature T, Y_{min} is the asymptotic value of the response variable in uninjured tissue, Y_{max} is the asymptotic value at maximum

low-temperature stress, k represents the steepness of the response curve, and T_m is the midpoint of the symmetrical curve (an estimate of LT_{50} only if there is complete mortality at Y_{max}). Sugar results were analyzed by factorial analysis of variance, with the three genera and two levels of relative LT tolerance as fixed main effects. Relative LT tolerance was based the mean value of Y_{max} from the curve-fit for each species, using a threshold value of 0.5 to classify each species as LT sensitive or insensitive.

Results

LT tolerance

REL response curves for the eight species in each genus varied between two extremes (Fig. 1; data for all species in Figures S1, S2, and S3) representing relatively LT sensitive (e.g., *Abies alba*, *Picea rubens*, *Pinus nigra*) and insensitive (e.g., *Abies balsamea*, *Picea glauca*, *Pinus sylvestris*) species. Curves for the relatively sensitive species were strongly sigmoid, with well-defined baseline (Y_{min}) and maximum (Y_{max}) REL regions connected by a steep response region. Mean T_m for these species ranged from about –27 to about –38°C (Table 1). In contrast, the most insensitive species showed relatively small increases in REL without pronounced baseline, maximum, and response regions. Although a sigmoid curve can be found for these insensitive species, the standard errors for the parameters are relatively large and in some cases (*Abies balsamea*, *Picea glauca, Pinus sylvestris*) the response appears nearly linear. The relatively low Y_{max} values for the insensitive species indicate that these species were not lethally stressed even at the lowest temperatures, as will be supported by comparison of electrolyte leakage and visual injury data below. Therefore, the T_m values for these species cannot be interpreted as LT_{50}s or directly compared to those of the sensitive species, as indicated in Table 1. Mean T_m values for species intermediate in sensitivity ranged from about –33 (*Abies veitchii*) to about –44°C (*Picea omorika* and *P. engelmanni*).

The three individual trees within some species differed in their responses to freezing stress (Fig. 1), with T_m estimates differing by as much as 15°C (*Pinus jeffreyi*, Figure S3), while in many species the response was more uniform. *Pinus strobus* included one relatively sensitive and two relatively insensitive trees (Figure S3). This tree-to-tree variability is expressed in the standard deviations given in Table 1. This variation in T_m estimates is partly an expression of variability within the species, but may also be due to error in the estimate of T_m where the fit of the curves is weak. Standard errors for the T_m parameter for individual trees, estimated by the curve-fitting procedure, ranged from about 0.5 to nearly 20°C, with the larger values generally associated with the weakly sigmoid curves for the insensitive species.

The slow-freezing LT response curves for the six species sampled in March were generally consistent with those of the same species in the February samples (Fig. 2), with the exception that the mean values for *Picea glauca* showed an intermediate type response, whereas in the previous sample collection the response was of the insensitive type (Fig. 1). As expressed in the higher variability in this species, this was a result of combining the data from three trees. One of the three trees followed the insensitive pattern seen in the boreal *Abies* and *Pinus* species, with REL always <0.4, while the data for other two trees followed the sensitive pattern, with REL >0.7 after slow freezing to the lowest temperature.

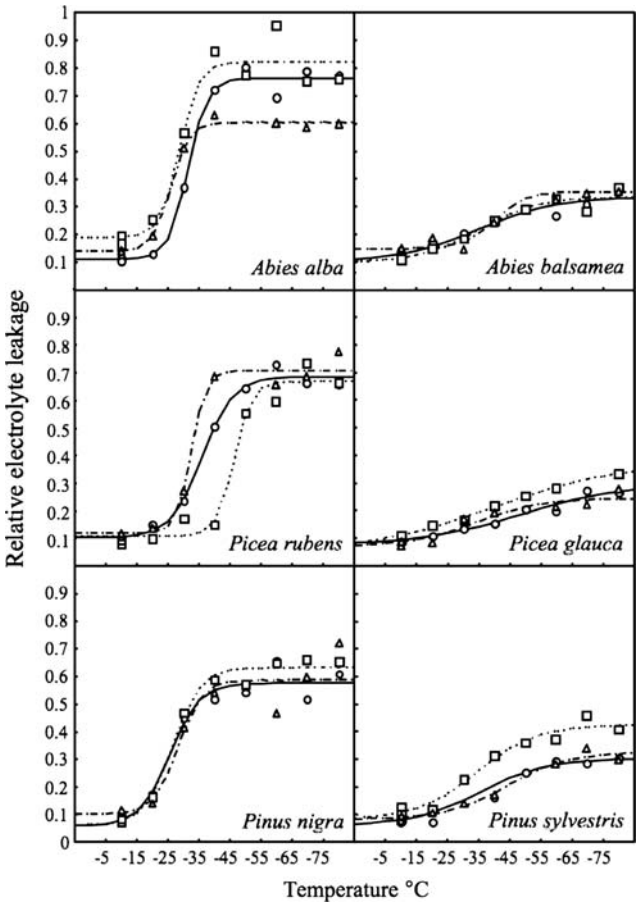

Fig. 1 Relative electrolyte leakage data and fitted temperature response curves of low temperature sensitive (*left*) and insensitive (*right*) foliage in three genera of Pinaceae. *Circle*, *square* and *triangle*: data points and curves for three individual trees within each species. The results for all 8 species in each genus are shown in Figures S1, S2, and S3

LN_2 quench tolerance

Comparison of the electrolyte leakage data from the boreal-temperate species pairs in each genus (Figs. 2) reveals two contrasting patterns of response to LT stress. As in the 24 species comparison, the relatively sensitive temperate species produced a generally sigmoid response to slow freezing stress, and additionally were always lethally stressed by LN_2 quenching regardless of pretreatment. The boreal species displayed a more limited response to slow freezing stress, were killed or more or less stressed by LN_2 at temperatures from 0 to –20, but survived LN_2 quenching from temperatures of about –30°C or lower. These results are in general agreement with those of Sakai and Weiser (1973) who demonstrated LN_2 quench tolerance of needles of several coniferous species including *Abies balsamea* and *Pinus banksiana*.

As in slow freezing, the higher variability in *Picea glauca* species reflects differences between the three trees sampled in each species. The two sensitive *P. glauca* trees developed REL >0.6 after LN_2 quenching at all temperatures, while REL for the third fell below 0.3 after quenching from –30°C or lower. As a result, the overall means suggest an intermediate response with high variability (Fig. 2).

Visual injury

The REL results are supported by image analysis of visual injury symptoms, which allows direct assessment of color changes associated with stress and mortality (Figs. 3, 4). Both chlorosis and the orange coloration associated with necrosis were detected and quantified.

Slow freezing in the LT sensitive species produced chlorosis at temperatures 5–10°C warmer than those associated with necrosis, with injury progressing to necrosis at lower temperatures. Under the conditions used to develop injury in our experiment, lethally injured *Abies alba* developed about 80% necrosis, with most of the remaining needle area classified as chlorotic (Fig. 3). Similar color changes were observed in *A. alba* samples subjected to LN_2 quenching at all temperatures (Fig. 4).

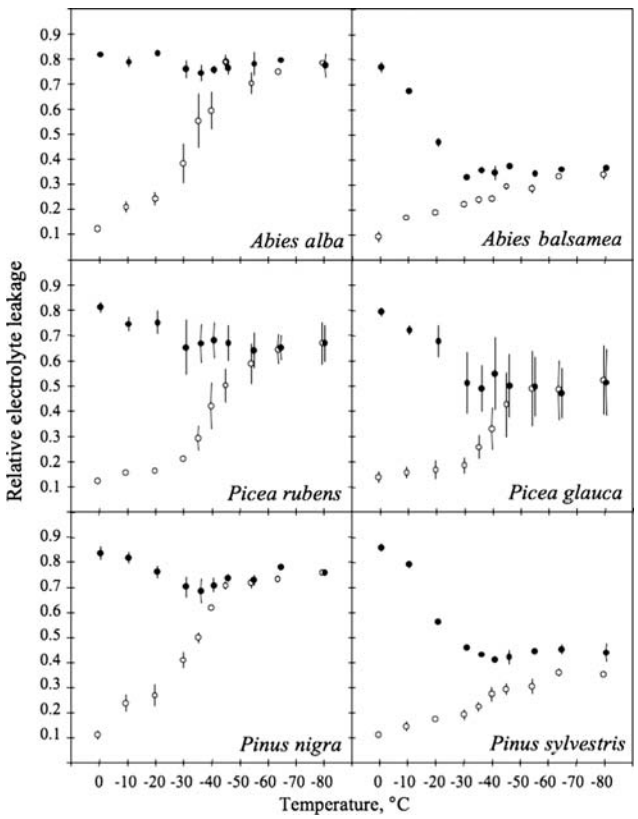

Fig. 2 Relative electrolyte leakage data after slow freezing and slow freezing followed by LN_2 quenching for foliage of six conifer species. *Open symbols*: mean ± standard error of three trees after slow freezing. *Closed symbols*: mean ± standard error of three trees after slow freezing to the indicated temperature followed by quenching in LN_2

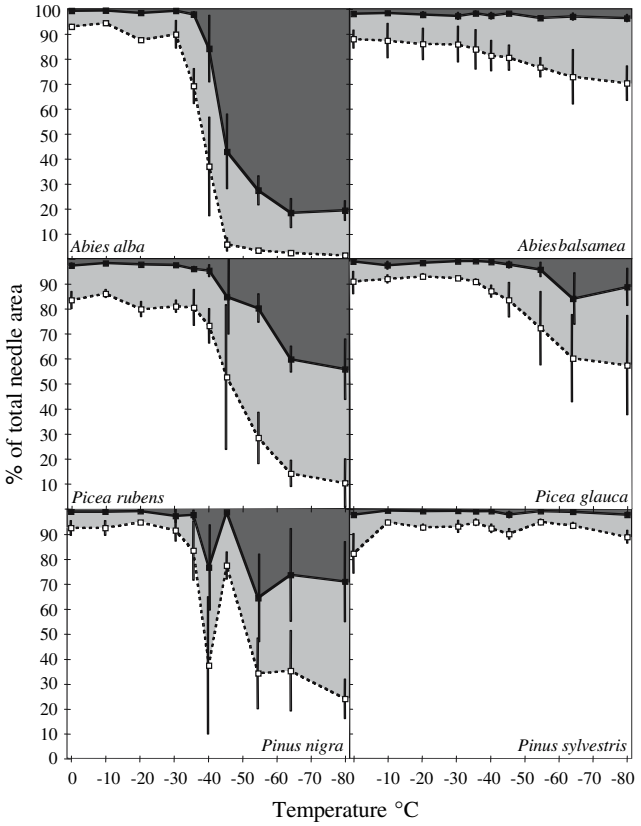

Fig. 3 Image analysis results of color changes in samples of needle sections after slow freezing of whole shoots from six conifer species. Open symbols: mean ± standard error (n = 3) of % green needle area. *Closed symbols*: mean ± standard error of % green + yellow needle area. *Dark shading* represents red and orange needle area, *medium shading* represents yellow needle area, and *white area* represents green needle area

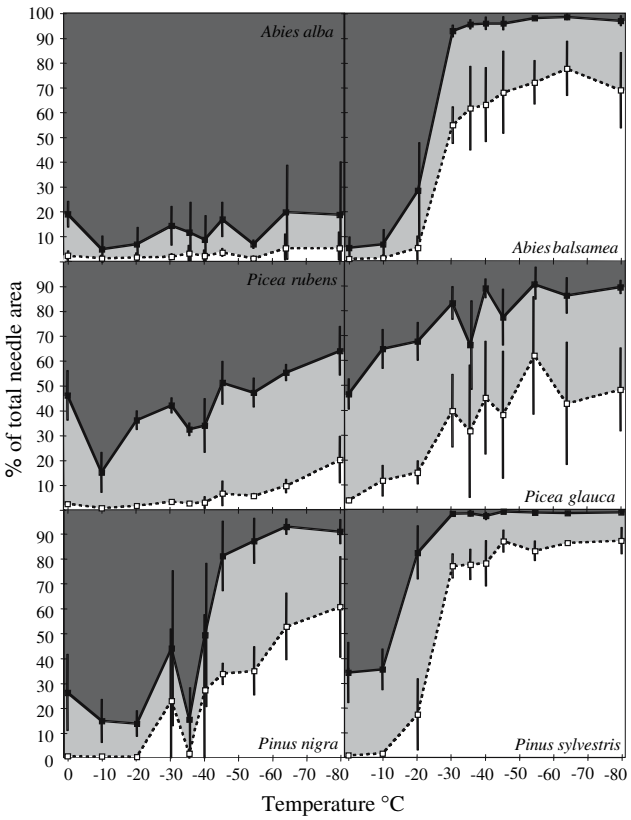

Fig. 4 Image analysis results of color changes in samples of needle sections after slow freezing followed by LN_2 quenching of whole shoots from six conifer species. *Symbols and shading* as in Fig. 3

Necrosis was less complete in *Picea rubens* and *Pinus nigra*, where extreme low temperature produced a mix of chlorosis and necrosis.

The boreal species developed up to about 30% chlorosis in response to slow freezing or LN_2 quenching from temperatures of –30°C or lower, with significant necrosis appearing only after LN_2 quenching from temperatures of –20°C or warmer. As in the REL data, visual injury symptoms in *Picea glauca* were more variable than in the other two boreal species, but agreed with the electrolyte leakage in that one tree appeared relatively insensitive while the other two responded strongly to LT stress, resulting in an overall intermediate pattern. In contrast to the REL results, the visual injury results for *Pinus nigra* suggest an intermediate pattern, but this may be an artifact of the method used to develop visual injury symptoms, as discussed below.

A comparison of the electrolyte leakage and visual injury data from slow freezing (Fig. 5) shows that up to about 65% chlorosis occurs over a broad range of REL values, with the higher percentages of chlorosis associated with REL values from about 0.4 to about 0.8. Necrosis occurred only at REL greater than about 0.5. While necrosis is the clearest symptom of lethal injury, substantial chlorosis

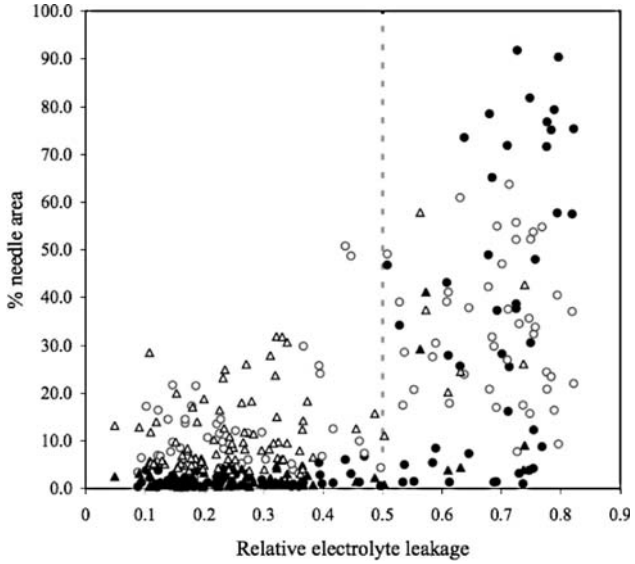

Fig. 5 Generalized relationship between REL and foliar chlorosis (*open symbols*) and necrosis (*closed symbols*), for temperate (*circles*) and boreal (*triangles*) species after slow freezing to temperatures from 0 to –80°C. Dashed line at REL = 0.5 shows threshold for development of necrosis

was associated with severe LT stress in some species (Figs. 3, 4), and this is reflected in Fig. 5. One hundred percent discoloration, expressed as the sum of chlorotic and necrotic needle area, occurred at REL as low as about 0.5, but mainly above about 0.6. These values give some estimates of REL associated with complete injury, and can be used to qualify the REL-based estimates of LT_{50} as discussed below.

Sugars

The sugar results are summarized by genus and LT sensitivity in Fig. 6. We found detectable amounts of sucrose, glucose, and raffinose in 23 species (the sample for *Abies alba* was lost during sugar extraction), fructose in 21 species, stachyose in 15 species (including all spruce species), and a trace amount of xylose in one species, *Abies lasiocarpa*. In analyses of variance for raffinose, sucrose, fructose, glucose, and total sugars, the 23 species were divided into 6 groups, three genera x two levels of LT sensitivity (based on a threshold value of $Y_{max} = 0.5$, Table 1). Despite the limited sample sizes and lack of replication within species, we found significantly lower sucrose ($P = 0.04$), raffinose ($P = 0.003$), and total sugars ($P = 0.014$) in sensitive than insensitive species, and significant differences between genera for raffinose ($P < 0.0001$) and total sugars ($P = 0.033$). There were no significant interactions. In a posteriori comparisons among genera (Tukey–Kramer HSD) *Pinus* had significantly lower ($P < 0.05$) raffinose and total sugar concentrations than *Picea* and *Abies*.

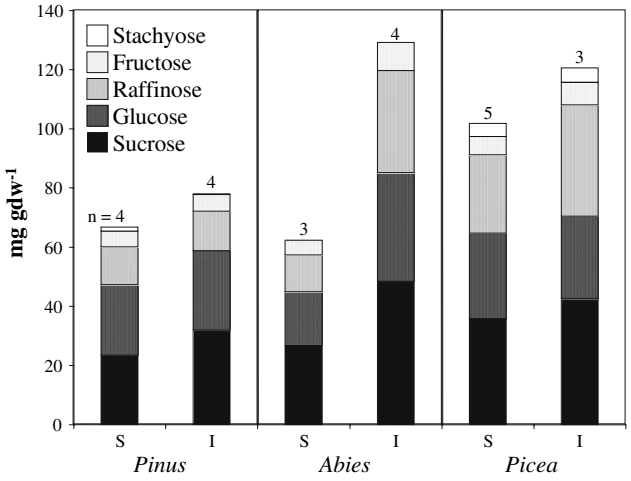

Fig. 6 Mean concentration of five sugars in needles of 23 LT sensitive (S, $Y_{max} > 0.5$) and insensitive (I, $Y_{max} < 0.5$) *Pinus*, *Abies*, and *Picea* species

Because T_ms of sensitive and insensitive species are not comparable, we tested for correlation between sugar concentration and T_m for the 12 sensitive species only. T_m correlated significantly with sucrose ($P = 0.042$), raffinose (Figure S5), and total sugar ($P = 0.017$) concentration.

Discussion

LT tolerance and climate

LT tolerance generally correlates with the regional climate for the main range of each species, especially when elevation is also taken into account (Table 1). The most sensitive *Abies* species and *Picea sitchensis* originate from regions with a temperate oceanic climate where proximity to the ocean moderates winter temperatures even at high elevation. The two most sensitive *Pinus* species (*P. jeffreyi* and *P. nigra*) grow in regions with Mediterranean climates where dry summers are a dominant factor even at high elevation, but also with moist and relatively mild winters (Trewartha and Horn 1971). *Abies alba*, the most sensitive fir species, also occurs in areas with Mediterranean climates in the southeastern portion of its range.

Most species from temperate continental climates and mountains in semi-arid regions were generally intermediate in sensitivity. While the individual trees of these species showed a sigmoid response, it was weaker than in the sensitive species. The upper REL values in some species remained below 0.5, the threshold REL value for severe injury based on comparison of REL and visual injury (Fig. 5), suggesting that these species were not completely or lethally injured at the lowest temperature. The exceptions are *Picea jezoensis* and *P. rubens*, among the most sensitive *Picea* species despite their distributions in regions with continental climates. This may be due to origins in regions with locally oceanic climates. *P. rubens* occurs at sea level at the northern end of its range, in the Canadian maritime provinces, and is found at increasingly higher elevations southward in the Appalachian Mountains. Paleoecological evidence suggests that it survived glaciation in coastal refugia and moved into montane habitats only during the last 2000 years (White and Cogbill 1992), so that its winter physiology may be better adapted to coastal than montane environments. *Picea rubens* is considerably more sensitive to LT than other species in Appalachian Mountain spruce-fir forests and in montane environments the foliage suffers frequent winter injury (DeHayes et al. 2001), a problem exacerbated by acid precipitation (Johnson et al. 1992). The mean T_m value reported here is somewhat warmer than those reported in many previous studies, but the values fall within the range of a thorough field study of 59 trees at three elevations (Schaberg et al. 1999). *P. jezoensis* occurs mainly in northern Japan, Korea, the Sakhalin and Kuril islands, along the southeast Siberian coast and up to a few hundred kilometers inland, in areas with locally oceanic or suboceanic climates (Krestov and Nakamura 2002), even though coarse mapping places it in temperate continental and even boreal regions. It has been described as "an ecological counterpart of Sitka spruce" (Earle 2006). Sakai (1983) found that *P. jezoensis* foliage from Hokkaido in Northern Japan could survive –70°C, making it one of the more LT tolerant conifers tested, but in an earlier study foliage of the southern variety *P. jezoensis* var. *hondoensis* foliage was killed at –25 to –30°C (Sakai and Okada 1971). The Ringve collection includes accessions of both southern and northern varieties, but individual trees were not identified to subspecies. It seems likely that the trees sampled in our study are the southern variety.

All the species with strongly boreal distributions were LT insensitive, with the notable exception of *Pinus banksiana*. This North American species has a strongly boreal distribution, but in our results appeared rather sensitive to low temperature, with Y_{max} suggesting a high level of injury at the lowest test temperatures. Previous studies have found it to be extremely LT tolerant (Sakai and Weiser 1973).

Two of the three *Picea glauca* trees sampled on 13 March followed a pattern of intermediate LT sensitivity like the temperate continental species, while the third tree followed the boreal pattern seen in *P. glauca* in the 13 February collection. The arboretum records show three different accessions for this species, one from southern Ontario, a second from Edmonton, Alberta, and the third from McKinley National Park, Alaska, representing a gradient from temperate continental to strongly boreal

climate conditions. We sampled different trees on the two different dates, so it is possible that the observed differences relate to geographic variation in low temperature tolerance associated with these climatic differences. Unfortunately, the seed source of individual trees in the collection cannot be identified (personal communication, Ane Guldahl, Ringve Botanical Garden).

While LT tolerance in all species was adequate to survive winter minimum temperatures in Trondheim, the LT insensitivity of the boreal species, in a climate that is much milder than in their native regions, indicates that the level of LT acclimation in these species is largely predetermined, presumably by gene expression pathways triggered by short days and/or low temperature. This opens the question as to whether there may be tradeoffs between the ability to survive extreme cold and the metabolic costs of acclimation and carbon gain or loss during relatively warm intervals in the cold season.

Chlorosis and necrosis

Field observations and various laboratory studies have shown that conifer foliage typically turns a distinctive orange-brown color (necrosis) after lethal LT stress and subsequent exposure to light (DeHayes 1992). In histological studies in *Picea rubens* this is associated with a progressive loss of intracellular structure ending with cytoplasmic remnants adhering to the walls of the dead cell (Adams et al. 1991). Although this color change is often assessed using a rank scoring system, image analysis gives quantitative results and may also be used to detect and quantify more subtle color changes associated with sublethal stress. This applies especially to the slight to severe yellowing or chlorosis we have observed here, which is difficult to score if the predominant symptom of injury is orange-brown necrosis.

Reversible chlorosis under winter conditions has been observed in *Pinus sylvestris* (Baronius et al. 1991), *Picea rubens* (Adams 1996), and more anecdotally in numerous other conifer species, and appears to be related to light stress (Adams 1996). Under the conditions we used to develop visual injury, apparently lethally injured needles may develop a mixture of necrosis and chlorosis in some species (e.g., *Picea rubens*, *Pinus nigra*), whereas LT insensitive species (*Pinus sylvestris*, *Abies balsamea*) may develop up to 30% chlorosis without substantial necrosis. We interpret the latter cases as reversible chlorosis, but suggest that some or all of the chlorosis in the sensitive species may be a result of incomplete development of injury symptoms. When *Picea rubens* is severely injured under natural conditions, the necrosis that develops in current-year shoots in spring and early summer is often complete, so given more time or under higher light intensity we predict more complete necrosis following severe LT stress in this species. In the case of *Pinus nigra*, many of the needles were cut in order to fit the shoot into a test tube for freezing, so that the samples may have desiccated during exposure to light, thereby preventing the development of typical necrosis. In situ desiccation of *Picea rubens* results in the development of a dull green color rather than the necrosis associated with LT stress (Strimbeck et al. 1991). In support of this interpretation, we observed that even the green parts of the needles in some *P. nigra* samples appeared dry.

Reversible chlorosis is generally interpreted as a result of a combination of high light intensity and LT, because light absorption and charge separation in chlorophyll is temperature insensitive, while rates of electron transport and other downstream reactions in photosynthesis are reduced at low temperature. This can result in the generation of free radicals and/or breakdown of chlorophyll molecules (Olszowka et al. 2003). Our results show that light exposure subsequent to LT stress can result in chlorosis, and this is also associated with limited increases in REL. This shows that even extremely LT tolerant species such as *Abies balsamea* and *Pinus sylvestris* have limited, reversible responses to LT stress, and that the nonlethal cellular disruptions associated with moderate increases in REL may also affect sensitivity to subsequent light stress.

Sugars and LT tolerance

Our results augment those of numerous other studies that demonstrate a general association between sugar content, notably sucrose and raffinose, and LT tolerance. This relationship is most often seen because sugar content changes in tandem with LT acclimation and deacclimation (e.g., Hinesley et al. 1992), but has also been shown in a sample of 59 *Picea rubens* trees sampled on a single date in midwinter (Schaberg et al. 1999). Our results show that this general relationship also occurs in the sample of 23 species for which we have both LT tolerance and sugar data. These species vary widely in needle size and structure, a possible source of the significant generic differences and the high variability in the sugar–LT tolerance relationship. Sugar concentration is typically expressed in terms of tissue dry weight, and different types of needles or even needles within a single species may vary in the proportion of dry weight in extracellular structures such as cuticle and cell walls. If sugars act as cytoplasmic cryoprotectants, then the concentration of sugars in the cytoplasm or ratio of sugars to other cytoplasmic components, although difficult to measure, would be more meaningful. Using water content to express sugar concentrations in terms of total tissue water may provide a more pertinent reference for comparison, but sugars may accumulate preferentially in the

cytoplasm (Koster and Lynch 1992), while most intracellular water is in the vacuole, and an additional fraction of the total tissue water is apoplastic.

Mechanism

Our results confirm those of Sakai (Sakai 1960; Sakai and Okada 1971; Sakai and Weiser 1973) showing that tissues of boreal conifers can survive LN_2 quenching from temperatures of –30°C or lower. One explanation for these observations is that the cell solution undergoes a glass transition or steep increase in viscosity at temperatures between –20 and –30°C. The glassy state is mechanistically defined in terms of viscosity: a glass is as an amorphous material with a viscosity greater than 10^{12} Pa s (Franks 1985), making it a kind of molecular 'suspended animation' involving a more or les complete loss of translational and rotational mobility that results in a kinetic barrier to both degradative chemical reactions and further dehydration.

Vitrification is employed in preservation of frozen or dried foods, drugs, cells, and tissues, and has been proposed as a general mechanism of extreme dehydration tolerance in both plant and animal cells (Burke 1985). Glass transitions have been more or less convincingly demonstrated by DCS, NMR, and ESR methods in dried seeds and other tissues with low water contents (e.g., Buitink and Leprince 2004; Koster 1991; Leprince and Waltersvertucci 1995), but they may be particularly difficult to detect in frozen plant tissues because: (1) the majority of the water in the tissue is in the form of ice and would act to dilute the heat capacity signal so the change in heat capacity due to vitrification of the cell solution is extremely small, and (2) glass transitions in complex solutions may be extremely broad, so that they appear as a nearly linear change in heat capacity rather than the step change that appears in simpler solutions. Existing evidence for glass transitions in frozen plant tissues relies on rather subjective interpretation of subtle inflections in differential scanning calorimetry (DSC) scans (e.g., Hirsh 1987; Hirsh et al. 1985).

The vitrification hypothesis also offers an explanation for the association between sugars and LT tolerance. Partially frozen sucrose solutions vitrify readily even at relatively slow freezing rates, for example 0.2°C min^{-1}, at a maximum temperature of about –41°C and concentration of 80.9% (Liesebach et al. 2003). Interactions of sucrose with raffinose and other cryoprotective substances, including some proteins associated with LT tolerance such as dehydrins or LEA proteins (Buitink and Leprince 2004), could raise intracellular glass transition temperature to –30°C or higher.

Vitrification may be a necessary but not sufficient component of extreme LT survival (Crowe et al. 1998). Sugars and other cryoprotectants are also thought to interact directly with membranes and macromolecules to protect them from the effects of dehydration (Arakawa and Timasheff 1982; Crowe et al. 1984). Although there are alternative explanations, because we have shown that tolerance of freezing stress in boreal species stabilizes at –30°C, our data are consistent with the hypothesis that vitrification is a component of survival at temperatures below –30°C.

References

Adams GT (1996) Wintertime photostress in red spruce foliage. Master's Thesis, Department of Botany, University of Vermont, Burlington

Adams GT, Perkins TD, Klein R (1991) Anatomical studies on first-year winter injured red spruce foliage. Am J Bot 78:1199–1206

Anderson JA, Kenna MP, Taliaferro CM (1988) Cold hardiness of 'Midiron' and 'Tifgreen' bermudagrass. HortSci 23:748–750

Arakawa T, Timasheff SN (1982) Stabilization of protein structure by sugars. Biochem 21:6536–6544

Bannister P, Neuner G (2001) Frost resistance and distribution of conifers. In: Bigras FJ, Colombo SJ (eds) Conifer cold hardiness, vol 1. Kluwer, Dordrecht, pp 3–21

Baronius G, Fiedler HJ, Montag HG (1991) Comparative investigations by means of Munsell-color charts and the Cielab Color System on the winter chlorosis of *Pinus sylvestris* L. in the Pollution Area of the Dueben Heath. Forstwiss Centralbl 110:263–277

Becwar M, Rajashekar C, Bristow K, Burke M (1981) Deep undercooling of tissue water and winter hardiness limitations in timberline flora. Plant Physiol 68:111–114

Buitink J, Leprince O (2004) Glass formation in plant anhydrobiotes: survival in the dry state. Cryobiology 48:215–228

Burke MJ (1985) The glassy state and survival of anhydrous biological systems. In: Leopold AC (ed) Membranes, metabolism, and dry organisms. Comstock Publishing Associates, Ithaca, pp 358–363

Crowe JH, Crowe LM, Chapman D (1984) Preservation of membranes in anhydrobiotic organisms—the role of trehalose. Science 223:701–703

Crowe JH, Carpenter JF, Crowe LM (1998) The role of vitrification in anhydrobiosis. Annu Rev Physiol 60:73–103

DeHayes DH (1992) Winter injury and developmental cold tolerance of red spruce. In: Eagar C, Adams MB (eds) Ecology and decline of Red Spruce in the Eastern United States, vol 96. Springer, New York, pp 295–337

DeHayes DH, Schaberg PG, Strimbeck GR (2001) Red spruce (*Picea rubens* Sarg.) cold hardiness and freezing injury susceptibility. In: Bigras FJ, Colombo SJ (eds) Conifer cold hardiness, vol 1. Kluwer, Dodrecht, pp 495–529

Earle CJ (2006) The gymnosperm database. http://www.conifers.org/index.html. Accessed 5 February 2007

Franks F (1985) Biophysics and biochemistry at low temperatures. Cambridge University Press, Cambridge

Hawkins BJ, Davradou M, Pier D, Shortt R (1995) Frost hardiness and winter photosynthesis of Thuja-Plicata and Pseudotsuga-Menziesii seedlings grown at 3 rates of nitrogen and phosphorus supply. Can J Forest Res 25:18–28

Hinesley L, Pharr D, Snelling L, Funderburk S (1992) Foliar raffinose and sucrose in four conifer species: relationship to seasonal temperature. J Am Soc Hort Sci 117:852–855

Hiratsuka Y, Zalasky H (1993) Frost and other climate-related damage of forest trees in the prairie provinces. Forestry Canada, northwest Region, Northern Forestry Centre, Edmonton, p 26

Hirsh A (1987) Vitrification in plants as a natural form of cryoprotection. Cryobiology 24:214–228

Hirsh A, Williams R, Meryman H (1985) A novel method of natural cryoprotection. Intracellular glass formation in deeply frozen *Populus*. Plant Physiol 79:41–56

Johnson AH, McGlaughlin SB, Adams MB, Cook ER, DeHayes DH, Eagar C, Fernandez IJ, Johnson DW, Kohut RJ, Mohnen VA, Nicholas NS, Peart DR, Schier GA, White PS (1992) Synthesis and conclusions from epidemiological and mechanistic studies of red spruce decline. In: Eagar C, Adams MB (eds) Ecology and decline of Red Spruce in the Eastern United States, vol 96. Springer, New York, pp 365–411

Koster K (1991) Glass formation and desiccation tolerance in seeds. Plant Physiol 96:302–304

Koster K, Lynch D (1992) Solute accumulation and compartmentation during the cold acclimation of Puma rye. Plant Physiol 98:108–113

Krestov PV, Nakamura Y (2002) Physosociological study of the Picea jezoensis forests of the far east. Folia Geobotanica 37:441–473

Leprince O, Waltersvertucci C (1995) A calorimetric study of the glass-transition behaviors in axes of bean-seeds with relevance to storage stability. Plant Physiol 109:1471–1481

Liesebach J, Rades T, Lim M (2003) A new method for the determination of the unfrozen matrix concentration and the maximal freeze-concentration. Thermochim Acta 401:159–168

Moen A (1999) National Atlas of Norway: Vegetation. Norwegian Mapping Authority, Hønefoss

Ögren E, Nilsson T, Sundblad L (1997) Relationship between respiratory depletion of sugars and loss of cold hardiness in coniferous seedlings over-wintering at raised temperatures: indications of different sensitivities of spruce and pine. Plant Cell Environ 20:247–253

Olszowka D, Maksymiec W, Krupa Z, Krawczyk S (2003) Spectral analysis of pigment photobleaching in photosynthetic antenna complex LHCIIb. J Photochem Photobiol B-Biol 70:21–30

Pukacki P (1987) Deep supercooling of shoot and bud tissues of *Picea abies*. For Ecol Manage 20:97–103

Sakai A (1960) Survival of the twigs of woody plants at –196 °C. Nature 185:393–394

Sakai A (1978) Low temperature exotherms of winter buds of hardy conifers. Plant Cell Physiol 19:1439–1446

Sakai A (1983) Comparative-study on freezing resistance of conifers with special reference to cold adaptation and its evolutive aspects. Can J Bot-Revue Can De Bot 61:2323–2332

Sakai A, Larcher W (1987) Frost survival of plants: responses and adaptations to freezing stress. Springer, Berlin

Sakai A, Okada S (1971) Freezing resitance of conifers. Silvae Gentica 20:91–97

Sakai A, Weiser CJ (1973) Freezing resistance of trees in North America with reference to tree regions. Ecology 54:118–126

Schaberg PG, Shane JB, Hawley GJ, Strimbeck GR, Cali PF, Donnelly JR, DeHayes DH (1996) Physiological changes in red spruce seedlings during a simulated winter thaw. Tree Physiol 16:567–574

Schaberg PG, Shane JB, Cali PF, Donnely JR, Strimbeck GR (1998) Photosynthetic capacity of red spruce during winter. Tree Physiol 18:271–276

Schaberg PG, Strimbeck GR, Hawley GJ, DeHayes DH, Shane JB, Murakami PF, Perkins TD, Donnely JR, Wong BL (1999) Cold tolerance and photosystem function in a montane red spruce population: physiological relationships with foliar carbohydrates. J Sustainable For 10:173–180

Strimbeck GR, Vann DR, Johnson AH (1991) In situ experimental freezing produces symptoms of winter injury in red spruce foliage. Tree Physiol 9:359–367

Strimbeck GR, Schaberg PG, DeHayes DH, Shane JB, Hawley GJ (1995) Midwinter dehardening of montane red spruce during a natural thaw. Can J For Res 25:2040–2044

Trewartha GT, Horn LH (1971) An introduction to climate. MGraw Hill International, London

White PS, Cogbill CV (1992) Spruce-fir forests of eastern North America. In: Eagar C, Adams MB (eds) Ecology and decline of red spruce in the Eastern United States, vol 96. Springer-Verlag, New York, p 417