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Frost resistance and ice nucleation in leaves of five woody timberline species measured in situ during shoot expansion

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Summary Frost resistance and ice nucleation temperatures of leaves, from bud swelling until after full expansion, were measured in situ for five major woody timberline species with recently developed field freezing equipment. Frost resistance determined in situ on leaves of attached twigs was significantly higher than values determined on detached leaves in laboratory tests (e.g., the temperature at which incipient frost damage was observed (LT_i) was 1.2 °C higher for detached leaves than for attached leaves of *Picea abies* (L.) Karst.). Frost resistance of leaves of all species changed significantly during shoot expansion (e.g., changes of 7.2 and 11 °C for *Rhododendron ferrugineum* L. and *Larix decidua* Mill., respectively). Expanding leaves (between 0 and 60% of full expansion) were the most sensitive to frost, with LT_i values ranging from –3.4 °C in *R. ferrugineum* to –6.3 °C in *L. decidua*. Among the studied species, *P. abies* and *R. ferrugineum* were the most frost sensitive throughout the shoot elongation period. In situ freezing patterns of leaves of attached twigs also differed from those of leaves of excised twigs. During leaf expansion, two distinct freezing exotherms were always registered in situ. The first freezing event (E1, high-temperature exotherm) was recorded at –1.5 ± 0.2 °C and reflected extracellular ice formation. Exposure of leaves to temperatures at which E1 occurred was, in all cases, noninjurious. The low-temperature exotherm (E2) mostly coincided with frost damage, except for some stages of leaf expansion in *R. ferrugineum* and *P. abies*, indicating that in situ freezing exotherms were not accurate estimators of frost damage in these species.

Keywords: field freezing equipment, freezing exotherms, Larix decidua, Picea abies, Pinus cembra, Rhododendron ferrugineum, Sorbus aucuparia.

Introduction

Low temperature is an important environmental constraint on plant distribution (cf. Sakai and Larcher 1987). At the timberline ecotone, freezing stress affects the development and growth of adult conifers (Tranquillini 1979, Sakai and Larcher 1987, Gross et al. 1991) and particularly contributes to the distorted growth of timberline trees (Tranquillini 1979, Larcher 1985). Although frost resistance in timberline trees is generally sufficient during winter (Tranquillini 1979), frost resistance decreases in spring to values that are insufficient to protect newly developed tissues against episodic frosts in late spring and summer (Kerner 1869, Matuszkiewicz 1977, Christersson et al. 1987). Natural frost damage to new leaves of *Picea abies* (L.) Karst., *Rhododendron ferrugineum* L., and a range of other subalpine woody species has been repeatedly observed in June at timberline on Mt. Patscherkofel, near Innsbruck (Kerner 1869), including after night frosts of –2.6 °C in 1999 and –4.3 °C in 2001 (D. Taschler and G. Neuner, unpublished observations). Damage caused by late episodic frost events could be aggravated by climate warming. Rising global mean temperatures can induce earlier bud break (Murray et al. 1989, Hänninen 1991, 1996), and climate warming is also anticipated to increase the frequency of unpredictable, episodic frost events (Katz and Brown 1992).

Although winter frost resistance of leaves of timberline conifers has been extensively studied (cf. Bannister and Neuner 2000), much less is known about the summer frost resistance of young leaves during the period from bud swelling until full leaf expansion. New leaves of woody timberline species tolerate frosts between –2 and –10 °C (cf. Tranquillini 1979, Sakai and Larcher 1987).

In most studies of summer frost resistance, little distinction has been made between different developmental stages during shoot expansion, and investigations have been carried out in the laboratory. Laboratory investigations have several shortcomings and are often inaccurate predictors of field frost survival (Pellet et al. 1981). Often, seedlings and young plants are used, which may differ significantly in frost resistance from adults (Sakai and Larcher 1987). Similarly, results obtained with excised plant parts may be unreliable indicators of behavior in the field because ice nucleation temperatures of excised plant parts generally decrease as a result of artifactual supercooling (Ashworth et al. 1985, Sakai and Larcher 1987, Robberecht and Junttila 1992, Flinn and Ashworth 1994, Neuner et al. 1997, Pearce 2001). Furthermore, only frosts occurring in the plant's natural environment allow study of the recuperation and further development of frost-injured plants or plant parts under natural conditions. Because ice nucleation temperatures are affected in laboratory freezing tests, we hypothesized that ice nucleation temperature would also affect frost

resistance, particularly during shoot elongation. Therefore, we frosted plants at their natural growing site in the field using recently developed field freezing equipment that can measure changes in freezing patterns and frost resistance of attached shoots. Because little is known about changes in frost resistance during shoot expansion, we conducted our measurements from bud swelling until full leaf expansion to assess the developmental stage most susceptible to frost damage.

Materials and methods

Plant species and experimental site

In situ freezing experiments were conducted at the timberline ecotone (1950 m a.s.l.) on the northwest-facing slope of Mt. Patscherkofel near Innsbruck, Austria (47°12′ N, 11°27′ E). All major timberline tree species of the European Alps are common at this site. Investigations were carried out on three coniferous tree species, *Larix decidua* Mill., *Picea abies* and *Pinus cembra* L., and two woody angiosperm species frequently found in the subalpine belt: *Sorbus aucuparia* L., a deciduous tree, and *Rhododendron ferrugineum*, an evergreen dwarf shrub. Frost treatments were conducted in situ on twigs of at least four adult trees of each species from May until August over two successive growing periods (2001 and 2002). These periods covered the complete development of the current year's growth.

For comparative purposes, the development of current-year growth from buds to fully expanded leaves was classified in eight developmental stages. Measurements were started on resting buds (s1) and continued during bud swelling (s2) and bud break (s3). During leaf expansion, three stages were identified: the onset of expansion marked by the loss of bud scales (0–30% of full leaf expansion; s4), the main expansion phase (30–60% of full leaf expansion; s5) and full expansion (60–100% of full leaf expansion; s6). Additional measurements were conducted from July 23 to 30 (s7) and from August 1 to 10 (s8) after full leaf expansion had been reached.

In situ frost treatment

Attached shoots were exposed to controlled freezing temperatures with a recently developed field portable freezing system (MCC-6, BK-Elektronik, Natters, Austria; http://www.bk-elektronik.com/pages/e_develop.htm). The freezing system consists of a control unit and six freezing chambers that can each be programmed independently. In contrast with other field frost equipment (cf. Neuner et al. 1997), our system measures frost resistance in situ by exposing plants to six different freezing temperatures in parallel. In each freezing chamber, air temperature is measured at high frequency (8 Hz) with an NTC temperature sensor (SEMI 833 ET, Hygrotec, Titisee-Neustadt, Germany) and recorded with a programmable data logger (CR10X micrologger, Campbell Scientific, Logan, UT). The data logger acts as a control unit and compares air temperature in the freezing chamber with the set-point temperature of a user-defined freezing program. Depending on the result, the switched control port of the data logger either sends a signal to the relay units or no signal is sent. As a result, a Peltier cooling unit (MAA 050T-12, Melcor, Trenton, NJ) mounted on top of each freezing chamber is turned on or off. The system can be powered by either a 12 V DC or a 230 V AC supply. The maximum temperature difference that can be maintained between ambient air and that inside the freezing chamber is –28 °C.

Each freezing chamber (interior diameter: 11 × 11 × 15 cm) is made of aluminum sheets insulated with 3-cm-thick Styrodur. The base of the chamber opens to permit insertion of a shoot while the shoot remains attached to the plant. A lid equipped with a slot that shuts allows twigs to be inserted and seals the freezing chamber during measurements. To prevent stratification of air inside the freezing chamber, a small ventilator is mounted on the cold side of the Peltier cooling unit.

Controlled in situ freezing programs followed constant cooling and thawing rates of 2 °C h^{-1} and 4-h exposure times (Figure 1). Deviations from the preprogrammed set-point temperatures were less than ± 0.2 °C. Rates of 2 °C h^{-1} were chosen because they approach naturally occurring freezing rates in subalpine environments and are in accordance with standard laboratory freezing procedures (Sakai and Larcher 1987). The exposure temperatures were selected such that the highest test temperature would cause no damage (LT_0) and the lowest temperature would kill all leaves (LT_{100}). The difference between adjacent target temperatures was less than 1.5 °C.

Laboratory freezing test

For comparative purposes, frost resistance during shoot expansion was determined on leaves of excised twigs of *P. abies*. Twigs (20 cm) were collected at the site and transported in a moistened and temperature-insulated container to the Institute of Botany in Innsbruck within 1 h of detachment. Shoots were placed on wet paper towels inside polythene bags and exposed to six target freezing temperatures in computer-controlled commercial freezers (Huber, Innsbruck, Austria). Freezing

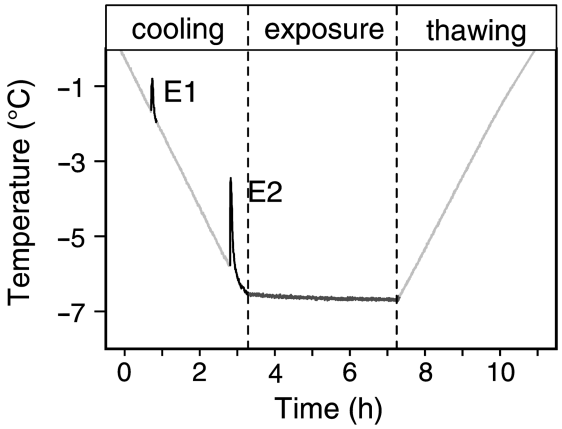

Figure 1. Controlled in situ freezing treatment (cooling and thawing rates = 2 °C h^{-1}; exposure time = 4 h) of expanding *R. ferrugineum* leaves (s5) at 1950 m a.s.l. during the night of July 5, 2002. Two distinct exotherms (E1, E2) were recorded during shoot expansion.

programs were identical to those used for the in situ frost treatments.

Viability assay

Frost damage was assessed in situ 3 days after the end of each frost treatment. In summer, during shoot expansion, we observed that 100% frost damage to leaves coincided with 100% frost damage to the whole current-year shoot. The assessment of frost damage therefore focused on leaves. Leaves of all species showed either no frost damage, initial damage, or were completely killed. Partial frost damage was rarely observed. Therefore, the percentage of the leaf area damaged was easily evaluated visually at the field site. Damage ratings were then plotted against treatment leaf temperatures. A classic logistic function was fitted to the data with P-Fit software (Biosoft, Durham, NC). Values of LT_{50}, i.e., the temperature causing 50% frost damage, were read directly from the fitted curve. The LT_0 value represents the lowest treatment temperature sustained without frost damage, LT_i denotes the temperature at which incipient frost damage was observed, and LT_{100} denotes the highest temperature causing 100% tissue death.

Ice nucleation temperatures

Concurrently with the in situ frost treatment and the laboratory freezing test, ice nucleation temperatures were measured with type T copper constantan fine-wire thermocouples (welding spot diameter: 0.127 mm). Temperatures were recorded every 12 s with a Campbell Scientific CR10X micrologger. Thermocouples were fixed to the leaves with lightweight, thermally insulated leaf clips during the in situ freezing treatment. During progressive lowering of leaf temperature (2 °C h^{-1}), one to several exotherms were recorded. Ice nucleation temperatures were determined graphically from the temperature record (see Figure 1). During shoot expansion, two different exotherms were usually recorded, a high-temperature exotherm (E1) and a low-temperature exotherm (E2).

Statistical data analysis

Frost resistance and ice nucleation temperatures were determined on leaves of randomly chosen shoots of at least three individuals per species. During each field freezing test, 36 ice nucleation temperatures were recorded concurrently, and six samples were taken for the estimation of frost resistance. For each developmental stage (s1–s8), mean values from at least 20 measurements were calculated. After the data had passed the Kolmogorov-Smirnov test, the significance of differences between frost resistance and ice nucleation temperatures at different stages in each species and between species were determined by analysis of variance (ANOVA) and the Bonferroni test ($P < 0.01$) using SPSS software (SPSS, Chicago, IL).

Results

Attached versus excised shoots

Attached shoots of *P. abies* were significantly more frost resistant than excised shoots from bud break until the end of the summer period (i.e., LT_i was 1.2 °C higher for detached leaves than for attached leaves) (Table 1), indicating a potential underestimation of frost resistance in laboratory freezing tests. Freezing patterns also differed between attached shoots and excised shoots, with a single exotherm recorded for leaves of excised shoots and two exotherms (E1, E2) usually recorded for attached *P. abies* shoots.

Changes in frost resistance during shoot elongation

Significant differences ($P < 0.01$) in frost resistance measured in situ were observed between stages of development of the current year's growth for the species tested (Figure 2). The observed changes were species-specific. The most frost-susceptible developmental stages were encountered after the loss of bud scales (s4; *R. ferrugineum*, *S. aucuparia*, *P. cembra*) and during the main expansion phase (s5; *L. decidua*, *P. abies*). Initial frost damage (LT_i) occurred between –3.8 and –6.5 °C depending on the species. A further drop in temperature of usually 2 °C killed the new shoot completely. After full leaf expansion, frost resistance increased again, except in *R. ferrugineum* and *P. abies*. These two species were, at nearly all stages, the most frost-susceptible of the species examined. *Larix decidua* had a higher (usually significantly higher) frost

Table 1. Comparison of frost resistance and ice nucleation temperatures (°C) measured during shoot expansion in leaves of *P. abies* on excised twigs in a laboratory freezing test and on attached twigs in an in situ freezing test. Values are means of at least $n = 30$ observations. Significance of differences between mean values of ice nucleation temperatures was tested by ANOVA and the Bonferroni test ($P < 0.01$). Significant differences are indicated by different letters. The significance of differences between mean values of frost resistance was tested with Students *t*-test ($P < 0.01$) and is indicated by an asterisk. Abbreviations: E1 = high-temperature exotherm; E2 = low-temperature exotherm; LT_0 and LT_{50} = temperatures causing 0 and 50% frost damage, respectively; and LT_i = temperature at which incipient frost damage was observed.

Developmental stage	Attached twigs (in situ)				Excised twigs		
	Ice nucleation		Frost resistance		Ice nucleation	Frost resistance	
	E1	E2	LT_i	LT_{50}		LT_i	LT_{50}
Resting bud	–4.6 a	–12.5 b	–12.8	–13.4	–13.9 c	–12.5	–13.6
Bud swelling	–4.1 a	–11.6 b	–11.7	–12.0	–12.9 c	–11.4	–12.0
Bud breaking	–2.2 a	–6.8 b	–6.9	–7.3 *	–10.1 c	–7.3	–8.2 *
Leaf expansion	–1.8 a	–5.5 b	–5.2 *	–5.6 *	–6.1 b	–4.0 *	–4.9 *

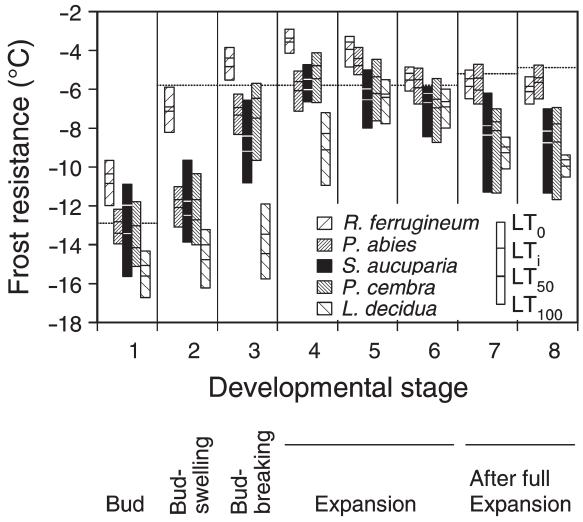

Figure 2. Changes in frost resistance measured in situ during the development of the current year's growth in five woody timberline species during two successive growing periods (2001 and 2002) at 1950 m a.s.l. The LT_0, LT_{50} and LT_{100} values are the temperatures causing 0, 50 and 100% frost damage, respectively. The LT_i value is the temperature at which incipient frost damage was observed. The dotted line indicates the absolute minimum air temperature recorded over a 30-year period at Mt. Patscherkofel at 2045 m a.s.l. in May (s1), June (s2–s6), July (s7) and August (s8). The mean sample size per species for each developmental stage was 45.

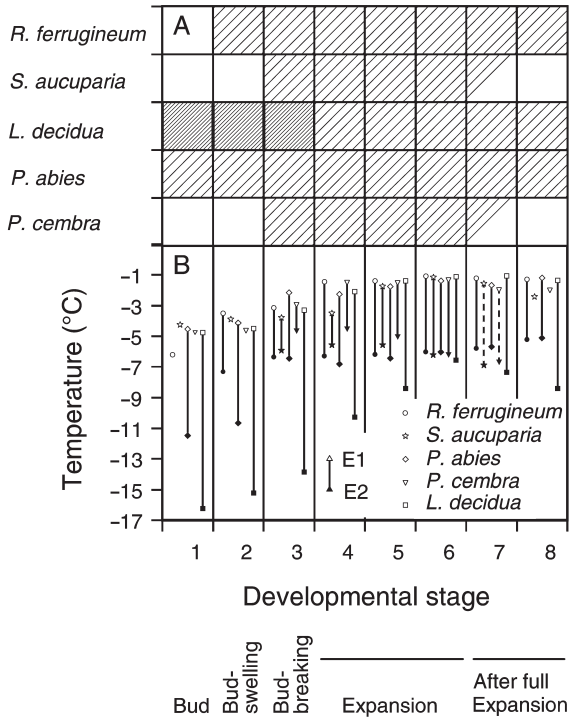

Figure 3. Freezing patterns (A) and ice nucleation temperatures (B) measured in situ at 1950 m a.s.l. in five woody plant species during the development of the current year's growth over two successive growing periods (2001 and 2002). (A) Different freezing patterns are indicated as follows: white = freezing with one exotherm; wide-hatched = freezing with two exotherms (one high-temperature exotherm (E1) and one low-temperature exotherm (E2)); and narrow-hatched = freezing with multiple exotherms (one E1 and multiple E2s). (B) The E1 (open symbol) is connected to the E2 (closed symbol) with a solid line. The dotted lines indicate the progressive disappearance of E2s after full expansion of leaves of *S. aucuparia* and *P. cembra*. The mean sample size per species for each developmental stage was 40.

resistance than other species throughout its development. All species could potentially be frost damaged at the investigation site because absolute air temperature minima (30-year temperature record) are lower than frost resistance during the most susceptible stages of each species.

Freezing patterns and ice nucleation temperatures during shoot elongation

Two freezing exotherms were registered for attached shoots in all species at most stages of shoot development (Figure 3A). High-temperature exotherms (E1) were triggered at mean leaf temperatures between –1.1 and –2.3 °C (mean ± SE; –1.5 °C ± 0.2 °C) for all species except *S. aucuparia* in s4 (–3.5 ± 0.6 °C) (Figure 4B). The low-temperature exotherm (E2) occurred below –4.4 °C and, except for *P. abies* and *L. decidua*, remained constant throughout shoot elongation. Expanding buds of *L. decidua* displayed so-called discontinuous freezing with multiple E2s (Sakai and Larcher 1987), i.e., one E1 at about –4.7 °C followed by a series of small E2s at much lower temperatures (around –16.5 °C). Each E2 marked the killing of one leaf in the bud. Thin layers of basal leaf tissue responsible for leaf dropping (Napp-Zinn 1966) may act as ice barriers. During bud break, the range of E2s diminished and drifted to higher temperatures (data not shown) until, in s4, a single E2 at –8.4 °C marked LT_{100} for the whole shoot (–7.8 °C). In *S. aucuparia* and *P. cembra*, E2s were observed only during bud break and the expansion period.

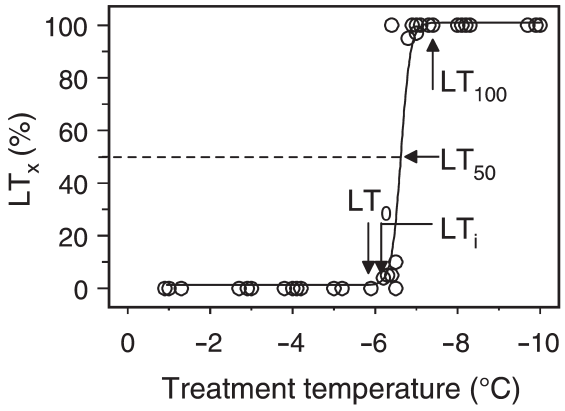

Figure 4. Frost damage was determined by plotting percentage damage against frost treatment temperature. The LT_{50} values, i.e., the temperature causing 50% frost damage, can be read directly from the fitted curve. The LT_0 value is the lowest treatment temperature sustained without frost damage, LT_i is the temperature where incipient frost damage was observed, and LT_{100} is the highest temperature causing 100% tissue death.

Relationship between freezing exotherms and frost damage

For all species at all developmental stages, E1 occurred at a significantly higher leaf temperature than initial frost damage (see Figure 5 for *R. ferrugineum*). Immediate removal of samples at E1 temperatures revealed extracellular freezing, as tissues were stiffly frozen and were infiltrated after thawing but were never frost damaged. Thus, all developmental stages of all species investigated tolerated extracellular ice formation during summer.

For *R. ferrugineum*, from bud swelling onward, a single E2 was recorded at a mean of –6.2 °C. In contrast, significant changes in frost resistance were measured. In several developmental stages, E2 coincided with frost damage, but during the expansion period, E2 occurred significantly below (1.7 °C lower) the lethal temperature (LT_{100}). Therefore, in these stages (s4 and s5), there was no causal relationship between E2 and frost damage.

In the other species, the relationship between E2 and frost damage was somewhat different (Figure 6). As in *R. ferrugineum*, some expanding *P. abies* needles (s5) were killed at temperatures higher than the E2. In *S. aucuparia*, E2s corresponded with LT_i or LT_{100}, and in *P. cembra*, with temperatures causing initial frost damage. In *L. decidua*, the E2 matched killing temperatures at all developmental stages except after full expansion when it corresponded with LT_0 or LT_i.

Discussion

In situ frost resistance and freezing pattern

In our in situ freezing experiments, all conifers tested were significantly more frost resistant (0.5–2.2 °C) than indicated in earlier laboratory freezing experiments (Table 2). This finding is probably associated with differences between in situ and laboratory freezing patterns. Our findings on detached shoots of *P. abies* corroborate results of earlier laboratory freezing experiments where usually only a single freezing exotherm at distinctly lower temperatures than E1 was recorded (Ashworth et al. 1985, Flinn and Ashworth 1994, Neuner and Bannister 1995, Neuner et al. 1997, Fuller and Wisniewski 1998). In contrast, two pronounced freezing exotherms were recorded in our field freezing experiments for all plant species investigated, at most stages of development.

First freezing event

After bud break, E1s were recorded in leaves of all species at mean temperatures of between –1.1 and –2.3 °C (–1.5 ± 0.2 °C), except for *S. aucuparia* in s4 (–3.5 ± 0.6 °C). These values correspond well with observations in earlier field freezing experiments where E1s were registered between –0.6 and –2.6 °C (for review see Pearce 2001). This first freezing exotherm reflects extracellular ice formation. In woody plants, the presence of intrinsic nucleators has been confirmed by infrared video thermography, and intrinsic nucleators have been shown to be active near subzero temperatures (Wisniewski et al. 1997, Fuller and Wisniewski 1998, Workmaster et al. 1999, Carter et al. 2001, Pearce and Fuller 2001). Exposure of leaves to temperatures at which these first freezing exotherms were registered was in all cases non-injurious. Thus, leaves of all investigated species tolerated at least some extracellular ice and subsequent cellular dehydration because cell viability was fully retained despite the presence of extracellular ice at E1. This is in accordance with earlier findings (Pearce 1988) that extracellular freezing is not only a phenomenon of freezing-tolerant plants but also occurs in non-acclimated leaves such as barley and in leaves with no capacity for cold acclimation (Pearce 2001). In freezing-sensitive dicotyledonous species and freezing-tolerant cereals, all tissues within leaves are

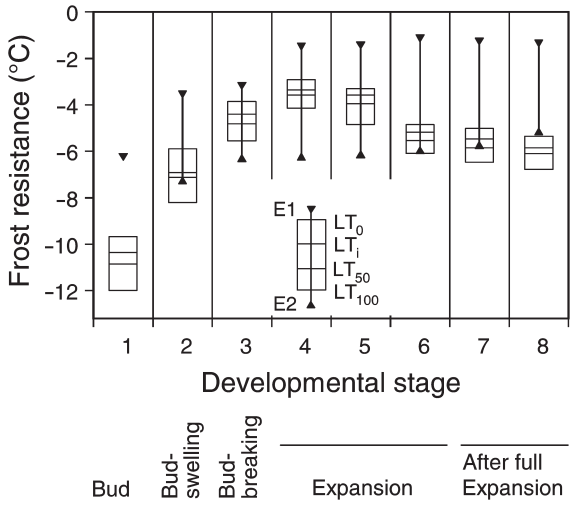

Figure 5. Relationship between freezing exotherms (▼ = E1 and ▲ = E2) and frost damage (bars: LT_0, LT_i, LT_{50} and LT_{100}) in leaves of *R. ferrugineum* measured in situ at 1950 m a.s.l. during the development of the current year's growth over two successive growing periods (2001 and 2002). The mean sample size for each developmental stage was 50.

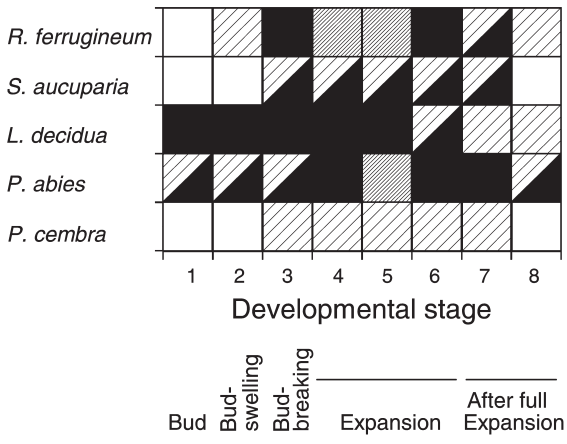

Figure 6. Relationship between low-temperature exotherm (E2) and the extent of frost damage. Symbols: wide-hatched, E2 is not significantly different from LT_0 or LT_i; black, E2 is not significantly different from LT_{50} or LT_{100}; and narrow-hatched, E2 is significantly lower than LT_{100}. White fields indicate the absence of E2s. The mean sample size per species for each developmental stage was 40.

Table 2. Frost resistance of immature leaves of woody timberline species measured by different authors compared with the minimum frost resistance determined in our field freezing experiments. Abbreviations: LT_0 and LT_{50} = temperatures causing 0 and 50% frost damage, respectively.

Species	Degree of damage	Frost resistance data from other authors	Frost resistance measured in situ
Rhododendron ferrugineum	LT_{50}	−3.8[1], −4.0[2]	−3.6
Larix decidua	LT_{50}	−5.0[3]	−6.4
Picea abies	LT_{50}	−3.0[3], −3.5[4]	−4.8
Pinus cembra	LT_0	−2.0[5], −3.5[6]	−4.1

[1] Neuner et al. 1999; [2] Schwarz 1970; [3] Christersson et al. 1987; [4] Repo 1992; [5] Tranquillini 1979; [6] Pisek and Schiessl 1947.

freeze-dehydrated (Pearce 1988, Pearce and Ashworth 1992, Ashworth and Pearce 2002). However, there are also species, such as maize, where certain leaf tissues, such as epidermal and bundle sheath cells, can supercool (Ashworth and Pearce 2002). Although we found an E1 reflecting extracellular ice formation in all leaves, suggesting tolerance of freeze dehydration, supercooling cannot be completely excluded, at least for certain leaf tissues.

Second freezing event and frost damage

All developmental stages showed initial frost damage significantly below E1, indicating that the main mechanism of frost damage, with the possible exception of some supercooling tissues, must be freezing-induced cell dehydration and its consequences. Most E2s measured in situ during shoot expansion matched frost damage (LT_i to LT_{100}) temperatures. These E2s indicate a lethal freezing process. Low-temperature exotherms usually mark the onset of intracellular ice nucleation caused by rupture of cell membranes (Pearce and Willison 1985) or homogeneous ice nucleation after supercooling (Olien 1978, 1981). Although cell membranes are a target for frost damage in freezing-tolerant species, the subcellular target in sensitive species (or stages) has not been identified (Pearce 2001). In expanding shoots of *R. ferrugineum* (s4–s5) and some *P. abies* (s5) shoots, where killing occurred at freezing temperatures higher than E2, the injurious event does not appear to be cell membrane damage because this should have immediately triggered an E2, whereas in *R. ferrugineum* during s4, E2s were not recorded until 2 °C below LT_{100}. These exceptions indicate that freezing exotherms are not a reliable estimator of frost damage.

The difference in frost resistance of up to 2.2 °C that we observed for the conifers between the in situ and laboratory tests can be a crucial point for survival of episodic late spring or summer frosts at the timberline ecotone. After loss of current-year growth because of frost damage, which has been repeatedly observed (Kerner 1869; D. Taschler and G. Neuner, personal observations), conifers do not foliate again until the next growing season. Consequently, frost damage contributes to the distorted growth of trees at the tree line, although it does not appear to be a real threat to survival (Tranquillini 1979, Larcher 1985). In *R. ferrugineum*, frost damage to current-year growth can disorder sexual reproduction and flower development for years even though it does not threaten tree survival (J. Wagner, University of Innsbruck, Austria, personal communication).

Climate warming potentially increases the frequency of unpredictable, episodic frost events (Katz and Brown 1992) and concomitantly promotes earlier bud break (Cannel and Smith 1986, Murray et al. 1989, Hänninen 1991, 1996, Guak et al. 1998). Bud burst is an irreversible process and new shoots may frost-harden to a limited extent only in response to low temperatures. Thus, the risk of frost damage to sprouting leaves may increase with climate warming, as shown for *Pinus sylvestris* L. (Repo et al. 1996). Additionally, elevated atmospheric CO_2 concentration (Lutze et al. 1998, Beerling et al. 2001, Obrist et al. 2001) increases ice nucleation temperatures in some species. Thus, in terms of frost survival, global changes in climate are likely to have a major impact on plant performance at the alpine timberline ecotone.

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