22 Going to Extremes: Low-temperature Tolerance and Acclimation in Temperate and Boreal Conifers

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Introduction

Despite global warming, temperatures in the continental interiors of Canada and Siberia can still fall below −60°C and can remain below −40°C for weeks at a time (Latysheva *et al.*, 2007). These extreme temperatures occur not in barren tundra regions, but taiga forests dominated by species of spruce (*Picea*), fir (*Abies*), pine (*Pinus*) and larch (*Larix*). While other plant and animal species may receive some protection from snow cover, the above-ground parts of trees, including the foliage of evergreen trees, must survive the full brunt of the winter environment. Sakai (Sakai, 1960; Sakai and Weiser, 1973) first showed that the stems, buds and evergreen needles of various boreal conifers and other woody species can survive immersion in liquid nitrogen (LN_2) at −196°C, provided they are first slowly cooled to an intermediate temperature, usually in the range from −15 to −40°C. For all practical purposes, these plants have achieved absolute low-temperature (LT) tolerance, far beyond that of most crop species, fruit trees and model species that have (for good reasons) been the focus of the majority of studies of plant LT tolerance. Many temperate-zone conifer species, including *Picea*, *Abies* and *Pinus* species, reach their lower limit of LT tolerance in the −20 to −50°C range. A fundamental understanding of the cryoprotective mechanisms of extreme LT tolerance and the acclimation process in boreal species may be of value in improving the LT tolerance of less hardy species, in developing technologies for the cryopreservation of food, drugs, cells and whole organs, and in predicting the effects of global warming on boreal forest ecosystems.

The Ringve 'Experiment'

The Ringve Botanical Garden in Trondheim, Norway, is owned and maintained by the Trondheim Natural History Museum, a branch of the Norwegian University of Science and Technology. It includes a small arboretum with 52 conifer and about 70 deciduous tree and shrub species from temperate and boreal regions around the northern hemisphere. These were planted shortly after the founding of the arboretum in 1975, with many accessions from other plantings in Norway, so that the ultimate seed source of many species and individual trees is, unfortunately, not known. Nevertheless, under the assumption that all genotypes can be traced to somewhere within the original range of each species, the collection presents an opportunity for comparative studies of LT tolerance and acclimation in conifers growing in a common environment, albeit one with a relatively mild winter climate. We hope that these comparative studies will

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help us to identify and isolate biochemical components and biophysical processes that contribute to extreme LT tolerance.

The arboretum is located just a few hundred metres from Trondheimsfjord and, like most of coastal Norway, the local climate is moderated by the North Atlantic Drift, a poleward-flowing branch of the Gulf Stream. The local climate is classified as southern boreal and moderately oceanic in the Norwegian system (Moen, 1999) or more generally as temperate oceanic in global climate classifications (Trewartha and Horn, 1971). Despite a latitude of 63°25′N, in December to February mean temperatures are in the range of −2 to −3°C, with (increasingly) frequent periods of mild (temperature >0°C) weather throughout the winter, and minimum temperatures are about −20°C, although these have become less common in recent years.

We compared midwinter LT responses of foliage from several species in each of the genera *Abies*, *Picea* and *Pinus*, and profiled LT acclimation and deacclimation in a boreal–temperate species pair in each genus. Our methods included controlled freezing to temperatures ranging from 0 to −80°C at relatively slow rates, quenching in LN_2 after slow cooling to intermediate temperatures, and assessment of injury by the well-established relative electrolyte leakage (REL) method or an image analysis method for quantifying visible symptoms of LT stress and injury (Strimbeck *et al.*, 2007). We also measured concentrations of mono-, di- and oligosaccharides in many of our samples, and used differential scanning calorimetry (DSC) to look for evidence of glass transitions in conifer needle tissue. Here we review our results along with some of the pertinent literature to develop a composite picture of the patterns of LT tolerance and acclimation in conifers, with some discussion of possible mechanisms.

Midwinter Low-temperature Tolerance

Much of the current understanding of LT tolerance in conifers dates to the exhaustive work of Akira Sakai, who identified the minimum survival temperature in 10°C classes for twig, bud and needle tissues of nearly 150 conifer species, mainly temperate and boreal species and varieties from the northern hemisphere (Sakai and Okada, 1971; Sakai and Weiser, 1973; Sakai, 1983) but also including some species from warmer regions and the southern hemisphere. Conifers have also been approximately grouped into US Department of Agriculture hardiness zones based on their natural distributions (Bannister and Neuner, 2001). While these data give an excellent 'big picture' of the overall range of LT tolerance among conifers, it does not shed much light on differences in the LT responses of different species or groups, which might help differentiate mechanisms involved in extreme LT tolerance.

Using material collected at the Ringve arboretum, we compared the midwinter LT responses of needles from eight conifer species in each of the genera *Picea*, *Abies* and *Pinus* (Strimbeck *et al.*, 2007). We used a non-linear procedure to fit symmetrical, sigmoid curves to electrolyte leakage data for each species (Fig. 22.1), using four parameters to describe the shape of the curve: (i) Y_{min}, the baseline or uninjured conductivity; (ii) Y_{max}, the maximum conductivity produced by slow freezing (−0.1°C/min); (iii) k, a measure of the steepness of the curve in the response region between Y_{min} and Y_{max}; and (iv) T_m, the midpoint of the curve. As in many studies involving deeply LT-tolerant species, relative tolerance cannot always be expressed as a simple LT_{50} if slow freezing never results in 100%, or in some cases even 50%, injury; however, T_m can be interpreted as an LT_{50} if Y_{max} corresponds to 100% lethal injury. The ratio of T_m to Y_{max} provides a composite index of relative LT tolerance that includes both a measure of response to LT stress (T_m) and a measure of maximum LT injury (Y_{max}). This index is especially useful in distinguishing the extremely tolerant boreal species, which have low but variable Y_{max} values.

In general, species originating in temperate oceanic climates or warm temperate mountains with sporadic frost had relatively high Y_{max} and T_m values, with slow freezing producing lethal injury generally in the −20 to −40°C range (Table 22.1). Species from boreal regions generally had lower T_m and Y_{max}, indicating not only that lower temperatures are required to stress or injure the needles, but that injury is incomplete even at −80°C. Species from

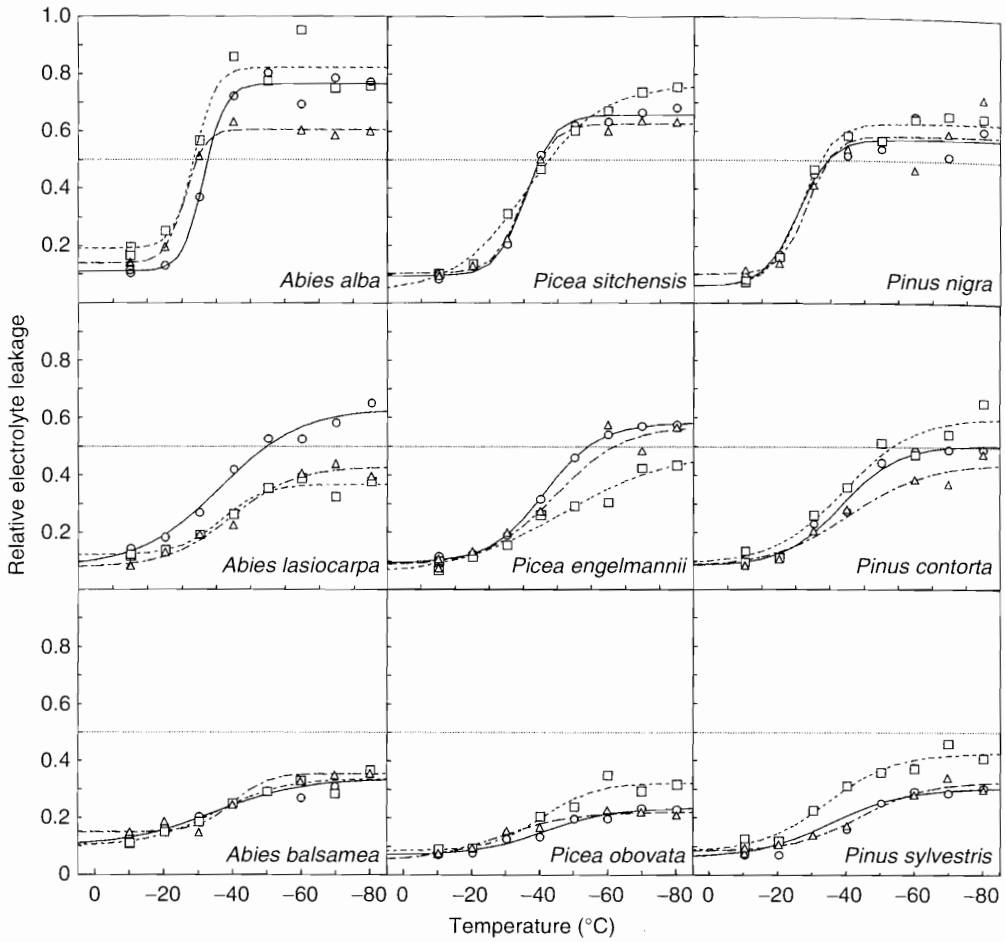

Fig. 22.1. Examples of temperature–relative electrolyte leakage curves for oceanic or warm temperate (top), temperate continental and montane (middle) and boreal (bottom) conifer species. The different symbols and lines represent three different trees of each species.

temperate continental climates showed intermediate responses. The T_m/Y_{max} ratio was generally >−60 for temperate oceanic and warm temperate montane species, −60 to −100 for continental species, and <−100 for boreal species. Thus, the LT tolerance of various conifer species correlates reasonably well with the climate in their region of origin, especially when elevation is taken into account, even when they are grown under common and relatively mild climatic conditions. Exceptions to this pattern include species with boreal (*Pinus banksiana*, *Abies sibirica*) and continental distributions (*Picea jezoensis*, *Picea rubens*) that are more LT-sensitive than expected, and this may be related to the ecological history of the species, seed source of trees in the collection, or specific responses to the local climate and growing conditions.

Quantification of Visible Symptoms

One of the problems with using REL as a response variable is that it leaves some uncertainty as to how it correlates with specific symptoms of LT injury, such as the orange-brown necrotic coloration characteristic of

Table 22.1. Original range, climate region and low-temperature tolerance parameters for foliage of 24 conifer species collected at Ringve Botanical Garden, Trondheim, Norway, on 13 February 2006.

Species	Main range (continent[a])	Elevation (m)	Regional climate[b]	T_m (°C)	Y_{max}	T_m/Y_{max}
Abies alba	Alps & Pyrenees (EU)	300–1950	Do, Dca	–28.8	0.73	–39.7
Abies procera	Pacific north-west mountains (NA)	60–2700	Do (H)	–32.4	0.68	–47.6
Abies veitchii	Mountains, central Japan (AS)	1600–1900	Dca (Cf)	–32.6	0.55	–59.6
Abies amabilis	Pacific north-west coast (NA)	1000–2300	Do	–37.7	0.63	–59.7
Abies sibirica	Siberia (AS)	?–2400	E (Dcb)	–30.6	0.45	–69.3
Abies homolepis	Mountains, southern Japan (AS)	700–2200	Cf	–33.6	0.47	–72.2
Abies lasiocarpa s.l.[c]	Rocky Mountains (NA)	600–3700	BSk (H)	–37.4	0.48	–83.0
Abies balsamea	Eastern Canada NA)	0–1700	E, Dcb	–36.7	0.34	–107.1
Picea jezoensis	Japan and adjacent mainland (AS)	40–1000	Dcb, Dca, E	–32.7	0.70	–46.5
Picea sitchensis	Pacific north-west coast (NA)	0–1000	Do	–35.7	0.68	–52.7
Picea rubens	Appalachian Mountains and north-east coast (NA)	0–2000	Dcb	–38.3	0.69	–56.1
Picea engelmanni	Rocky Mountains (NA)	1000–3000	BSk (H)	–44.0	0.54	–81.7
Picea omorika	Balkans (EU)	400–1700	Dca	–43.8	0.53	–83.4
Picea abies	Northern Europe (EU)	0–2200	E, Dcb	–47.9	0.43	–112.7
Picea glauca	Canada (NA)	0–2100	E	–41.0	0.31	–136.1
Picea obovata	Siberia (AS)		E	–37.0	0.26	–144.6
Pinus jeffreyi	Sierra Nevada (NA)	2000–3100	Cs (H)	–30.4	0.68	–45.0
Pinus nigra	Mountains in Mediterranean region (EU)	200–2000	Cs, Dca	–26.8	0.60	–44.9
Pinus banksiana	Eastern Canada (NA)	0–800	E, Dcb	–27.3	0.55	–50.5
Pinus strobus	North-east US and south-east Canada (NA)	0–1500	Dcb, Dca	–36.6	0.49	–81.1
Pinus contorta	Rocky Mountains & Pacific north-west coast (NA)	0–3500	BSk (H), Do	–39.5	0.52	–78.0
Pinus koraiensis	Korea and Japan (AS)	600–1800	Dca	–35.2	0.46	–77.5
Pinus sylvestris	Northern Europe to Siberia (EU, AS)	0–1000	E	–38.1	0.35	–111.5
Pinus cembra	Alps and Carpathians (EU)	1300–2400	Dca	–42.9	0.37	–118.0

[a]AS, Asia; EU, Europe; NA, North America.

[b]Climate in original range estimated from global map in Trewartha and Horn (1971). E, boreal; Dc, temperate continental; Do, temperate oceanic; Cf, subtropical humid; Cs, subtropical dry summer (Mediterranean); BS, steppe or semi-arid; (H), montane, follows climate of surrounding region.

[c]*Abies lasiocarpa* s.s. includes only populations in coastal mountain ranges; Rocky Mountain populations have been segregated as *Abies bifolia* (Earle, 1997–2008). The provenance of the trees in the arboretum is unknown, so they might have come from either region.

LT-injured needles under field conditions (Friedland *et al.*, 1984). Exposing whole shoots to LT stress, then placing them under bright light in cool conditions for about two weeks gives symptoms of injury very similar to those observed in the field. We scanned samples of needle sections after development of injury symptoms and used an image analysis procedure to quantify the relative amounts of necrotic (red-orange), chlorotic (yellow) and healthy green tissue in boreal–temperate pairs of *Abies*, *Picea* and *Pinus* species (Strimbeck *et al.*, 2007). In agreement with the REL results, the temperate species developed up to 100% necrosis after LT stress at temperatures ranging from −40 to −60°C (Fig. 22.2). Some samples from the temperate species maintained up to 40% chlorosis even when severely injured; we think this is because these samples desiccated rapidly so that the tissue died and dried out before necrosis was complete. In temperate species, chlorosis is also an intermediate stress symptom, appearing after exposure to temperatures 5 to 10°C warmer than those resulting in necrosis. In boreal species, LT treatment produced up to 30% chlorosis but little necrosis. Winter chlorosis in conifer foliage is thought to be a result of the destruction of chlorophyll by a combination of LT and bright light, and appears to be reversible as chlorotic needles can regain a normal green colour in the spring (Baronius *et al.*, 1991).

We also measured REL in sub-samples of the shoots used to evaluate visible symptoms, giving paired REL and colour measurements for these samples. While there was no straightforward correlation between relative conductivity and chlorosis or necrosis across all six species, necrosis occurred only in temperate species at REL values above 0.5 (Fig. 22.3),

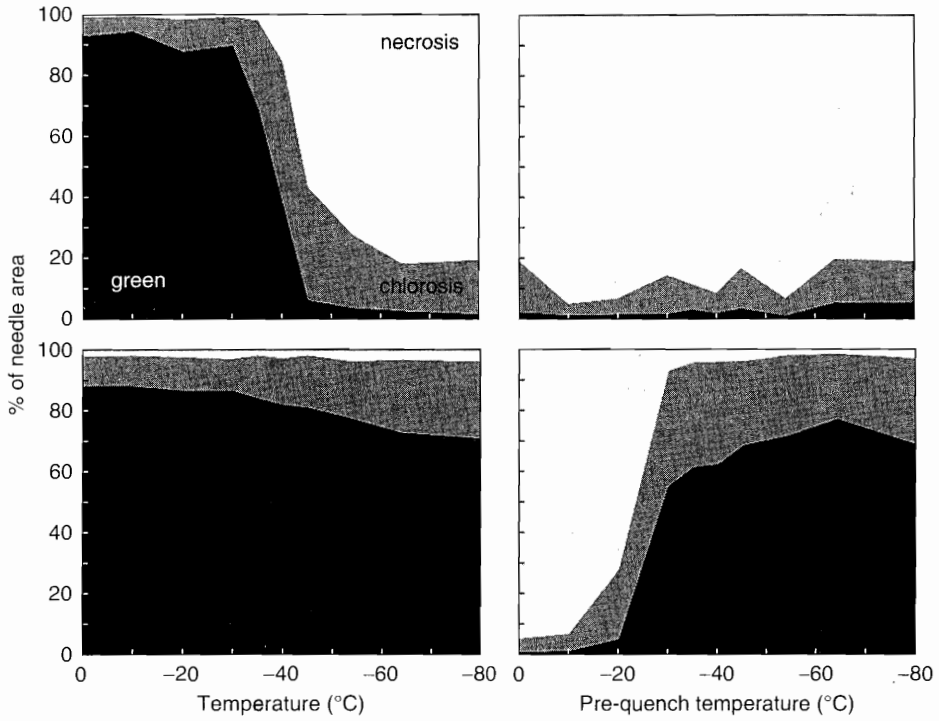

Fig. 22.2. Visible low-temperature stress and injury symptoms in *Abies alba* (top), a species from warm-temperate region mountains, and *Abies balsamea* (bottom), a boreal species, after slow freezing (left) and slow freezing followed by quenching in liquid nitrogen (right). Samples were collected at Ringve Botanical Garden, Trondheim, Norway (63°25′N), 13 March 2006.

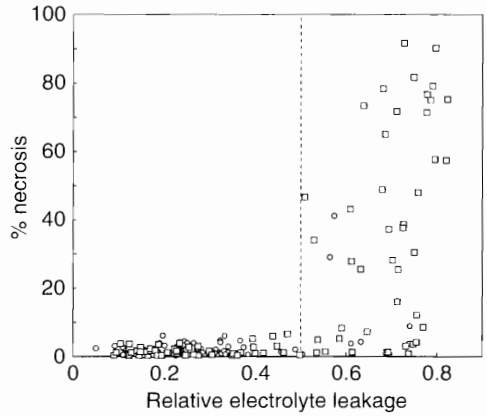

Fig. 22.3. Relative electrolyte leakage and necrosis in needles of boreal (○) and temperate (□) *Abies*, *Picea* and *Pinus* species after slow cooling to temperatures from 0 to −80°C.

giving a minimum value of REL associated with the development of necrosis, the main visible symptom of lethal injury. We now use an REL of 0.5 as a threshold value for determining if a sample is lethally stressed by LT treatment, and where Y_{max}>0.5 we refer to the corresponding T_m as LT_{50}, an estimate of the temperature giving 50% cell death. As REL of foliage from most boreal and some temperate continental and montane species does not exceed this value at temperatures as low as −80°C, we conclude that they can survive these extreme low temperatures.

Liquid Nitrogen Quench Tolerance

Sakai (1960) first showed that twig and bud tissues of boreal willows could survive quenching in LN_2 at −196°C provided they are first slowly cooled to some intermediate temperature in the −15 to −30°C range. He later showed that tissues of boreal conifers can survive similar treatment, and suggested that the highest pre-quench temperature that a tissue can survive be used as an index of relative LT tolerance in extremely hardy species (Sakai and Okada, 1971; Sakai and Weiser, 1973; Sakai, 1983).

To confirm and build on these results, we quenched whole shoots of six boreal and temperate conifer species in LN_2 after slow cooling to temperatures ranging from 0 to −80°C, and measured both REL and visible symptoms (Strimbeck *et al.*, 2007). The temperate species were always lethally injured by LN_2 quenching, with REL values in the same range as Y_{max} for the same species, and developed a mixture of chlorosis and necrosis comprising usually 90% or more of needle area (Fig. 22.2). In contrast, boreal species were lethally injured by LN_2 quenching from temperatures between 0 and −20°C, but maintained REL values <0.5 and a mixture of chlorosis and green tissue with <5% necrosis when quenched from −30°C or lower. Our results confirm that the foliage of boreal conifers can survive LN_2 quenching if it is first cooled to −30°C, and we can conclude that the cells undergo a transition during slow cooling between −20 and −30°C that confers the capacity to survive further slow cooling and LN_2 quenching.

Acclimation Patterns

The acclimation process has been studied in various conifer species in both natural and controlled environments (reviewed in Bigras *et al.*, 2001). Acclimation in conifers follows many of the general patterns established for other species. Early acclimation is triggered by a critical night length, and reinforced by chilling temperatures. It involves desaturation of fatty acids and changes in membrane lipid composition (e.g. Senser, 1982; Martz *et al.*, 2006), and accumulation of sucrose and oligosaccharides (e.g. Parker, 1959; Hinesley *et al.*, 1992; Schaberg *et al.*, 2000).

We used our REL method to profile acclimation and deacclimation in boreal–temperate pairs of *Abies*, *Picea* and *Pinus* species growing in the Ringve Botanical Garden from August 2006 to April 2007 (Strimbeck *et al.*, 2008). Figure 22.4 shows the results for the Siberian species *Picea obovata* and the temperate rainforest species *Picea sitchensis*. In August, all six species had Y_{max} values >0.75 and LT_{50} values around −10°C. Despite the relatively mild winter climate of Trondheim and unusually warm conditions in early winter, the three boreal species acclimated rapidly, becoming tolerant of temperatures below

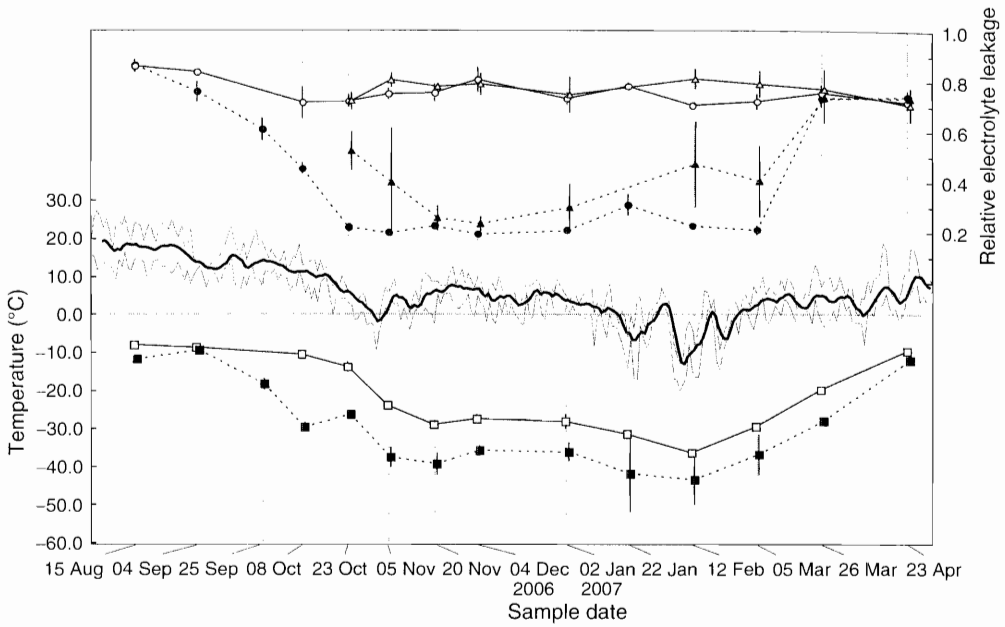

Fig. 22.4. Y_{max} (●, ○), relative electrolyte leakage after quenching in liquid nitrogen (LN_2) from −30 °C (▲, △) and T_m (■, □) of foliage of *Picea sitchensis* (○, △, □) and *Picea obovata* (●, ▲, ■) growing in the Ringve Botanical Garden during acclimation and deacclimation. Maximum and minimum (-----) and 5-day mean air temperature (——) at Værnes International Airport (Trondheim) in the middle. Error bars are standard errors for parameters of non-linear curves fitted to three trees of each species (Y_{max} and T_m) or standard deviations for three trees (LN_2 quenching). Some symbols are offset by +1 day for clarity.

−40°C by late October, with only one night where the temperature dropped a few tenths of a degree below freezing, probably not low enough to induce freezing in the plant. By late November, foliage from these species survived LN_2 quenching from −30°C, although daytime temperatures remained above freezing with only sporadic night frost for most of the month. The temperate species acclimated only to around −15°C by late October, then more rapidly to temperatures in the −20 to −30°C range by late November, where they remained throughout the mild early winter period. They reached minimum LT_{50} ranging from about −28 to −36°C following a period of lower temperatures in late January and early February. Although temperatures were similar to those in November, all species deacclimated during March and April.

These results suggest that acclimation in boreal species follows a rigid program that is relatively unaffected by environmental temperatures, while temperate species maintain some responsiveness to temperature throughout the winter. It is interesting to note, however, the parallel course of T_m values in both *Picea* species over the midwinter period (Fig. 22.4); the changes in Y_{max} are the dominant factor in the differences between the two species.

Changes in temperature response curves during acclimation reveal different styles of acclimation in the boreal and temperate species (Fig. 22.5). In temperate species, acclimation can be described as 'parallel slope retreat', a shift of the sigmoid curve towards a low temperature while it maintains its steepness and amplitude. In these species, T_m can always be interpreted as LT_{50}, and this parameter alone is more or less adequate to describe the changes in LT tolerance. Acclimation in boreal species can be described as 'slope decline', where there is both a shift of T_m towards lower temperatures and a decrease in maximum REL (Y_{max}) until it falls below the 0.5 REL

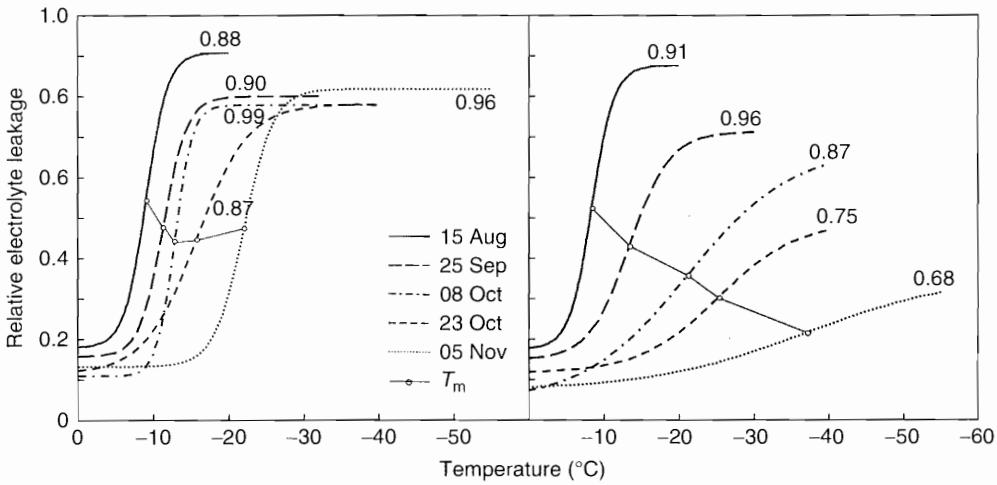

Fig. 22.5. Temperature–relative electrolyte leakage curves for temperate (*Pinus jeffreyi*, left) and boreal (*Pinus sylvestris*, right) *Pinus* species over five sample dates during acclimation. Curves fit to three trees of each species, values are model R^2.

threshold we use to distinguish lethal LT stress. This also involves a decrease in slope characterized by a third parameter, k, but T_m and Y_{max} together give an adequate picture of the changes in LT tolerance in extremely hardy species. In both groups, T_m drops after periods of temperatures below 0°C and may increase slightly during prolonged mild periods, and thus seems somewhat responsive to environmental temperature.

These parameters may represent different mechanisms of LT tolerance. T_m is the midpoint of some process that results in an increase in electrolyte leakage. One explanation for this would be a temperature-dependent phase change in cell membranes, with the transition temperature depending on membrane lipid composition, another factor known to change during LT acclimation. Fatty acid desaturation and changes in lipid composition that occur during acclimation can affect membrane phase change temperatures and stability during freezing stress (Steponkus, 1984; Uemura and Steponkus, 1999). Regardless of the actual mechanism, the transition represented by T_m is ultimately lethal in temperate species, but a controllable stress response in boreal species. Thus, the lowering of Y_{max} may represent a different process that prevents lethal injury.

Sugars and Low-temperature Tolerance

Sugar accumulation is closely associated with LT tolerance in numerous plant species, including conifers, and *in vitro* studies show that sugars have cryoprotective effects on proteins and membranes (Arakawa and Timasheff, 1982; Caffrey *et al.*, 1988; Leborgne *et al.*, 1995; Santarius and Franks, 1998). While sucrose is typically the most abundant sugar in acclimated plants, raffinose is also common, especially in conifers (Little, 1970; Hinesley *et al.*, 1992; Schaberg *et al.*, 1999), and smaller amounts of stachyose may also appear during acclimation.

We measured dry weight concentrations of sucrose, raffinose, stachyose, glucose, fructose and xylose in foliar samples drawn from the same bulk samples we used in our LT tolerance testing in both the midwinter 24 species comparison and the seasonal profile of six boreal and temperate species. In the midwinter study, we found significant correlations between T_m and sucrose (R^2=0.35, P=0.042, n=12) and raffinose (R^2=0.46, P=0.015, n=12) in a subgroup of relatively LT-sensitive temperate and continental species (defined by Y_{max}>0.5), but no significant relationship in the remaining continental and boreal species, where T_m is an

incomplete descriptor of LT tolerance (Strimbeck *et al.*, 2007). Thus, the relationships between LT tolerance and sugars found within species also hold across species, despite likely variation in the proportion of dry weight invested in cuticle and cell walls in different genera and species, which may affect the sugar/dry weight ratio.

Studies of seasonal variation in sugar content in conifers have shown both sustained winter increases in montane populations of *Picea rubens* (Schaberg *et al.*, 2000) and fluctuations that correlated with environmental temperature in paired cold- and warm-temperate *Pinus* and Cupressaceae species (Hinesley *et al.*, 1992). In the latter study, raffinose was again singled out as closely associated with LT tolerance. In our seasonal study (Strimbeck *et al.*, 2008), total sugar concentrations, driven principally by sucrose, fluctuated dynamically throughout the measurement period, with increases generally following periods of freezing temperatures and gradual decreases during prolonged periods of mild weather (Fig. 22.6). Decreases in sucrose may be due to some combination of respiratory

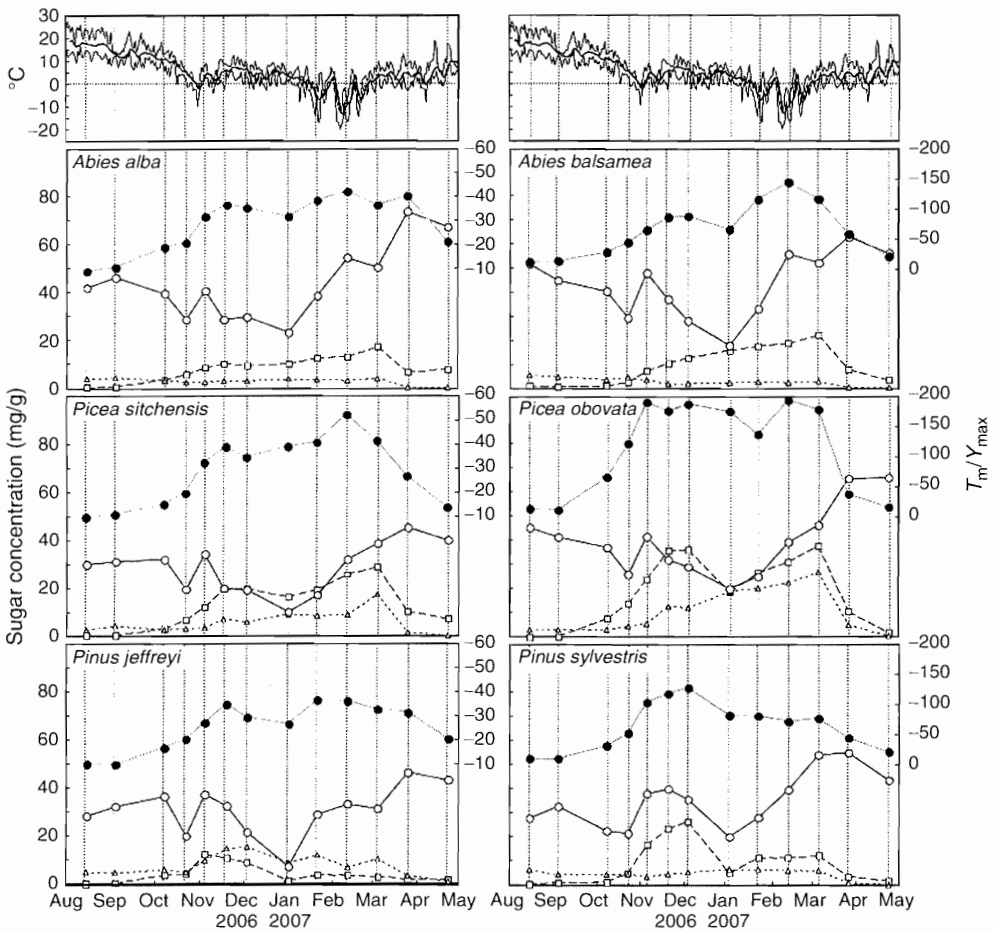

Fig. 22.6. Top panels: daily maximum and minimum temperature (-----) and 5-day running mean (——) at Værnes International Airport (Trondheim). Other panels: T_m/Y_{max} (●) and concentrations of sucrose (○), raffinose (□) and stachyose ×5 (Δ). T_m and Y_{max} from non-linear curves fit to relative electrolyte leakage data for three trees of each species, sugar concentrations are mean of three trees. X-axis tick marks on the first day of each month.

depletion, phloem export or perhaps conversion to starch, while increases may depend on the reverse processes. Raffinose and, in some species, stachyose first appeared in significant quantities early in the acclimati on process, increased monotonically through the winter, and decreased rapidly during deacclimation. Glucose and fructose were also found in variable quantities throughout the winter, with glucose increasing temporarily in all species during deacclimation (data not shown). There were no clear patterns in total sugar fluctuations unique to the boreal and temperate species groups.

As in previous analyses, simple correlations between LT tolerance parameters and sugars suggest a key role for raffinose in LT tolerance in both the boreal and temperate groups (Table 22.2). Correlations between raffinose and both T_m and Y_{max} are negative, indicating that higher sugar concentrations are associated with lower midpoint temperatures and lower maximum REL values in both groups. Over all dates, raffinose concentrations are significantly higher in boreal than temperate species as indicated by a significant main effect of group in analysis of variance (Strimbeck *et al.*, 2008). Stachyose also correlates significantly and negatively with Y_{max} in the boreal group and T_m in both groups. Overall stachyose concentrations are higher in the boreal than the temperate *Picea* species, but higher in *Pinus jeffreyi* than *Pinus sylvestris*, so there is no clear association of this sugar with extreme LT tolerance. Despite its association with LT tolerance in our and others' previous studies, sucrose correlates weakly with LT tolerance variables in the seasonal study. Sugar concentrations, notably stachyose and raffinose, are intercorrelated, including significant negative correlations between glucose and raffinose and stachyose in the boreal group, making multiple regression models of LT tolerance parameters on sugar concentrations unstable.

Cytoplasmic Vitrification

Intracellular vitrification has long been implicated as a general mechanism of extreme LT and desiccation tolerance in plant tissues (Burke, 1985; Hirsh, 1987). According to this model, cytoplasm goes through a glass transition as a result of dehydration (in desiccation-tolerant organisms) or a combination of LT and dehydration in frozen tissues. Dehydration concentrates sugars and other cellular components, and both processes result in increased cytoplasmic viscosity and ultimately in a complete loss of rotational and translational molecular mobility, so that all molecules are more or less locked in place in an amorphous glassy matrix. This will slow further dehydration by several orders of magnitude, and prevent deleterious interactions between proteins, membranes and other cell components. Effectively, the glassy state is a kind of molecular suspended animation.

Table 22.2. *R* values of correlations among low-temperature tolerance parameters and sugar concentrations for the boreal and temperate species, based on measurements of data for nine trees in each group on 13 sample dates. T_m and Y_{max} for some trees in the boreal species group could not be determined on some dates due to poor curve fits.

Temperate species (n=117)	Boreal species (n=106 for T_m and Y_{max}, n=117 between sugars)						
	T_m	Y_{max}	Stachyose	Raffinose	Sucrose	Glucose	Fructose
T_m		0.704***	−0.272**	−0.677***	0.092	0.153	0.039
Y_{max}	0.283**		−0.377***	−0.819***	0.187*	0.342***	−0.022
Stachyose	−0.263**	−0.151		0.576***	−0.195*	−0.373***	−0.047
Raffinose	−0.687***	−0.414***	0.339***		−0.094	−0.315***	0.119
Sucrose	0.008	0.081	−0.354***	−0.053		0.457***	−0.017
Glucose	0.105	−0.165	−0.151	0.020	0.214*		−0.042
Fructose	−0.091	0.122	0.213*	−0.101	−0.161	0.276**	

*P<0.05, **P<0.01, ***P<0.001.

Our LN_2 quenching results confirm that the foliage of boreal conifer species can survive temperatures as low as −196°C, and show that some transition occurs as the tissue is slowly cooled to about −30°C that allows the tissue to survive lower temperatures without further loss of membrane integrity or development of post-freezing necrosis. Although there may be other interpretations, these observations are consistent with the hypothesis that the concentrated cytoplasm in the freeze-dehydrated cells undergoes a glass transition.

The well-characterized vitrification behaviour of sucrose and oligosaccharides provides a link between sugar accumulation and cytoplasmic vitrification. Under slow freezing, maximally freeze-concentrated sucrose solutions vitrify at about −41°C, without any apparent eutectic crystallization of the concentrated sucrose (Goff and Sahagian, 1996; Goff *et al.*, 2003). Anhydrous raffinose and stachyose have glass transition temperatures as much as 40°C higher than sucrose, and can be expected to elevate glass transition temperatures in solutions as well (Buitink *et al.*, 2000). The relatively high concentrations of these sugars in extremely LT-tolerant tissues suggests that vitrification is possible, if not probable, as tissues cool and cells are dehydrated by extracellular ice. Other cytoplasmic components, notably stress-associated proteins such as dehydrins (Buitink and Leprince, 2004), are also potential contributors to cytoplasmic vitrification.

While vitrification has been convincingly demonstrated in dried orthodox seeds that remain viable for long periods at about 5% water content (Koster, 1991; Buitink and Leprince, 2004), the evidence for vitrification in frozen tissues is less persuasive (Hirsh, 1987) because it relies on rather subjective interpretation of DSC scans (Wolanczyk, 1989). A glass is functionally defined as a fluid with a viscosity $>10^{12}$ Pa s (Franks, 1985), but there are no known methods for directly measuring viscosity in the extremely dehydrated protoplasts, surrounded by cell walls and buried in extracellular ice, that would be found in frozen conifer needles or other plant tissues at −30°C. DSC is the most commonly used method for studying glass transitions, and relies on identifying a step change in heat capacity as molecules lose rotational and translational mobility in the glass transition. This is relatively clear in single materials such as plastics or in simple two-component systems such as aqueous sucrose solutions, but has proved much more difficult to detect in frozen tissue samples. More recently, modulated temperature DSC (MT-DSC) has been employed to distinguish changes in heat capacity from other thermal events that take place during a thermal scan, giving a much clearer picture of glass transitions (e.g. Goff and Sahagian, 1996).

We employed MT-DSC to search for glass transitions in conifer needles and other extremely LT-tolerant plant tissues. We have detected glass transitions in a few samples from boreal species (Fig. 22.7) and they occur in the expected temperature range, but they are weak and not fully repeatable, so at best our direct evidence for LT-induced vitrification is equivocal. Because MT-DSC can only give the heat capacity of the whole sample and the freeze-dehydrated cells comprise only a small fraction of the total mass, we think that any change in cytoplasmic heat capacity may be masked by the stronger signal from extracellular ice and other components. Furthermore, while glass transitions in single materials or two-component systems occur over a range of a few degrees, in complex mixtures the shift in heat capacity would occur over a broader temperature range as different classes of molecules lose mobility. Other methods, such as electron spin resonance and NMR spectroscopies, which rely on detecting a relative change in molecular mobility, may suffer from similar problems when applied to frozen plant tissues. Cooling and freeze-concentration of cytoplasm may result in a more continuous change in viscosity and heat capacity, with the cytoplasm increasingly out of thermodynamic equilibrium with extracellular ice at progressively lower temperatures (Wolfe *et al.*, 2002).

Conclusion

LT tolerance in the foliage of conifers from north temperate and boreal regions varies according to the climate in the region of origin. Species from warm temperate mountains die at midpoint temperatures of −25 to −35°C,

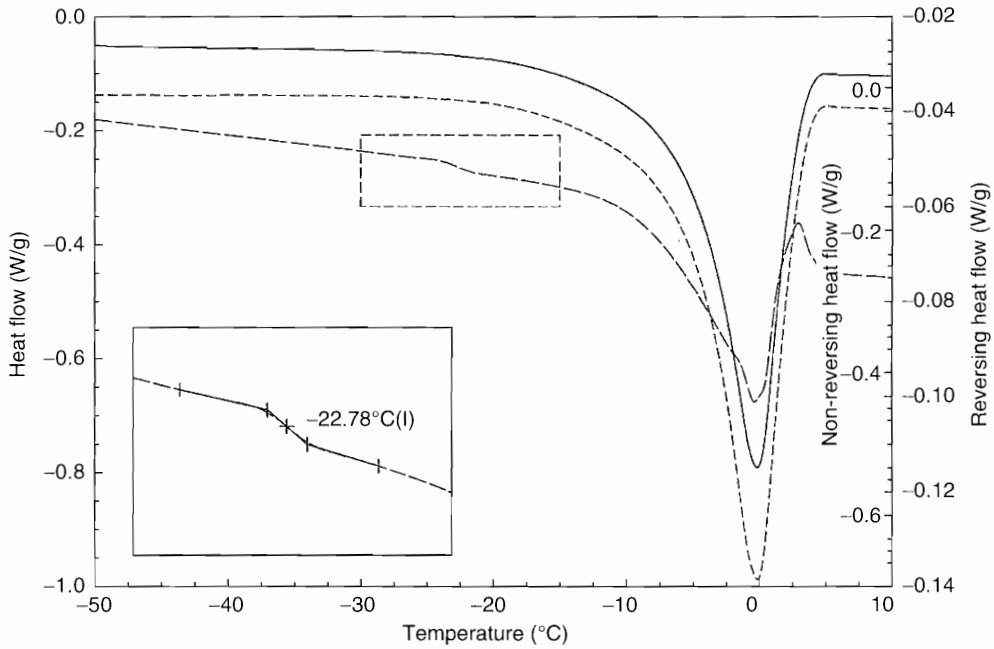

Fig. 22.7. Modulated-temperature differential scanning calorimetry scan of four 5 mm *Picea abies* needle sections, sampled 3 March 2004, cooled slowly to −60°C, then scanned during warming at 0.2°C/min with temperature modulation of ±0.318°C in 60 s (Q1000 MT-DSC; TA Instruments, New Castle, Delaware, USA). Shown are total (——), non-reversing (- - -) and reversing (– – –) heat flows. Reversing heat flow corresponds to heat capacity, and the step change shown in the inset is a glass transition, measured using the inflection point.

while boreal species can survive slow freezing to −80°C or lower and quenching in LN_2 after slow freezing to −30°C. Injury due to LT stress is expressed as electrolyte leakage of LT-stressed cells and as chlorosis and necrosis developed after exposure to light. Acclimation in temperate species involves a shift of the temperature response curve towards lower temperature, while in boreal species there is both a temperature shift and a decrease in the maximum amount of injury produced by LT stress. Foliar concentrations of the oligosaccharides raffinose and stachyose correlate strongly with LT tolerance, indicating an important cryoprotective role for these sugars. During slow cooling from −20 to −30°C, boreal conifers undergo a transition, possibly cytoplasmic vitrification involving oligosaccharides, sucrose or other cytoplasmic components, that allows them to survive extreme LT stress.

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Plant Cold Hardiness

From the Laboratory to the Field

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