

## Seasonal differences in freezing stress resistance of needles of *Pinus nigra* and *Pinus resinosa*: evaluation of the electrolyte leakage method

MARJA-LIISA SUTINEN,<sup>1</sup> JIWAN P. PALTA<sup>2</sup> and PETER B. REICH<sup>3,4</sup>

<sup>1</sup> Finnish Forest Research Institute, Rovaniemi 96300, Finland

<sup>2</sup> Department of Horticulture, University of Wisconsin, Madison, WI 53706, USA

<sup>3</sup> Department of Forestry, University of Wisconsin, Madison, WI 53706, USA

<sup>4</sup> Present address: Department of Forest Resources, University of Minnesota, St. Paul, MN 55108-1027, USA

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### Summary

Seasonal changes in freezing stress resistance of needles of red pine (*Pinus resinosa* Ait.) and Austrian pine (*Pinus nigra* Arnold) trees were measured by an electrolyte leakage method and by visual observation. During most of the year, freezing stress resistance determined by the two methods gave similar results. The electrolyte leakage method provided a good estimate of seasonal changes in freezing stress resistance except for red pine needles in their most winter-hardy state. To obtain a reliable estimate of freezing stress resistance in winter-hardy red pine needles it was necessary to combine the electrolyte leakage method with visual observations. When red pine needles survived exposure to  $-80^{\circ}\text{C}$  or lower, electrolyte leakage was never more than 30% even when the needles were exposed to a slow freeze-thaw stress of  $-196^{\circ}\text{C}$ . However, rapid freezing of red pine needles to  $-196^{\circ}\text{C}$  resulted in electrolyte leakage of over 80%. Red pine needles attained a much higher freezing stress resistance during the winter than Austrian pine. Red pine needles also acclimated and deacclimated faster than Austrian pine needles. An index of injury was developed based on the electrolyte leakage method  $((R_2 + R_1)/2)$ , where  $R_1$  is the minimum % electrolyte leakage from noninjured tissue and  $R_2$  is the maximum % electrolyte leakage at the highest injury) that reliably predicted freezing stress resistance of pine needles for most of the year. Important aspects for developing a successful index of injury for pine needles are: use of cut needles, vacuum infiltration and shaking during incubation in water.

We conclude that: (1) during cold acclimation the cell wall properties of the pine needles changed and these changes, which appeared to differ in the two species, might explain the very low leakage of electrolytes from winter-hardy needles of red pine; (2) pine needles survive winter by developing the ability to tolerate extracellular ice formation, because after rapid freezing the needles were severely injured; and (3) red pine is adapted to a shorter growing season and colder winters than Austrian pine.

### Introduction

Because northern conifers experience a climatic rhythmicity, they must adjust their metabolic activities well in advance of seasonal environmental changes. Minimum air temperatures can vary from as high as  $25^{\circ}\text{C}$  in midsummer to as low as  $-40^{\circ}\text{C}$  in midwinter. Freezing stress resistance is correlated to a rhythmicity of growth (Levitt 1980, Sakai and Larcher 1987); it is lowest during the growing season and at a maximum during the dormant winter period. Dormancy prevents growth and the accompanying loss of freezing stress resistance even during relatively warm winter days (Fuchigami et al. 1982).

Cold acclimation and deacclimation of northern conifers are induced in response to changes in photoperiod, air temperature and soil water status (Levitt 1980). The timing of cold acclimation and deacclimation and the extent of seasonal changes in freezing stress resistance vary with species, provenance and variety (Levitt 1980, Sakai and Larcher 1987). Thus the annual growth rhythm and the expression of freezing stress resistance appear to be genetically controlled. Of the northern conifers, *Pinus* is a widely distributed genus. Freezing stress resistance of various pine species ranges from slight to extreme (Sakai and Larcher 1987).

Many studies investigating the freezing stress resistance of pines have utilized the artificial freeze test of seedlings (Glerum 1973, Christersson 1973, Rook et al. 1974, Aronsson 1975, Christersson 1978, Menzies and Holden 1981, Greer and Warrington 1982, Smit-Spinks et al. 1985, Berrang and Steiner 1986, Rikala and Repo 1987, Burr et al. 1990). Some studies have utilized artificial acclimation of twigs of adult pine trees before measurement of freezing stress resistance (Sakai and Okada 1971, Sakai and Weiser 1973, Oohata and Sakai 1982, Sakai 1983). However, apart from visual observations of winter survival *in situ* (Kolb et al. 1985), very limited data are available on seasonal changes in freezing stress resistance of adult pine trees. Furthermore, in most studies of adult pine trees, the measurement of freezing stress resistance has been limited to about  $-50\text{ }^{\circ}\text{C}$  (Schwarz 1968, Aronsson 1980, Koski 1985, Repo et al. 1990).

In nature, plants experience slow freeze-thaw rates (Levitt 1980, Steffen et al. 1989). Because atypical plant responses have been observed in response to atypical freeze-thaw rates (Steffen et al. 1989) it is important to simulate the conditions associated with freeze-thaw stress in natural environments. The critical points are freeze-thaw rate, the duration of freezing (Christersson and Krasavtsev 1972) and ice nucleation at or near  $0\text{ }^{\circ}\text{C}$  to prevent supercooling and flash freezing (Steffen et al. 1989). Steffen et al. (1989) have demonstrated that increasing the cooling rate from  $1\text{ }^{\circ}\text{C h}^{-1}$  to about 3 or  $6\text{ }^{\circ}\text{C h}^{-1}$  greatly increases membrane damage in potato leaves.

It has been shown that the accuracy of freezing stress resistance estimation can be increased by simultaneously using two or more viability tests and by combining the results of these tests (Palta et al. 1978). Measurement of electrolyte leakage following a freeze-thaw stress has been used to estimate freezing stress resistance and the ability of tissues to recover following freezing injury (Wilner 1960, Palta et al. 1977a, 1977b, Warrington and Rook 1980, Palta et al. 1982, Colombo et al. 1982, Hallam and Tibbitts 1988, Murray et al. 1989, Burr et al. 1990).

Electrolyte leakage is usually expressed as the ratio of electrolyte leakage from freeze-injured tissue to electrolyte leakage from killed tissue. To correct for electrolyte leakage from the noninjured (unfrozen) control, Flint et al. (1967) developed an index of injury in which the percent electrolyte leakage from the unfrozen control sample is given a value of 0% and the percent electrolyte leakage from killed tissue is given a value of 100%. The temperature giving an index of injury value of 50% is termed the  $LT_{50}$ -value (e.g., Burr et al. 1990). The basic assumption in this index is that severely freeze-injured tissues will give values close to 100%. We have found that electrolyte leakage from severely freeze-injured midwinter samples of pine

needles has a maximum value of 30% using this index. Thus an arbitrary value of 50% for the index of injury will not correctly estimate freezing stress resistance. We present here an alternative procedure for estimating freezing stress resistance that can correct for this seasonal variation in electrolyte leakage from severely injured tissues.

The purpose of this study was: (1) to standardize an electrolyte leakage method to detect seasonal changes in freezing stress resistance of pine needles frozen either slowly or quickly to the temperature of liquid nitrogen; (2) to relate the electrical conductivity method of assessing injury in needles during the post-thaw period to visual observations; and (3) to determine seasonal changes in freezing stress resistance of needles of two closely related pine species (*Pinus resinosa* Ait. and *Pinus nigra* Arnold) grown in the field.

## Materials and methods

### *Plant material*

Needles from the youngest, fully expanded annual cohort were collected from five different 45-year-old Austrian pine (*Pinus nigra* Arnold) and red pine (*Pinus resinosa* Ait.) planted trees (lat. 43°03'9 N, long. 89°25' W, alt. 300 m, University of Wisconsin-Madison Arboretum, Wisconsin, USA) at 2- or 4-week intervals from March 7, 1988 to May 31, 1989. On June 28, 1988 the new (1988) current-year needles were taken instead of the 1987 cohorts, because the former were fully expanded. At each sampling, needles were collected from four separate southern exposed branches of each tree and pooled to yield a single sample. Thus one sample per tree was collected and kept separately for all measurements.

### *Freeze-thaw treatment*

For slow freezing and thawing, two sets of five fascicles per freezing temperature from each sample were wrapped with moist filter paper containing crushed ice. To avoid desiccation during the experiment, the samples were wrapped in plastic-lined paper. To slow down the cooling rates, samples were transferred to thermosbottles (800 ml, Thermos) before being subjected to low temperatures. Needle temperature was monitored in each bottle with a copper-constantan thermocouple placed in the middle of the needle sample. The needles were first frozen in a freezer in steps first to  $-25^{\circ}\text{C}$ , then to  $-75^{\circ}\text{C}$ , and then cooled to  $-196^{\circ}\text{C}$  by immersing the thermosbottles in liquid nitrogen. The maximum cooling rate was  $1.8^{\circ}\text{C h}^{-1}$  for cooling to  $-25^{\circ}\text{C}$ ,  $5.5^{\circ}\text{C h}^{-1}$  for cooling from  $-25$  to  $-75^{\circ}\text{C}$  and  $26.1^{\circ}\text{C h}^{-1}$  for cooling from  $-75^{\circ}\text{C}$  to the temperature of liquid nitrogen. The control samples were kept in a thermosbottle at  $4^{\circ}\text{C}$ .

After a desired freezing temperature was reached the thermosbottles were removed from the freezer and the samples were thawed slowly. Samples frozen from  $-5$  to  $-25^{\circ}\text{C}$  were thawed overnight by transferring the thermosbottles to  $4^{\circ}\text{C}$ . Samples frozen from  $-30$  to  $-75^{\circ}\text{C}$  were first thawed overnight to  $-25^{\circ}\text{C}$  and then transferred to  $4^{\circ}\text{C}$  for complete thaw. Samples frozen to liquid nitrogen temperature

were first thawed overnight to  $-75\text{ }^{\circ}\text{C}$  and then thawed to  $-25\text{ }^{\circ}\text{C}$  and finally to  $4\text{ }^{\circ}\text{C}$ . The rate of temperature rise was  $15.6\text{ }^{\circ}\text{C h}^{-1}$  during thawing from the temperature of liquid nitrogen to  $-75\text{ }^{\circ}\text{C}$ ,  $3.5\text{ }^{\circ}\text{C h}^{-1}$  for thawing from  $-75$  to  $-25\text{ }^{\circ}\text{C}$  and  $1.5\text{ }^{\circ}\text{C h}^{-1}$  for thawing from  $-25$  to  $4\text{ }^{\circ}\text{C}$ .

For the fast freeze-thaw treatment, samples in plastic bags were transferred from  $4\text{ }^{\circ}\text{C}$  to liquid nitrogen. The maximum cooling rate in this treatment was about  $100\text{ }^{\circ}\text{C h}^{-1}$ . These samples were thawed by transferring them from liquid nitrogen to  $4\text{ }^{\circ}\text{C}$ . The maximum thawing rate was  $45\text{ }^{\circ}\text{C h}^{-1}$ .

#### *Electrical conductivity method*

One set of five fascicles per tree per freezing temperature was used to measure electrical conductivity. After thawing, fascicles were cut into  $0.8\text{ cm}$  pieces and transferred to  $20\text{ ml}$  of distilled water in  $50\text{-ml}$  test tubes. The samples were vacuum infiltrated by subjecting them 3 times to  $-0.15\text{ Pa}$  pressure for  $2\text{ min}$ . After shaking for  $20\text{ h}$  ( $250\text{ rpm}$ , gyratory shaker, New Brunswick Scientific, Model G 10), the electrical conductivity of the effusate was measured ( $C_i$ ) with a conductivity salt bridge (YSI conductivity meter, Model 32, Yellow Springs Instruments). The needles were then killed by autoclaving the samples for  $15\text{ min}$  at  $250\text{ }^{\circ}\text{C}$ . After cooling the samples to room temperature and shaking for  $30\text{ min}$ , electrical conductivity was remeasured ( $C_k$ ). Percent electrolyte leakage ( $R$ ) was calculated as  $C_i/C_k \times 100$ . The temperature representing the freezing stress resistance was estimated from the plot of % electrolyte leakage (Figure 1) as follows: % electrolyte leakage at FSR =  $(R_1 + R_2)/2$ , where  $R_1$  = % electrolyte leakage from the noninjured tissue and  $R_2$  = % electrolyte leakage at the highest level of injury.

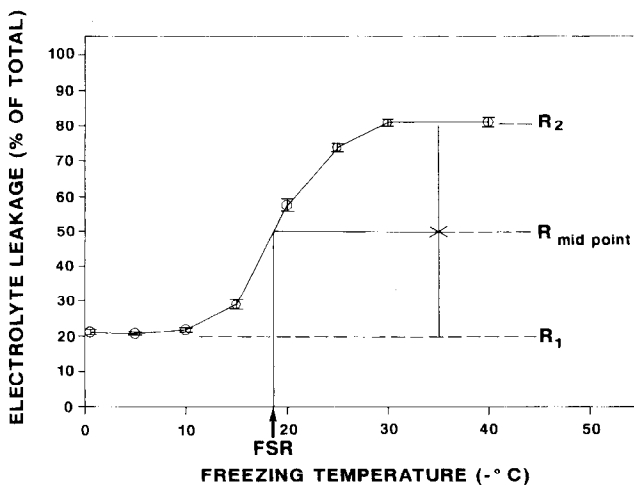


Figure 1. Estimation of freezing stress resistance (FSR,  $^{\circ}\text{C}$ ) for the needles of two pine species from the % (of total) electrolyte leakage versus freezing temperature curve. Percent electrolyte leakage at FSR =  $(R_1 + R_2)/2$ , where  $R_1$  = % electrolyte leakage from the noninjured tissue and  $R_2$  = % electrolyte leakage at the highest level of injury.

### Visual observations

A second set of five fascicles was used for visual observations of needle injury. The thawed needles were kept moist in beakers for up to 2 weeks at room temperature (about 22 °C) and an irradiance of about 25  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Needles were observed for browning and the extent of injury was estimated from the length of the brown area compared to the total needle length. Freezing stress resistance was calculated as the temperature yielding needles having 50% brown needle length.

To determine seasonal changes in freezing stress resistance of Austrian and red pine needles, the electrolyte leakage method was used except for the midwinter samples of red pine needles (November–February) when estimation was done by visual observations.

### Statistical analyses

For both Austrian and red pine needles, the correlation between the electrolyte leakage method and visual observations was tested by using BMDP-programs (Biomedical programs, UCLA, Los Angeles, CA). Significant differences in freezing stress resistance values during deacclimation in the springs of 1988 and 1989 were tested by the pairwise *t*-test ( $n = 5$  per species).

## Results

### Evaluation of the conductivity method

Compared with intact needles that were incubated without shaking, there was a threefold increase in electrolyte leakage when needles were cut into 0.8 cm segments and incubated with shaking to facilitate diffusion of ions from the cut surface (Table 1).

During most of the year, the values of freezing stress resistance determined by the electrolyte leakage method and by visual observation were highly correlated ( $R = 0.973$  for both species pooled, red pine samples from November–February were excluded when regression equation was determined, Figure 2). The regression

Table 1. Influence of needle length on electrolyte leakage (% of total) from control and freeze-thawed (to  $-28$  °C) red pine needles. After freeze-thaw, the needles were used intact or cut into pieces of different lengths. The needles were vacuum infiltrated ( $-0.15$  Pa) in 20 ml of distilled water. Needles were shaken at 20 °C for 18 h. Values are mean of 15 replicates  $\pm$  SD.

Sample	Needle length (cm)	% Electrolyte leakage			
		With shaking		Without shaking	
		Control (C)	Frozen (F)	Control (C)	Frozen (F)
Intact needle	12–15	7.0 $\pm$ 0.6	9.8 $\pm$ 0.9	5.6 $\pm$ 1.8	5.6 $\pm$ 0.8
Needle segments	4–5	10.4 $\pm$ 0.9	31.0 $\pm$ 1.8	10.6 $\pm$ 0.8	12.8 $\pm$ 3.5
Needle segments	0.8	21.8 $\pm$ 1.1	58.2 $\pm$ 0.8	21.4 $\pm$ 1.2	30.6 $\pm$ 2.4

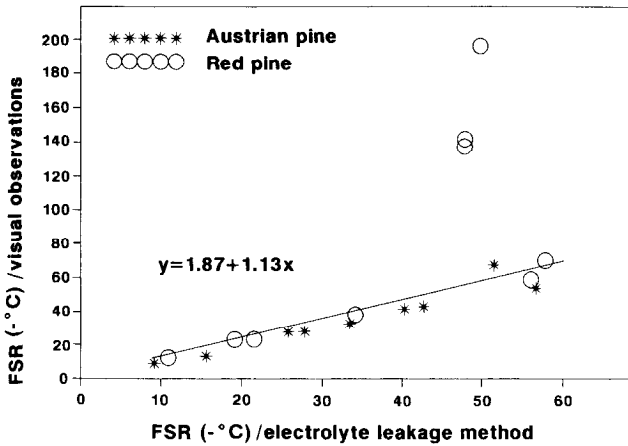


Figure 2. The relationship of freezing stress resistance of Austrian and red pine needles estimated by the electrolyte leakage method and visual observations. The sampling was between September 6, 1988 and March 28, 1989.

relationships (slope and intercept) for the two species (excluding the red pine midwinter values) were not statistically different ( $P > 0.05$ ) and the values of freezing stress resistance obtained by the two methods were usually within 0.2 to 2 °C. However, when red pine needles survived  $-80$  °C or lower (according to visually assessed damage), the relative electrolyte leakage was never more than 30% even when the needles were slowly frozen to the temperature of liquid nitrogen (Figure 3). Longer shaking times (up to 48 h) or shorter needle segments (0.5 cm) did not result in any increase in % electrolyte leakage in injured needles compared to controls (data not shown). During midwinter (November–February), the freezing

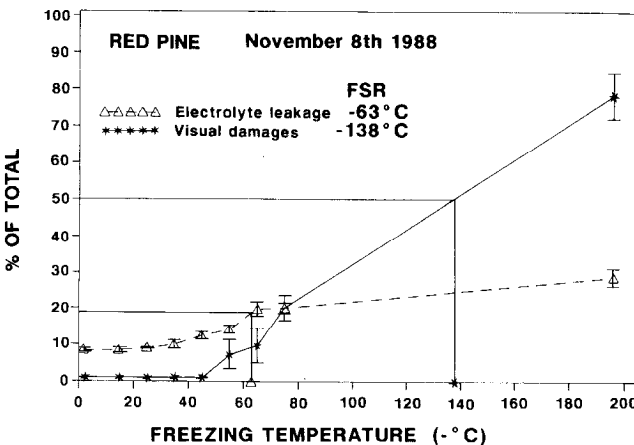


Figure 3. Freezing stress resistance of red pine needles estimated by the electrolyte leakage method (FSR =  $-63$  °C) and by visual observations (FSR =  $-138$  °C). Freezing stress resistance by the electrolyte leakage method was estimated according to Figure 1.

stress resistance value of red pine needles was about  $-50\text{ }^{\circ}\text{C}$  when determined with the conductivity method, whereas visual observation of the needles gave freezing stress resistance values of  $-196\text{ }^{\circ}\text{C}$ . Thus for red pine, freezing stress resistance values from the electrolyte leakage method correlated well with those estimated by visual observation to about  $-80\text{ }^{\circ}\text{C}$  (Figure 4), but not below that temperature. For Austrian pine needles, the freezing stress resistance values for electrolyte leakage method and visual method (neither of which fell below  $-80\text{ }^{\circ}\text{C}$ ) correlated well throughout the year (Figure 2).

*Seasonal changes in freezing stress resistance of needles of two pine species*

The freezing stress resistance of both species tracked the mean minimum air temperature throughout the year (Figure 4). During midsummer, the freezing stress resistance in both species was about  $-6\text{ }^{\circ}\text{C}$ . During the fall, when the minimum air temperature was consistently below  $0\text{ }^{\circ}\text{C}$ , there was a sharp increase in the freezing stress resistance of red pine needles (Figure 4), whereas the freezing stress resistance

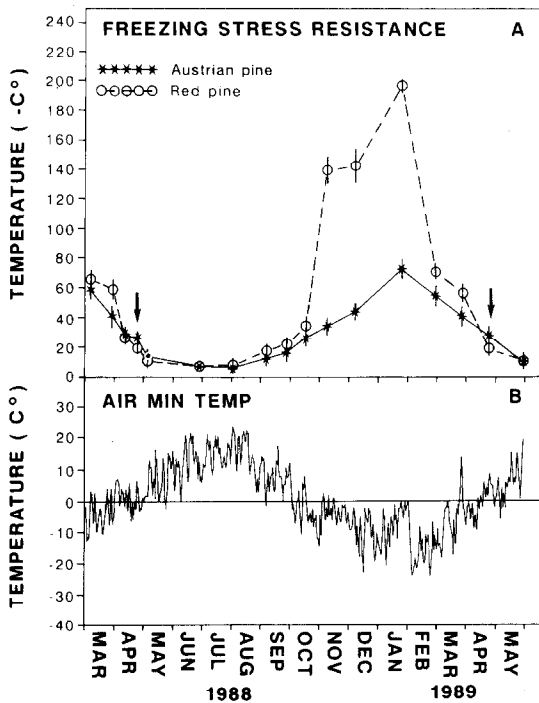


Figure 4. (A) Seasonal changes in freezing stress resistance of Austrian and red pine needles. The sampling was done between March 7, 1988 and May 31, 1989. Current-year needles were used except in June (for details see "Materials and methods"). In July, the newest needles were used when they were fully expanded. Freezing stress resistance was estimated by the electrolyte leakage method (see Figure 1 for details) except during winter (November–February), when it was estimated by visual observation. The arrows show the period when red pine needles are deacclimating faster than Austrian pine needles. (B) The daily minimum air temperature (2 m above ground level) at the experimental site.



of Austrian pine needles changed gradually.

During the winter, red pine needles attained a much greater freezing stress resistance than Austrian pine needles. In January, the red pine needles survived (visually assessed damage less than 50%) freezing to the temperature of liquid nitrogen, whereas the freezing stress resistance of Austrian pine needles was close to  $-70\text{ }^{\circ}\text{C}$  (Figure 4).

During spring, deacclimation in red pine needles was slightly faster than in Austrian pine (Figure 4A and Figures 5A–5C). In the early spring (March 7, 1988), the freezing stress resistance of Austrian pine and red pine was  $-59$  and  $-66\text{ }^{\circ}\text{C}$ , respectively (Figures 4A and 5A). In mid-spring (April 15, 1988), the freezing stress resistance of the needles of both species was similar,  $-26$  to  $-28\text{ }^{\circ}\text{C}$  (Figure 5B). However two weeks later (April 28, 1988), the freezing stress resistance of red pine and Austrian pine needles was  $-19$  and  $-25\text{ }^{\circ}\text{C}$ , respectively (Figure 5C). A similar difference was also observed during spring 1989 (see Figure 4A). These differences in the late-spring freezing stress resistance of the two pine species were statistically significant ( $t = 7.75$ ,  $DF = 5.4$ ,  $P = 0.0004$ ).

The shape of the electrolyte leakage versus freezing temperatures curves differed at various times of the year (Figures 5A–5C). During winter, electrolyte leakage increased gradually over a  $20$ – $25\text{ }^{\circ}\text{C}$  temperature range (roughly from  $-45$  to  $-65\text{ }^{\circ}\text{C}$ , Figure 5A). However, in late spring, electrolyte leakage increased dramatically over a narrow ( $5$ – $10\text{ }^{\circ}\text{C}$ ) range of freezing temperatures (Figure 5C).

#### *Influence of cooling rate*

Very fast freeze-thaw rates greatly influenced the extent of needle damage in both species (Table 2). Fast freezing (immersion in liquid nitrogen) resulted in electrolyte leakage of more than 80% of the total even in very winter-hardy needles and in red pine needles that were otherwise hardy to liquid nitrogen temperatures when frozen slowly (January 24, 1989 samples). In response to slow freezing, visually assessed damage in red pine needles in the December and January samples was 64 and 40%, respectively, whereas in response to fast freezing visually assessed damage was 100% in both samples (Table 2). Electrolyte leakage was only about 20% in needles in the December and January samples when they were frozen slowly to the temperature of liquid nitrogen, whereas electrolyte leakage was over 80% when comparable needles were frozen quickly (Table 2).

## **Discussion**

#### *Evaluation of the electrolyte leakage method*

The electrolyte leakage method can be used to determine freezing stress resistance of Austrian and red pine needles except for red pine needles during the midwinter months (November–February) (Figures 2 and 3). Freezing stress resistance determined by the electrolyte leakage method and by visual observations correlated well ( $R = 0.973$ ) during all times of year except from November to February when the



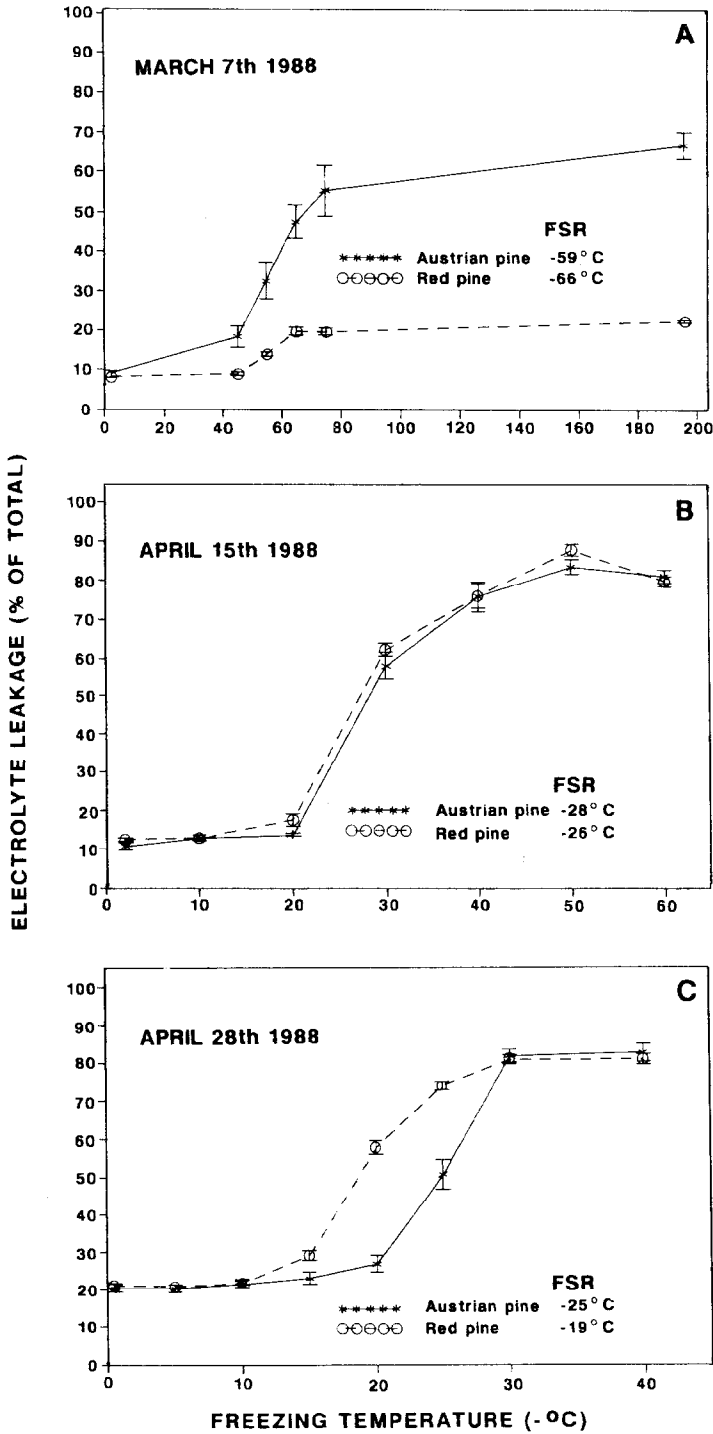


Figure 5. Changes in the relationship between percent electrolyte leakage and freezing temperatures during deacclimation in spring in Austrian and red pine needles.

Table 2. Influence of freeze-thaw rate on needle injury in Austrian and red pine as estimated by electrolyte leakage and visual observation. In the slow freeze-thaw treatment, the maximum rate of temperature change between 4 and  $-75\text{ }^{\circ}\text{C}$  was  $5.5\text{ }^{\circ}\text{C h}^{-1}$  and between  $-75\text{ }^{\circ}\text{C}$  and the temperature of liquid nitrogen it was about  $70\text{ }^{\circ}\text{C h}^{-1}$ . In the fast freeze-thaw treatment, the maximum rate of temperature change between  $4\text{ }^{\circ}\text{C}$  and the temperature of liquid nitrogen was about  $100\text{ }^{\circ}\text{C h}^{-1}$ . Values are means of five replicates  $\pm$  SD.

Date	Freeze-thaw rate			
	Slow	Fast	Slow	Fast
	Leakage (% of total)		Browning of needles (% of total length)	
<i>Austrian pine</i>				
September 6	–	89.8 $\pm$ 2.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
September 27	87.6 $\pm$ 1.8	94.6 $\pm$ 2.1	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
October 17	84.8 $\pm$ 1.0	94.0 $\pm$ 1.5	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
November 8	74.9 $\pm$ 0.5	80.4 $\pm$ 0.9	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
December 7	67.7 $\pm$ 4.7	89.5 $\pm$ 1.1	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
January 24	73.5 $\pm$ 3.7	84.9 $\pm$ 2.1	93.1 $\pm$ 2.0	100.0 $\pm$ 0.0
February 28	74.0 $\pm$ 2.3	88.6 $\pm$ 1.7	95.5 $\pm$ 3.3	100.0 $\pm$ 0.0
March 28	74.7 $\pm$ 2.5	85.8 $\pm$ 0.6	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
<i>Red pine</i>				
September 6	–	81.9 $\pm$ 0.6	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
September 27	79.1 $\pm$ 1.6	86.8 $\pm$ 0.3	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
October 17	77.5 $\pm$ 3.1	89.8 $\pm$ 1.2	91.8 $\pm$ 7.2	100.0 $\pm$ 0.0
November 8	29.0 $\pm$ 2.6	72.0 $\pm$ 1.3	85.8 $\pm$ 4.8	100.0 $\pm$ 0.0
December 7	19.9 $\pm$ 1.1	85.7 $\pm$ 0.7	64.2 $\pm$ 2.4	100.0 $\pm$ 0.0
January 24	23.9 $\pm$ 0.8	81.6 $\pm$ 0.7	40.5 $\pm$ 1.2	100.0 $\pm$ 0.0
February 28	25.6 $\pm$ 1.2	85.1 $\pm$ 2.2	86.4 $\pm$ 4.0	100.0 $\pm$ 0.0
March 28	63.1 $\pm$ 2.1	81.3 $\pm$ 1.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0

electrolyte leakage method underestimated the freezing stress resistance of red pine needles (Figure 3). During this period, although visual observations indicated substantial needle injury, maximum electrolyte leakage was only 30% (Figures 3 and 5A).

The reasons for the low electrolyte leakage from winter-hardy red pine needles with substantial injury are unclear. Electrical conductivity was measured shortly after complete thawing when the needles appeared normal. Visually assessed damage was apparent only during the post-thaw period after about 2 weeks. Thus visually assessed damage was the manifestation of some injury that was not apparent immediately after thawing. Secondary damage during the post-thaw period has been investigated in other plant systems (Palta and Li 1980, Arora and Palta 1988). An increase in electrolyte leakage following thawing is known to be a reliable indicator of membrane injury, but this measurement does not necessarily reflect the final condition of the tissue (Palta et al. 1977b).

There is evidence that cell wall properties change during cold acclimation (Griffith and Brown 1982), with an increase in lignification and suberization of cell walls

during the fall. The low electrolyte leakage from winter samples of red pine needles might be due to changes in cell wall properties that provide resistance to diffusion of electrolytes from the cells of needle to the extracellular water. The observation that winter-hardy red pine needles only exhibit high electrolyte leakage after rapid freezing in liquid nitrogen suggests that physical disruption of the tissue is required for the ions to diffuse out. Austrian pine needles had high electrolyte leakage even after slow freezing in liquid nitrogen indicating that changes in the cell wall properties during winter must differ in the two species.

Although the electrolyte leakage method has been used in many studies to estimate freezing stress resistance of needles (Aronsson and Eliasson 1970, Johnson and Gagnon 1988, Burr et al. 1990) and other conifer tissues (Warrington and Rook 1980, Colombo et al. 1982, Kolb et al. 1985, Pukacki and Pukacka 1987, Murray et al. 1989), there have been no reports of difficulties associated with measuring electrolyte leakage of winter samples. This may be because in all of these studies not even the very hardy needles were exposed to freezing temperatures below  $-80\text{ }^{\circ}\text{C}$  and the needles were not frozen slowly to  $-80\text{ }^{\circ}\text{C}$ . Fast freezing, which is almost always lethal, results in high electrolyte leakage immediately after thawing (Table 2). When estimating freezing stress resistance of plant material the artificial freeze-thaw procedure should closely simulate field conditions, where the rate of change in air temperature below  $0\text{ }^{\circ}\text{C}$  rarely exceeds  $1\text{--}2\text{ }^{\circ}\text{C h}^{-1}$  (Steffen et al. 1989). The results of this and many other studies (Sakai 1960, Aronsson and Eliasson 1970, Christersson and Krasavtsev 1972, Warrington and Jackson 1981) clearly demonstrate a relationship between the amount of damage and the freeze-thaw rate.

Electrolyte leakage has been expressed as the ratio of electrolyte leakage from injured tissue to heat-killed tissue, as an index of injury (Flint et al. 1967), as a differential % leakage (Zhang and Willison 1986), or as a rate of electrolyte leakage (Murray et al. 1989). None of these methods gave satisfactory results for seasonal changes of freezing stress resistance in pine needles. For most of the year, a reliable estimate of freezing stress resistance of red pine and Austrian pine needles was achieved by expressing the freezing stress resistance as the midpoint of leakage between noninjured and freeze-damaged tissues (Figure 1). By using this approach we were able to correct for seasonal changes in the quantity of electrolytes released from unfrozen tissue as well as from freeze-killed tissue. Furthermore, our approach allowed the measurement of leakage within a day after thawing, whereas some approaches necessitate incubation for up to 5 days (Murray et al. 1989), which increases the risk of microbial contamination (Aronsson and Eliasson 1970).

For midwinter needles (November–February) of red pine, electrical conductivity was not a reliable method for estimating injury and freezing stress resistance. Electrolyte leakage is not directly related to cell death (Palta et al. 1977*a*, 1977*b*). Depending on the extent of initial injury, the injury can either increase or decrease during the post-thaw period. Thus, if electrolyte leakage is below a threshold (when post-thaw recovery is possible) it is not a good indicator of freezing stress resistance.

### *Seasonal changes in freezing stress resistance of pine needles under natural conditions*

Red pine appeared better adapted to the conditions of early frost, severe winter and short growing season than Austrian pine. Red pine needles attained a significantly greater resistance to freezing stress during the winter than Austrian pine needles (Oohata and Sakai 1982, Sakai 1983).

Red pine needles also cold acclimated and deacclimated faster than Austrian pine needles (Figure 5). Sensitivity to freeze-thaw stress is an important factor limiting the distribution of plants (Sakai and Larcher 1987). In red pine's natural range there is a higher probability of a minimum temperature below  $-40^{\circ}\text{C}$  during the midwinter months than in the range of Austrian pine. A higher degree of freezing stress resistance in red pine during winter compared to the closely related Austrian pine appears to be related to the natural distribution of these two species. Red pine needles also exhibited high freezing stress resistance until late (end of March, Figure 5) into the spring that could be an adaptation to late-spring frosts, which are common in the northern latitudes where red pine is distributed (Critchfield and Little 1971). The rapid acclimation and deacclimation ability of red pine could be related to its shorter growing season.

### *Freeze-thaw stress and survival in pine needles*

Winter-hardy needles were able to tolerate extracellular ice formation (slow freezing) but not intracellular ice formation (fast freezing), indicating that, during acclimation, needles must develop tolerance to the stresses created by the extracellular formation of ice.

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