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Dynamics of low-temperature acclimation in temperate and boreal conifer foliage in a mild winter climate

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Summary To provide baseline data for physiological studies of extreme low-temperature (LT) tolerance in boreal conifers, we profiled LT stress responses, liquid nitrogen (LN_2)-quench tolerance, and sugar concentrations in foliage of boreal–temperate species pairs in the genera *Abies*, *Picea* and *Pinus*, growing in an arboretum in a temperate oceanic climate from August 2006 through April 2007. The boreal species acclimated more rapidly and deeply than the temperate species, acquiring LN_2-quench tolerance by late November, despite unusually warm conditions throughout the autumn and early winter. Maximum LT tolerance in the temperate species was in the –25 to –35 °C range, and was reached only after a period of freezing temperatures in late January and February. During LT acclimation in the temperate species, sigmoid temperature–relative electrolyte leakage (REL) curves shifted toward lower temperatures, whereas in boreal species there was both a temperature shift and a lowering of the maximum REL until it fell below a threshold associated with irreversible injury. These differences may reflect differences in mechanisms of LT acclimation and LT tolerance. The concentrations of total and individual sugars did not show a clear pattern that could differentiate the boreal and temperate groups. Raffinose and, in three of the six species, stachyose showed the closest association with LT tolerance. Sugar concentrations, principally sucrose, decreased during mild weather, perhaps because of respiratory losses or phloem export, and increased after periods of freezing temperatures. Low-temperature acclimation in boreal species appears to follow a rigid program that may affect their ability to avoid excessive respiratory losses in the event of continued climate warming in boreal regions.

Keywords: Abies, cold, frost, LN_2-quench hardiness tolerance, Picea, Pinus, raffinose, stachyose, sucrose, sugar.

Introduction

Although plant low-temperature (LT) tolerance (also referred to as cold or frost tolerance or hardiness) has been investigated for over a century, relatively little is known about the mechanisms of LT acclimation and survival in extremely LT-tolerant boreal woody plant species. Boreal forests are dominated by conifer species of the genera *Abies*, *Picea*, *Pinus* and *Larix*, which are well adapted to the severe winter climate, with minimum temperatures below –60 °C and prolonged periods at temperatures below –40 °C. Their survival of low temperatures depends on timely acclimation to LT stress and maintenance of the acclimated state throughout the winter. In the fully acclimated state, the aboveground parts of boreal conifers, including evergreen foliage, and of the deciduous species in the genera *Salix*, *Populus* and *Betula* survive immersion in liquid nitrogen (LN_2) at –196 °C (Sakai 1966, Sakai and Weiser 1973, Strimbeck et al. 2007), making them, for all intents and purposes, "perfectly" LT tolerant.

They may not, however, be immune to the effects of global warming. According to the consensus predictions of several general circulation models, under an intermediate scenario, mean winter temperatures in boreal forest regions of Canada and Siberia will rise by 4 °C to as much as 7 °C (Christensen et al. 2007). Changes in the timing of the onset of subfreezing temperatures or the frequency and duration of midwinter thaws could disrupt LT acclimation, maintenance of the acclimated state, or winter carbon balance in boreal plants, and may ultimately be an important factor in global-warming-induced changes in boreal forest ecosystems.

Controlled-environment studies of LT acclimation in a variety of woody plant species have shown that photoperiod and chilling (0 to 10 °C) or subfreezing temperatures control acclimation (e.g., Bigras et al. 2001, Smallwood and Bowles 2002, Li et al. 2004). Several field studies have tracked acclimation of extremely LT-tolerant species under natural conditions (e.g., Repo 1992, Sutinen et al. 1992, Sauter et al. 1996), but few studies of boreal species in controlled environments have produced the depth of acclimation that is observed for the same species in nature.

Temperature may affect maintenance of the acclimated state during winter. For example, a prolonged period with temperatures above 0 °C resulted in an average 9 °C loss of LT tolerance in *Picea rubens* Sarg. (Strimbeck et al. 1995), and warm

storage of this species can induce dehardening in as little as 18 h (DeHayes et al. 1990). *Picea rubens* is one of several temperate conifer species known to fix carbon during mild winter weather (Hawkins et al. 1995, Schaberg et al. 1995), indicating a possible tradeoff between LT tolerance and carbon balance (Ögren 1997).

Accumulation of di- and oligosaccharides is consistently associated with LT acclimation and tolerance (Sakai and Larcher 1987), and sugars are generally thought to act as cellular cryoprotectants through some combination of colligative effects or direct interaction with membranes or proteins (Arakawa and Timasheff 1982, Carpenter and Crowe 1988). Most studies on sugars involve cereal and other crops, fruit trees and *Arabidopsis*, with relatively few studies reporting on sugar accumulation in extremely hardy conifer or deciduous species (e.g., Hinesley et al. 1992, Sauter et al. 1996).

To provide baseline data for future studies of mechanisms of LT acclimation and LT tolerance in boreal conifers, we measured the LT tolerance of needles from six boreal and temperate conifer species growing from August 2006 to April 2007 in an arboretum with a temperate oceanic climate. Although buds are often more LT sensitive than needles (Burr et al. 1990), we used needles in our work because they can be collected in bulk with minimal disturbance to the tree, and they develop quantifiable visible injury symptoms that can be compared with electrolyte leakage data (Strimbeck et al. 2007). We measured foliar concentrations of mono-, di- and oligosaccharides over the same period to further investigate the role of these sugars in LT acclimation and tolerance. In keeping with recent global and local climate trends, during our study the first part of the winter was unusually mild, so our results provide additional insight into the LT-acclimation process under these mild conditions.

Materials and methods

Sample collection and climate data

The arboretum at the Ringve Botanical Garden (63°26′56″ N, 10°27′12″ E) in Trondheim, Norway, is located near sea level and just a few hundred meters from Trondheimsfjord, which remains unfrozen in winter and moderates the local climate. Winter temperatures rarely fall below –20 °C, and winters are punctuated by periods with temperatures above 0 °C, especially in recent years. The arboretum collection includes 52 species of temperate and boreal conifers from around the northern hemisphere, in groups of a few to as many as 30 trees per species. These were planted mostly during the early development of the arboretum in the 1970s, so they have attained sufficient size to produce cones. Many accessions in the arboretum have come from collections in Scandinavia or other local sources, but the provenance of most species is unknown or unverifiable.

We used daily maximum, minimum and mean temperatures at Værnes International Airport (eKlima 2008), located in Stjørdal, about 30 km to the east of the Ringve Botanical Garden and at a similar elevation and proximity to the fjord, as proxy temperature data.

Based on LT-tolerance data from the previous winter, we selected boreal–temperate pairs of species in the genera *Abies*, *Picea* and *Pinus* (Table 1) to compare the acclimation process in species originating in different environments. The temperate species originate in temperate coastal environments or mountains in Mediterranean climate regions with relatively mild winters and were among the least LT-tolerant species of these genera in the collection (Strimbeck et al. 2007). Three trees of each species were selected for repeated sampling during the autumn and winter. Samples were collected at 2- to

Table 1. Species information. Range and climate information adapted mainly from Earle (1997–2007).

Main geographic range	Elevation (m)	Latitude (° N)	Climate	Provenance
Abies alba Mill.				
Alps, Pyrenees, Carpathians	300–1950	38–50	Mountains in Mediterranean and warm temperate region	Besançon, France (47°15′ N)
Abies balsamea (L.) Mill.				
Eastern Canada, northeastern USA	0–1700	42–57	Boreal and cold temperate continental	Unknown
Picea obovata Ledeb.				
Siberia		54–72	Boreal continental	Tomsk, Russia (54°45′ N) Ufa, Russia (56°30′ N)
Picea sitchensis (Bong.) Carr.				
Pacific northwest coast, North America	0–1000	40–60	Temperate oceanic	Unknown
Pinus jeffreyi Grev. & Balf.				
Sierra Nevada, CA	2000–3100	30–43	Mountains in Mediterranean and warm temperate region	Unknown
Pinus sylvestris L.				
European northwest coast to Siberia	0–1000	50–70	Boreal continental to temperate oceanic	Tomsk, Russia (54°45′ N)[1] Rennebu, Norway (62°52′ N)

[1] We used the Tomsk provenance of *P. sylvestris*; in other cases, provenance could not be determined for individual trees.

4-week intervals from August 2006 to April 2007, with the greatest frequency during the acclimation period from September to early December. Samples consisted of lateral branch tips or, in the case of *Pinus jeffreyi*, needle clusters of sufficient size and number to provide the 2–5 g of first-year needles needed for the freezing tests and sugar analysis. The trees were large enough that the total sample material collected over the course of the experiment represented only a small fraction of the first-year foliage of each tree. Samples were transported to the laboratory in insulated containers at ambient temperature and stored at 0 °C for 1 to 4 h.

Low-temperature tolerance

The needles were cut into 5-mm sections, and about 0.2 g of needle sections from each tree were placed in a series of up to 16 vials for freezing treatments. One ml of 0.1% TritonX-100 solution containing a bacterial ice nucleator was added to each vial before freezing. One replicate was held at 4 °C, and the remaining samples were cooled to an initial subfreezing temperature (usually –10 °C) and held overnight before further freezing in steps to temperatures as low as –65 °C. Replicates were removed from the freezer at a series of predetermined test temperatures after a 30-min hold at each temperature and transferred to a 4 °C cold room in insulated containers and allowed to warm passively overnight. With passive warming, samples from lower temperatures warm more rapidly initially, but warming rates converge asymptotically as samples approach 0 °C, so that rates are similar when cells reabsorb most water from extracellular ice. Beginning in late October, additional replicates were quenched directly in liquid nitrogen from temperatures of –20, –30 or –40 °C. After thawing, 10 ml of 0.1% TritonX-solution at 4 °C was added to each vial, and the samples were held at 4 °C for 24 ± 0.5 h, then warmed and shaken for 30 min at 20 °C before measuring initial conductivity. Samples were then autoclaved at 120 °C for 20 min, and final conductivity was measured after cooling to room temperature and shaking for 30 min at 20 °C.

Relative electrolyte leakage (REL), the ratio of initial to final conductivity, corrected for the baseline conductivity of solution blanks, was used as an index of injury. Nonlinear curves of the form:

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

were fit to the data for the three trees of each species to give a composite temperature response curve for each species. The abbreviations Y_{min} and Y_{max} are the uninjured and maximum relative conductivity, respectively, k is a coefficient defining the steepness of the response curve, and T_m is the midpoint of the curve, which is an estimate of LT_{50} (temperature at 50% cell mortality) when Y_{max} represents complete mortality. Previous studies comparing REL with visible injury symptoms indicate that REL values below 0.5 are associated primarily with reversible chlorosis, whereas cell death followed by necrosis occurs only at REL > 0.5 (Strimbeck et al. 2007).

Sugars

Sugars were measured in subsamples drawn from the bulk samples prepared for LT-tolerance testing. Needle pieces were freeze-dried before sugar extraction. Cuticular waxes were removed with hexane, and then soluble sugars were extracted in 80% ethanol (Hinesley et al. 1992). The supernatant was filtered through a Waters C18 Sep-Pac Plus Cartridge (Waters Corporation, Milford, MA) 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 with 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^{-1}. Soluble sugar standards encompassing low, medium and high anticipated sugar concentrations were run before each sample set.

Relationships between the LT-tolerance parameters and the concentrations of the individual sugars were evaluated by linear models, with sugar concentration as the response variable; group (boreal or temperate) and genus as nominal factors; T_m and Y_{max} as continuous factors; first-order interactions between group, genus, T_m and Y_{max}; and tree within group and genus as a random factor to allow for the repeated measures component of the sampling scheme. Because the general linear model used to evaluate relationships between LT-tolerance parameters and sugar concentrations was constructed a posteriori, we used a conservative α value of 0.01 to determine statistical significance, and used P values to assess the relative importance of the different sources of variance. Use of mass-based measurements of sugars as the response variable allowed analysis of between-species and individual-tree variations in sugar concentration associated with differences in investment in structural materials such as cuticles and cell walls. Only trace amounts of xylose were found in some samples, so the xylose data were not analyzed.

Results

Temperature conditions

Following global and regional trends, 2006 was the warmest year on record for Norway, with numerous monthly mean and maximum temperature records set at stations throughout the country. In the Trondheim region, mean temperatures for August and December were the warmest in the period of record (since 1867), and September, October and November were also well above normal. Mean temperature for January was only slightly warmer than normal, but below normal in February, whereas March was again the warmest on record for the country as a whole.

In the Trondheimsfjord area, temperatures remained continuously above freezing through the first half of October (Figure 1), with a few nights of frost and one day of continuous below-freezing temperature in late October. Daytime temperatures remained mostly above freezing throughout November and December, with sporadic night frosts of a few degrees below the freezing point. Temperatures were lower in late January and February with the exception of a 4-day thaw in late

January. By early March, mean daytime temperatures remained above freezing, but with frequent night frosts occurring into the beginning of April.

Low-temperature acclimation

In August and September, foliage from all 18 trees was killed at the lowest test temperature ($Y_{max} > 0.5$, Figure 1), so T_m for all species on the first three sample dates can be interpreted as LT_{50} values. These were all above or near –10 °C in August and early September and decreased slightly in most species by late September, with the exception of *Picea obovata*, which had an LT_{50} of around –20 °C. By the first week of October, the three boreal species began hardening more rapidly, as shown by decreases in both Y_{max} and T_m (Figure 1). A value of $Y_{max} < 0.5$ for *P. obovata* indicated that, by October 8, foliage of this species was not killed by the lowest test temperature (–40 °C on that date), and by October 23, all three boreal species at least partially survived –40 °C, with the very low Y_{max} for *P. obovata* indicating 100% survival. High Y_{max} values for the temperate species indicated lethal injury at the minimum test temperature throughout the winter, with LT_{50} decreasing slowly to about –15 °C by late October. More rapid hardening in all species followed a cold period with several nights of frost in late October and early November. By late November, the temperate species reached LT_{50} values of –24 to –30 °C, and all boreal species survived the minimum test temperature of –62 °C.

In all species, T_m remained stable or increased slightly during the mild weather in November and December, then decreased in association with a cold period in late January, with the temperate species reaching minimum LT_{50} values of around –30 °C. *Abies alba* and *Picea sitchensis* continued acclimating during a 9-day period of cold weather in February, reaching their winter minimum mean LT_{50} of –34.5 and –36.5 °C, respectively, whereas the LT_{50} for *Pinus jeffreyi* remained above –30 °C. Both Y_{max} and T_m indicated that *Picea obovata* was the most LT-tolerant species overall throughout the winter, whereas *Pinus jeffreyi* was generally the least LT tolerant. During the first half of the winter, *Abies balsamea* appeared to be the least LT tolerant of the boreal species, as indicated by relatively high Y_{max} values, whereas after the cold period in late January and through the remainder of the winter, *Pinus sylvestris* had relatively high Y_{max} values and *Abies balsamea* became less LT sensitive.

All species began de-acclimating in early March, with Y_{max} and T_m approaching their summer values by late April. Daytime maximum temperatures remained above freezing through most of this period, with episodic night frost, with temperature falling on one night to about –8 °C.

LN_2-quench hardiness

The LN_2-quench hardiness depended directly on the temperature prior to quenching. None of the species survived quenching from –10 or –20 °C at any time, whereas the boreal species survived quenching from –30 or –40 °C, or both, during part or all of the winter period, with lower conductivity values being observed with a lower pre-quench temperature (Figure 2). *Picea obovata* survived LN_2 quenching from –30 °C by early

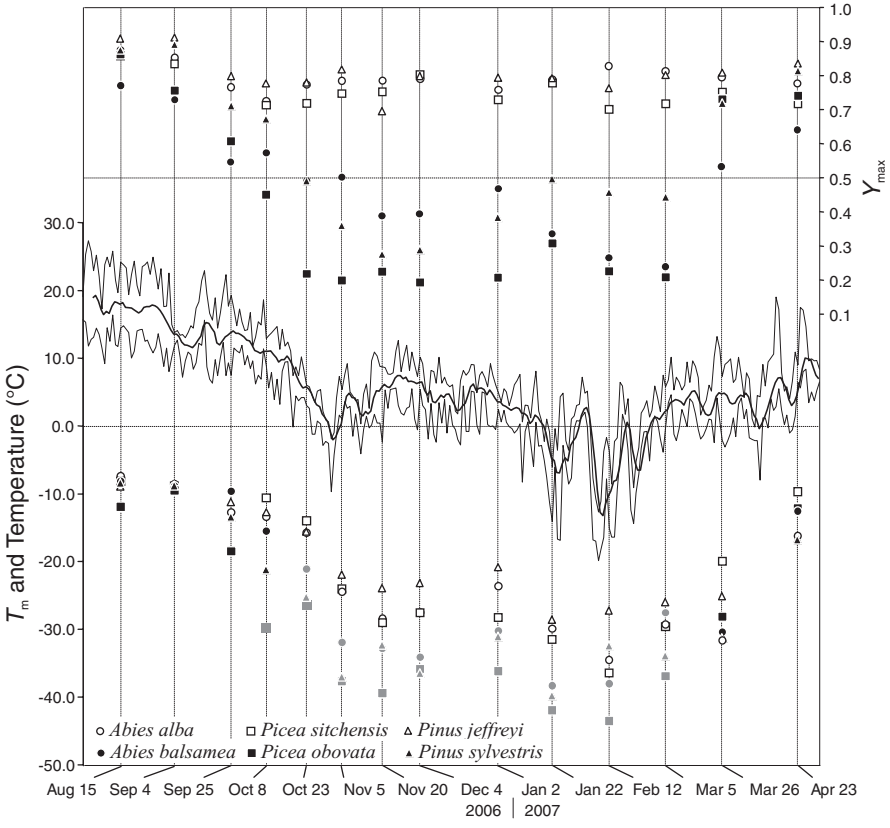

Figure 1. (Top) Maximum relative conductivity (Y_{max}) and (bottom) midpoint of the temperature response curve (T_m) in needles of six conifer species in the arboretum at the Ringve Botanical Garden, Trondheim, Norway, from August 2006 to April 2007. (Middle) minimum, maximum (thin lines), and 5-day mean (thick line) temperature at Værnes International Airport. Values of T_m and Y_{max} are means of three trees. The T_m is an estimate of LT_{50} except for the gray symbols, where $Y_{max} < 50\%$ indicates non-lethal injury at the lowest test temperature. Data for *Picea sitchensis* on September 25 are missing because the samples were lost.

November, with the other two boreal species surviving the same treatment two weeks later. *Pinus sylvestris* apparently lost some quench hardiness during the prolonged interval of mild weather in early winter; mean values of REL > 0.5 for LN_2 quenching from –30 °C indicated that leaves of this species were killed by this treatment by early January, and the trees never regained maximum quench hardiness even under colder conditions later in the winter. All three boreal species lost quench hardiness in March, concurrent with both above-freezing temperature and a decrease in tolerance to slow freezing (Figure 1).

Temperature response curves

The changes in LT tolerance illustrated in Figure 1 were accompanied by changes in the shape and position of the temperature response curves fit to the data for the three trees of each species (Figure 3). In general, all trees of a single species responded similarly, so that curves fitted to the three trees together often explained more than 90% of the variance in the temperature–REL relationship. Weaker fits occurred in some fully hardened boreal species where the overall mean REL was below 0.2, and in *Pinus sylvestris* on some dates where the responses of the individual trees differed markedly. Where variability or measurement error obscured the sigmoid response of individual trees, the sigmoid shape was usually clear in the three-tree composite data (Figure 4). These composite curves illustrate the different patterns of acclimation in the temperate versus boreal species. In the temperate species, acclimation resulted in a shift in T_m to a lower temperature, while the curves maintained a steep response region and a high Y_{max}, indicating complete mortality at the lowest test temperatures. Acclimation in the boreal species resulted in a decrease in both slope and Y_{max}, with T_m also shifting to lower temperatures. The decreased Y_{max} indicates that foliage of the boreal species was not killed at the lowest test temperatures, especially where $Y_{max} < 0.5$.

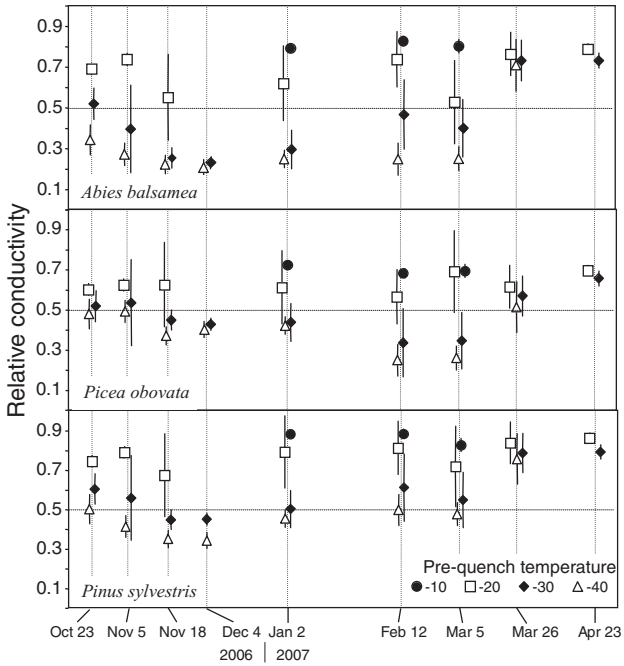

Figure 2. Relative electrolyte leakage in three boreal conifer species after slow freezing to –10, –20, –30 or –40 °C followed by quenching in liquid nitrogen. A 50% electrolyte leakage is the threshold for lethal injury. Values are means ± standard deviation of three trees. Some symbols have been offset by ± 1–2 days for clarity.

Sugar concentration

Needle sugar concentrations fluctuated during acclimation and throughout the winter, with no simple pattern evident in all species or uniquely to the temperate or boreal species groups (Figure 5). In all species, total sugar concentrations remained fairly constant or decreased during early acclimation through October 8, decreased during the next 2 weeks, then increased sharply in the following 2 weeks, between October 23 and November 5. This interval included the first frosts of the season

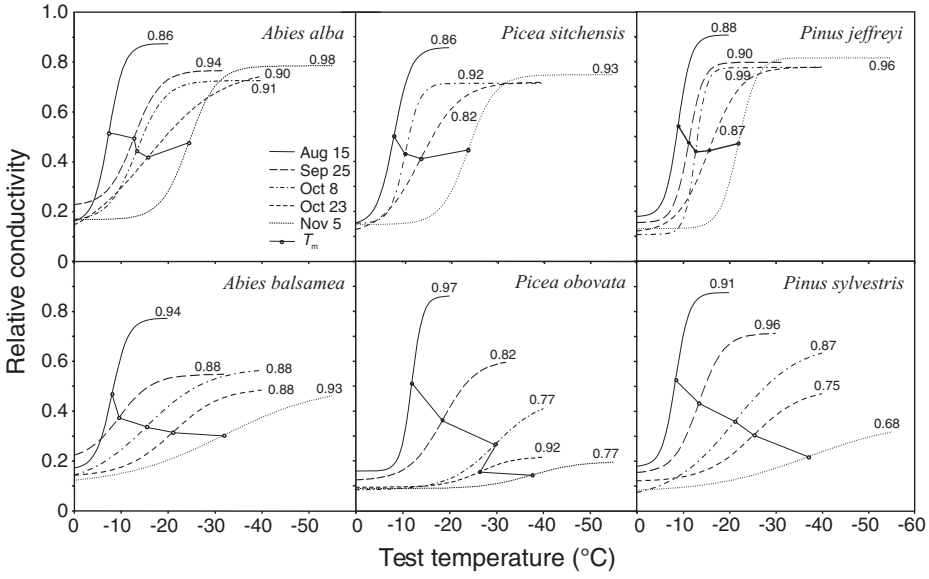

Figure 3. Temperature–relative electrolyte leakage curves for six conifer species during acclimation. Curves are fitted to the data for three trees of each species on each date. Model R^2 values are shown. Data for *Picea sitchensis* on September 25 are missing because the samples were lost.

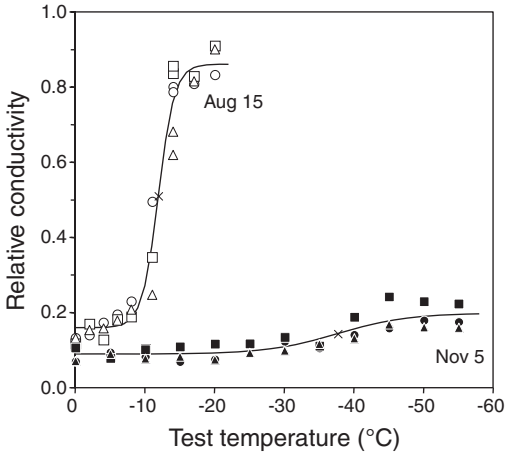

Figure 4. Temperature–relative electrolyte leakage data for *Picea obovata* before and after acclimation. Symbols represent means for three trees.

and a relatively steep decrease in T_m in all species (Figure 1). Total sugar concentrations decreased during the prolonged period of mild weather from late November through early January, then increased again in all species in late January, coincident with the return of subfreezing temperatures. Sugars remained relatively high or continued to increase during early de-acclimation in March, then dropped as LT tolerance approached summer values in late April. During the winter months, this overall pattern was driven largely by changes in sucrose and raffinose, the first and second most abundant of the five sugars measured, respectively.

Raffinose concentrations were highest in the two *Picea* species, followed by *Abies* and *Pinus*. It was essentially absent in all species on the first two sample dates, but appeared in measurable concentrations by October 8. Raffinose increased nearly continuously throughout the winter in all species except *Pinus jeffreyi* and *Picea obovata*, in which it reached maxima in early November and December, respectively. In *P. obovata*, raffinose decreased in December, then recovered to relatively high concentrations during the ensuing 2 months. Raffinose decreased in all species during de-acclimation. Measurable stachyose concentrations occurred in all species, but amounts greater than 2 mg g^{-1} were found only in the two *Picea* species and *Pinus jeffreyi*, where it followed similar trends to raffinose, that is a gradual increase in the *Picea* species and an early winter maximum in *P. jeffreyi*, and a decrease to values below 1 mg g^{-1} during de-acclimation.

Concentrations of fructose and glucose were relatively low in the *Abies* species and *Pinus sylvestris*. Fructose concentrations fluctuated up to about 25% of total sugar concentration in *Picea obovata* and *Pinus jeffreyi*. Glucose contributed as much as 30% of total sugar on most dates in *Picea sitchensis*, and increased in all species during de-acclimation in March. Only trace amounts of xylose were found in all species (data not shown).

Raffinose covaried strongly with both T_m and Y_{max} (Table 2), with significant group × T_m and genus × T_m interaction terms indicating that the boreal and temperate groups and the three genera differed in the slope of the T_m–raffinose relationship (Figure 6). There was also a significant linear relationship between Y_{max} and raffinose concentration, but the slope of this relationship did not vary significantly between groups and genera. Groups and genera differed significantly in overall mean raffinose concentration, above and beyond the LT-parameter effects. The significant linear relationship between stachyose and T_m differed among genera but not groups. The slopes of these relationships were negative, indicating that higher sugar concentrations were associated with greater LT tolerance, whereas the slope of the weaker but still significant Y_{max}–glucose relationship was positive. Mean concentrations of sucrose and glucose differed among genera and among genera within groups, but did not covary with the LT-tolerance parameters, and there were no strong relationships for fructose.

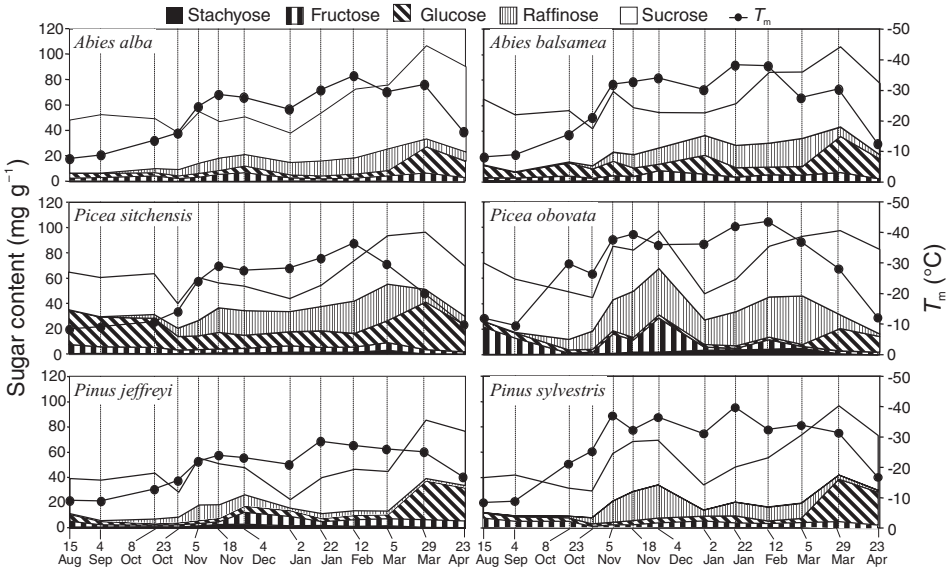

Figure 5. Dry-mass-based sugar concentrations and midpoint of the temperature response curve (T_m) values in needles of six conifer species from August 2006 to April 2007. Sugars are arranged in approximate order of abundance, and concentrations are cumulative, so that the top curve represents the total of the sugars measured. Temperature scale has been inverted to aid in interpretation.

Table 2. Probabilities from linear models using LT-tolerance parameters to predict sugar concentration, with group (boreal or temperate) and genus (*Abies*, *Picea* or *Pinus*) as additional factors. Significant *P* values ($\alpha = 0.01$) are in boldface.

Source	df	Raffinose	Stachyose	Sucrose	Glucose	Fructose
T_m	1	**< 0.0001**	**< 0.0001**	0.1857	0.8145	0.3739
Y_{max}	1	**< 0.0001**	0.3094	0.0994	**0.0036**	0.6698
$Y_{max} \times T_m$	1	**0.0012**	0.0144	0.4674	0.0155	0.0255
Group	1	**0.0004**	0.0424	0.0128	0.0453	0.8090
Group × T_m	1	**0.0003**	0.0291	0.6011	0.0155	0.0157
Group × Y_{max}	1	0.2616	0.5015	0.7485	0.4003	0.5925
Genus	2	**0.0002**	0.0130	**0.0001**	0.0339	0.0517
Genus × T_m	2	**< 0.0001**	**0.0014**	0.2400	0.1141	0.0643
Genus × Y_{max}	2	0.3014	0.1207	0.6351	0.0947	0.2086
Genus × Group	2	0.1519	0.1803	**0.0030**	**0.0042**	0.1149
Tree [Group, Genus]	12	0.0459	0.0015	0.6716	**0.0025**	0.4847
Model R^2		0.80	0.50	0.28	0.40	0.20

Discussion

Climate and acclimation

In recognition of the complexity of responses to LT stress, we discuss changes in T_m and Y_{max}, two parameters from the sigmoid curve-fitting procedure, representing the midpoint of the sigmoid curve and the maximum conductivity attained by slow freezing, respectively. Although most studies of plant LT tolerance rely on a single parameter (usually LT_{50} or equivalent) to describe relative tolerance, we have demonstrated more complex changes in responses to LT stress during acclimation. Similar changes have been observed in a few other species (Burr et al. 1990, Sutinen et al. 1992). Our comparative, high-resolution results point to fundamental differences in mechanisms of LT acclimation and LT tolerance in boreal and temperate conifer species.

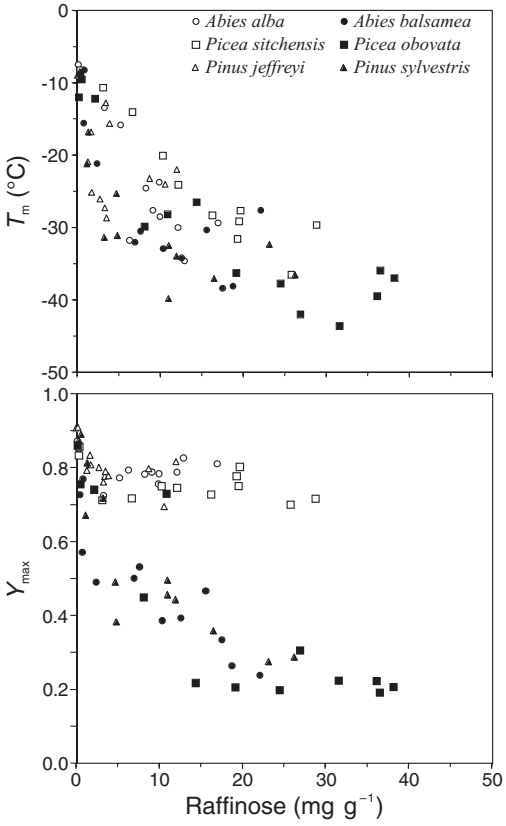

Figure 6. Midpoint of the temperature response curve (T_m; top) values, and maximum relative conductivity (Y_{max}; bottom) as functions of raffinose concentration in needles of six conifer species sampled before and during LT acclimation and through the winter and early spring of 2006–2007.

The decreases in Y_{max} and T_m between September 4 and 25 showed that all species began acclimating in September, as day length approached 12 h, regardless of climate or photoperiod in the species' home range. Numerous studies have shown that the onset of dormancy and the first stage of LT acclimation are triggered by short days in late summer (reviewed by Bigras et al. 2001). The early decrease in Y_{max} for *Abies balsamea* and *Picea obovata* may indicate that these boreal species, with main ranges centered on about 50 and 60° N, respectively, have longer day-length thresholds than the other species, as appropriate for the latitude and normal date of occurrence of the first freezing temperatures in their regions of origin.

All three boreal species acclimated more rapidly than their temperate counterparts, becoming at least partially tolerant to LN_2 quenching from –40 °C as early as September 25, and from –30 °C by mid-November. During this same period, LT_{50} of the temperate species decreased to the –20 to –30 °C range. The T_m of all species decreased after the first severe frosts of the year in late October, but the boreal species had already attained a high degree of LT tolerance, in some cases including at least partial LN_2-quench tolerance, in the absence of freezing (Figures 1 and 2), indicating that freezing may not be required for extreme, if not maximal, LT acclimation in these species. This conclusion is in agreement with the results of controlled environment studies indicating that night frost increases LT tolerance in temperate species such as *Thuja plicata* Donn ex D. Don, *Chamaecyparis nootkatensis* D. Don

and *Pinus radiata* D. Don, whereas boreal species such as *Picea glauca* (Moench) Voss acclimate rapidly in response to increased night length and chilling temperatures with no apparent freezing requirement (Greer and Warrington 1982, Silim and Lavender 1994). This rigid acclimation response may involve a tradeoff between rapid and timely acclimation versus the ability to fix carbon and thereby minimize respiratory energy losses during mild weather (Hawkins et al. 1995, Schaberg et al. 1995, Ögren 1997, Schaberg 2000), which could ultimately affect the growth and competitive abilities of boreal species under conditions of global warming (Busch et al. 2007). Many boreal species, including *Picea obovata* and *Pinus sylvestris*, are growing well in the Ringve botanical garden, indicating that, at least in noncompetitive situations, the mild local climate does not strongly affect the growth of these species. However, all the *Abies balsamea* trees in the collection have multiple leaders and dead branches, and some of the trees have died and been removed, so they seem susceptible to insect attack.

In both the temperate and boreal species, T_m responded to temperature, generally remaining stable or increasing slightly during periods of mild weather, and decreasing during or after prolonged periods with temperatures below 0 °C (Figure 1). In the temperate species, Y_{max} remained high, indicating 100% cell mortality after slow freezing, regardless of acclimation status. There was some fluctuation in Y_{max} in the boreal species that seemed largely independent of changes in T_m and environmental temperature. Thus T_m and Y_{max} are at least partly independent, suggesting that they represent different processes involved in LT acclimation, as discussed below.

Sugars

Our results complement those of numerous studies showing an association between sugar accumulation or concentration, especially of oligosaccharides, and LT acclimation or tolerance in conifers (e.g., Parker 1959, Hinesley et al. 1992, Ögren 1997, Schaberg et al. 1999, 2000). The strong statistical relationships between raffinose and the LT-tolerance parameters (Table 2, Figure 6) point to functional roles in maintaining membrane integrity during freezing and thawing. The significant interactions between groups and LT tolerance suggests that raffinose may function differently or take on additional roles in the extreme LT tolerance found in boreal species. Although significant amounts of stachyose were not found in all species, our results suggest that stachyose plays a role in LT tolerance in those species that do accumulate stachyose.

Some of the fluctuations in sugar concentration corresponded to fluctuations in environmental temperature, as reported by others (Hinesley et al. 1992). In our study, the relatively sharp increase in sucrose and raffinose after the first frost indicates that low temperature may stimulate sugar accumulation through changes in production, export or interconversions. The decreases in sucrose in late summer and during the early winter period of mild weather may be due to respiratory depletion, as observed in *Pinus* and *Picea* seedlings (Ögren 1997, Ögren et al. 1997), or phloem export. After the second decrease, the sucrose concentration increased again with the return of freezing temperatures, suggesting some mechanism for replenishing sugars, perhaps even in the frozen state as reported in *Salix* species (Sakai 1966).

Mechanism

The different patterns of acclimation in temperate and boreal species shown in Figure 3 indicate different but overlapping mechanisms of LT acclimation. The reduction in T_m in all species indicates a lowering of the temperature threshold for some event that is critical in the progress of cellular injury resulting in electrolyte leakage, such as a temperature- and dehydration-dependent liquid crystal to gel transition or other phase change in cell membranes (Steponkus 1984). This shift can be achieved by fatty acid desaturation or other changes in lipid composition, as has been documented in many species, including some conifers (Senser 1982, Martz et al. 2006), although the role of changes in lipid composition in the development of extreme LT tolerance has not been explored.

The decrease in Y_{max} found only in the boreal species may indicate that there is another protective mechanism that prevents a membrane phase change or, more generally, freeze dehydration effects on membranes and macromolecules, from being lethal to the cell. One strong possibility is that intracellular vitrification at low temperature acts to prevent further dehydration and deleterious interactions between membranes or macromolecules, or both. The theory of vitrification as a mechanism of extreme LT and desiccation tolerance is well supported (Wolfe and Bryant 1999) and is applied in cryopreservation (Fahy et al. 1987). In agreement with previous work (Strimbeck et al. 2007), we have shown that extremely LT-tolerant tissue can survive LN_2 quenching from –30 but not –20 °C, an observation consistent with the prediction that an intracellular glass transition occurs at some temperature in this range. Sakai reported similar results for needles of *Pinus banksiana* Lamb., and survival of bud tissues of boreal *Larix*, *Betula*, *Populus* and *Salix* species after LN_2 quenching from temperatures as high as –15 °C (Sakai 1966, Sakai and Weiser 1973).

In addition to direct cryoprotective effects, sugars may have a role in intracellular vitrification. Freeze-concentrated sucrose solutions vitrify readily at a minimum temperature of about –41 °C (Goff et al. 2003). Raffinose and stachyose are also excellent glass formers, with glass transition temperatures as much as 40 °C higher than sucrose at low levels of hydration (Buitink et al. 2000). Complex mixtures of sucrose, oligosaccharides, and other polymers, including protective proteins such as dehydrins or other LEA proteins (Allagulova et al. 2003), could raise glass transition temperatures into the –20 to –30 °C range (Hirsh 1987). We suggest that the boreal species, by some combination of sugars, other compatible solutes and proteins associated with LT or desiccation tolerance, raise the intracellular vitrification temperature above T_m as controlled by membrane or other properties, thereby achieving extreme LT tolerance. We have archived whole needle and RNA samples of *Picea obovata* and *Pinus sylvestris* over the entire time series and are studying these to identify proteins that accumulate during acclimation. We further plan to assay the cryopro-

tective effects and study glass transitions in solutions of key proteins alone and in combination with sugars.

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