Plant resistance to cold stress: Mechanisms and environmental signals triggering frost hardening and dehardening

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This introductory overview shows that cold, in particular frost, stresses a plant in manifold ways and that the plant's response, being injurious or adaptive, must be considered a syndrome rather than a single reaction. In the course of the year perennial plants of the temperate climate zones undergo frost hardening in autumn and dehardening in spring. Using Scots pine (*Pinus sylvestris* L.) as a model plant the environmental signals inducing frost hardening and dehardening, respectively, were investigated. Over 2 years the changes in frost resistance of Scots pine needles were recorded together with the annual courses of day-length and ambient temperature. Both act as environmental signals for frost hardening and dehardening. Climate chamber experiments showed that short day-length as a signal triggering frost hardening could be replaced by irradiation with far red light, while red light inhibited hardening. The involvement of phytochrome as a signal receptor could be corroborated by respective night-break experiments. More rapid frost hardening than by short day or far red treatment was achieved by applying a short period (6 h) of mild frost which did not exceed the plant's cold resistance. Both types of signals were independently effective but the rates of frost hardening were not additive. The maximal rate of hardening was – 0·93°C per day and frost tolerance of $< – 72°C$ was achieved. For dehardening, temperature was an even more effective signal than day-length.

1. Introduction

1.1 *Ecophysiological aspects of plant cold stress and acclimation*

About two thirds of the world's landmass is annually subjected to temperatures below the freezing point and about half of it suffers from temperatures below – 20°C (Larcher 2001). It is therefore not surprising that the impacts of cold stress on plant life have been comprehensively studied and many attempts have been undertaken, to improve cold resistance in particular of important crop plants. However, progress in achieving frost hardiness of plants either by classical breeding or by gene transfer (Jaglo-Ottonsen *et al* 1998; Kasuga *et al* 1999) is difficult, due to the fact that cold resistance is not a quality conferred by the product of one gene, but has turned out as a syndrome (Beck *et al* 1995; Fowler and Thomashow 2002), comprising many quite different traits of cell biology, such as fluidity of the biomembranes, synthesis and accumulation of low molecular weight (Hansen *et al* 1997, Nanjo *et al* 1999) and high molecular weight (Steponkus *et al* 1998) cryoprotectants, increase of the potential to cope with oxidative stress and others. The syndrome is even more complex because the various tissues of a plant are differently frost resistant, whereby meristematic cells are in general less frosthardy than mature tissues (Sakai and Larcher 1987). Another phenomenon that complicates the investigation of cold as a plant stressor is the seasonal change of frost hardiness of many perennial plants of temperate and

subarctic climates (Repo 1992; Silim and Lavender 1994; Beck *et al* 1995): Needles of the Central European Scots pine (*Pinus sylvestris* L.) are lethally damaged when exposed to – 10°C during the summer months, while in mid winter they survive exposure to – 80°C (Hansen J and Heim R, unpublished results). Frost hardening and dehardening of a plant are extremely slow processes which cannot be studied like metabolic reactions and which require special methods of investigation. Maximum rates of frost hardening of Scots pine needles under laboratory conditions as well as in the natural environment were below – 1°C per day, as will be shown later.

Low temperature may impose stress on a plant in a two-fold manner: By the effects of low temperature alone, and by dehydration of the cells and tissues when cellular water freezes. Several modes of how these stressors can affect a plant are shown in figure 1. Low temperatures above the freezing point are detrimental to many plants of the tropics and subtropics which can not acclimatize to cold. This kind of damage has been termed 'chilling' (Sakai and Larcher 1987) and results primarily from loss of function of biomembranes connected with a decrease of their fluidity and an inactivation or at least deceleration of the membrane-bound ion pumps. Light energy which is absorbed independently of the temperature, produces oxidative stress, if metabolism cannot keep pace with the energetization of the photosynthetic membranes. Freeze dehydration, on the other hand usually takes place at an unexpectedly high extent: More than 75% of the water of a frost-hardy evergreen leaf (*Pachysandra terminalis* Sieb. *et* Zucc.) was frozen to ice that was deposited in the intercellular spaces (Zhu and Beck 1991). Whether, and to which extent a plant becomes damaged by exposure to low temperature depends on many factors, such as its developmental stage, the duration and severity of frost, the rates of cooling (and rewarming) and whether ice

formation takes place intracellularly or extracellularly in the intercellular spaces. Intracellular ice formation, by disintegration of the cellular membranes, is known to be inevitably lethal. The bilayer structure of the biomembranes depends on the hydrophobic interaction with the aqueous cellular phase which cannot be replaced by ice (Gordon-Kamm and Steponkus 1994). An exception to this rule is the artificial vitrification, whereupon amorphous ice is formed due to an extremely rapid cooling (10·000 K × min^{-1}) of the sample (Sakai *et al* 1968). Frost hardiness or sensitivity is a quality of each individual plant and is governed by its genetic potential as well as by environmental factors and therefore usually changes with time.

As mentioned above frost hardening and dehardening are accomplished by thorough changes of a tissue's cell biology. Well known alterations affect the lipid composition of the biomembranes with respect to the maintenance of their fluidity (Quinn 1985; Senser and Beck 1982; Welti *et al* 2002; Williams 1990), the synthesis and accumulation of compatible solutes, the synthesis of cold acclimation induced proteins (Close 1997; Shinozaki and Yamaguchi-Shinozaki 2000), changes in the carbohydrate metabolism (Hansen and Beck 1994; Hansen *et al* 1997; Liu *et al* 1998; Frankow-Lindberg 2001) and the boosting of the radical scavenging potential of the cells (Tao *et al* 1998; Hernández-Nistal *et al* 2002; Baek and Skinner 2003). Less well studied are the signals that trigger frost hardening and dehardening in evergreen perennial plants, such as conifers and even less is known how plants sense such signals. Upregulation of gene expression following exposure to cold has been reported in many studies, mainly with mono- and dicotyledonous herbs (for review see Hughes and Dunn 1996; Shinozaki and Yamaguchi-Shinozaki 2000). Many studies have been performed with Arabidopsis, which, as an annual short-lived herb, may become frost tolerant to only some extent (Steponkus *et al* 1998; Takagi *et al* 2003). Nevertheless, it shows features of frost hardening when exposed to moderate cold and therefore studies with Arabidopsis have extended our knowledge of cold hardening to the level of molecular biology (Shinozaki and Yamaguchi-Shinozaki 2000; for review see Thomashow 2001). Common traits between resistance to cold and to drought have been identified in particular with respect to intracellular signal transduction (Shinozaki and Yamaguchi-Shinozaki 2000), which involves an increase of the cellular calcium level (Monroy *et al* 1993; Monroy and Dhindsa 1995) and the action of abscisic acid (Thomashow 1999; Ishitani *et al* 1997). However, understanding of the molecular biology of cold sensing (as a signal effective in Arabidopsis) is just at the beginning (Monroy *et al* 1997). While exposure to cold, salt or drought are possible signals triggering frost hardening of a short-lived annual herb that under natural con-
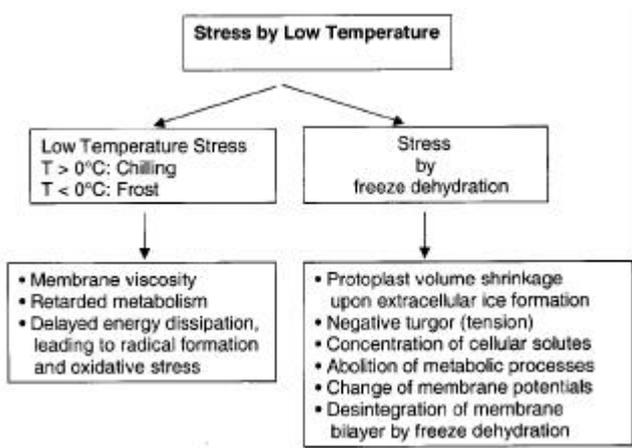

Figure 1. The cold stress syndrome of plants.

ditions does not experience cold, perennial, and in particular evergreen plants must perceive signals triggering frost hardening prior to the incidence of the first frost event. Shortening of the photoperiod together with a decrease of the average temperatures have been identified as effective signals for frost hardening of evergreens (Aronsson 1975; Bervaes *et al* 1978; Christerson 1978; Kandler *et al* 1979; Smits-Spinks *et al* 1985; Tremblay and Lalonde 1987). A prolonged exposure of already moderately frost tolerant plants to non-lethal subfreezing temperatures (Tumanov and Krasavtsev 1959) or a sequence of mild frost events (Greer and Warrington 1982) induces further hardening to the final stage of extreme frost tolerance. Realizing the molecular events following cold treatment of Arabidopsis (Thomashow 2001), questions arise about the efficacy of both types of environmental signals and whether the biochemical routes for achieving frost tolerance triggered by photoperiod or cold are identical. In this article we will concentrate on these questions using *P. sylvestris* (Scots pine) as experimental plant. Since Scots pine keeps its needles for two or three years, dehardening of the frost tolerant needles in spring is a likewise important phenomenon which in principle is understood as a reversal of the processes of frost hardening. This is in particular true for the restitution of the photosystems which have been degraded in the course of frost hardening (Vogg *et al* 1998a, b).

2. Materials and methods

2.1 *Plant material and growth conditions*

Four-year-old Scots pine (*P. sylvestris*) trees, grown in the Botanical Garden of the University of Bayreuth in 8 l containers in an 1 : 1 v/v mixture of sand and leaf mould were used for the outdoor experiments. The containers were completely embedded into a sandy soil bed for equilibration with the ambient soil temperature. For manipulation of the daily photoperiod under outdoor climatic conditions exposure cabinets were used consisting of light-tight, air-ventilated aluminium boxes each accommodating about 30 trees (Beck *et al* 1995). Automatic opening and closing of the lids of the cabinets could be set by a control unit. Three light regimes were applied: Short day (SD, 9 h light, and 15 h dark), long day (LD, 16 h light, 8 h dark) and the naturally changing photoperiod. For long day conditions fluorescent tubes (light colours 20 and 77 Fluora, Osram, FRG) were mounted into the lid of the cabinets and the trees received additional illumination with an immanent R/FR ratio (660 nm/ 730 nm) of 6·0 at a photon flux density of 100 µmol photons $\times$ m^{-2} $\times$ s^{-1} (at the top of the trees) for 7 h.

For climate chamber experiments 5-year-old potted plants which had been grown under ambient conditions were used. Four weeks prior to the red light treatment 6 trees were transferred from the field into the climate chambers and subjected to a + 12°C/ + 6°C day-/night temperature regime. During the main light period the trees were illuminated with white light (HQI 2000/Daylight, Osram, FRG) at a photon flux density of 500 µmol photons $\times$ m^{-2} $\times$ s^{-1} with an immanent R/FR ratio of 1·8.

2.2 *Red and blue light treatments*

R + FR was produced by 3 incandescent tubes (LIN 1604, 60 W, Osram, FRG) and filtered through a red Plexiglas filter with a lower edge at 610 nm. FR light was generated by filtering the red light of 4 incandescent tubes through Plexiglas filters with a lower edge at 710 nm. Irradiance was 12·0 µmol photons $\times$ m^{-2} $\times$ s^{-1} under the R + FR lamps with a R/FR ratio (660 nm/730 nm) of 1·2; under the FR source, irradiance was 7·3 µmol photons $\times$ m^{-2} $\times$ s^{-1} (calculated from the spectral flux distribution curves). Blue light was produced by 8 fluorescent tubes (L8/25, 8W, Osram, FRG) and filtered through a blue Plexiglas filter. Intensity of the blue light source between 425 and 515 nm was 1·5 µmol photons $\times$ m^{-2} $\times$ s^{-1}. In general the intensities of the additional light were too low for powering photosynthesis.

2.3 *Determination of frost hardiness*

Frost hardiness was determined with randomly mixed samples of 15 g (fresh weight) of current-year needles harvested from 20 trees in the field experiment and from 6 trees in the climate chamber experiments. The needle samples of each treatment were divided into 7 subsamples, wrapped into 4 layers of filter paper and enclosed in water-tight steel cylinders. The probes were cooled at a rate of 2°C per hour to the selected subfreezing temperatures in cooling baths, maintained at this temperature for 2·5 h and rewarmed at the same rate to + 4°C. After the freeze-/thaw-cycle the samples were kept at + 4°C for 4 days in the dark in Petri-dishes to allow frost damage to develop. One set of controls was kept at + 4°C for 4 days while the reference sample for maximal damage was killed by heating at 100°C for 10 min. Damage was quantified with the tetrazolium assay, which aims at the function of a plants' energy metabolism, using its reductive potential as criterion. In this assay the endogenous dehydrogenases reduce the artificial electron acceptor triphenyl-tetrazolium-chloride to the deeply coloured formazan (Steponkus and Lanphear 1967; Vogg *et al* 1998a, figure 2) which can be extracted with 96% ethanol and quantified by its absorption at 490 nm.

Correction for light scattering (measured at 780 nm) was made and the data were fitted by the least square method as described by Janácek and Prásil (1991). LT_{50} indicates the minimum temperature of the freeze-thaw cycle at which formation of formazan was 50% of the untreated controls.

Alternatively the photochemical efficiency of PS II instead of the TTC-reduction was measured. The ratio of variable to maximum chlorophyll fluorescence (F_v/F_m) was determined with a PAM 101 fluorometer (H Waltz, Effeltrich, FRG), F_m being measured with a saturating pulse (1 s) of white light (2500 µmol photons $\times$ m^{-2} $\times$ s^{-1}, Xenophot, Osram). A 50% decrement of a tissue's reduc-

ing power or photochemical efficiency after the freeze-thaw cycle is considered as threshold of frost hardiness (lethal temperature 50, LT_{50}).

3. Results

3.1 *Induction and progress of frost hardening*

Frost hardiness of current-year needles of 4-year-old Scots pine trees growing under natural outdoor conditions was determined with the TTC-assay in the course of two con-
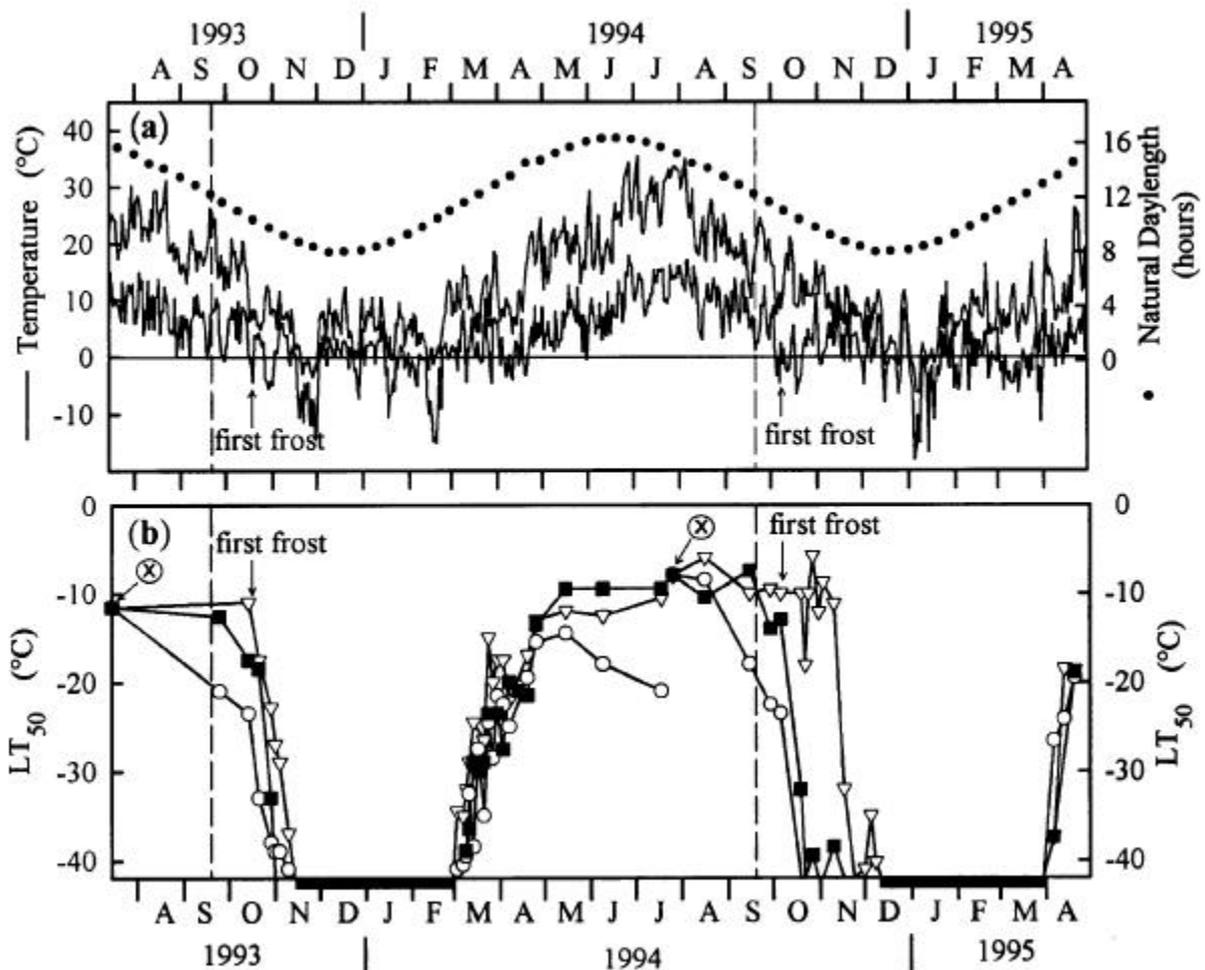

Figure 2. Seasonal course of natural day length and daily minimum and maximum air temperature (**a**), and frost hardiness (LT_{50}) (**b**) of current-year needles of 4-year-old Scots pine (*P. sylvestris*) trees. The trees were subjected to the natural (■), to a constant 9 h (O) and a constant 16 h (∇) daily photoperiod under otherwise outdoor conditions. Temperatures were measured at 2 m (upper curve) and 5 cm (lower curve) above ground level, respectively. The time of autumn equinox is indicated by vertical dashed lines. Arrows indicate transfer of the trees from natural day length to 16 h and 9 h day length (x), and the first incidence of frost temperatures (first frost), respectively. The period of time during which the needles possess extreme frost hardiness ($LT_{50} < -42°C$) is represented by the black bar. The climate data were recorded at the Bayreuth climate station (330 m) of the Deutscher Wetterdienst (Offenbach, FRG).

secutive years (figure 2). During summer and early autumn the needles were irreversibly damaged when exposed to temperatures below – 10°C. This stage was termed frost-sensitive. When natural day length fell below 12 h, frost hardening of the needles started at a moderate rate. After the incidence of the first frost about one month later, the rate of frost hardening increased dramatically and the needles acquired the stage of extreme frost hardiness (i.e. $LT_{50} < – 42°C$) within two weeks.

In order to separate the effects of day length and sub-freezing temperatures on the process of frost hardening, trees were grown under outdoor temperatures, however, under constant short-day (SD, 9 h) or long-day (LD, 16 h) light periods. Under LD conditions the trees remained frost-sensitive until the incidence of subzero temperatures (figure 2). Usually the very first frost events did not exceed the low degree of cold resistance of the needles and the immediately commencing rapid frost hardening prevented the needles from damage by increasing cold stress.

When the trees were subjected to a constant 9 h-photoperiod, induction of frost hardening started already in summer and thus much earlier than in trees growing under the natural day length (figure 2). Even at outdoor temperatures above the freezing point the needles reached frost tolerance of – 20°C to – 25°C. After the first frost events extreme frost resistance was acquired in about a week.

These results could be confirmed during the following year. Under natural conditions the induction of frost hardening was triggered by short day length, however, the degree of frost resistance which the needles attained, varied with the date of the first frost events in autumn (figure 2). Under the 16 h-photoperiod frost hardening was delayed until middle of November, in spite of the fact that the first frost events had occurred already at the end of September (1994). These results clearly demonstrate that in Scots pine both environmental factors, the length of the daily photoperiod and moderate subfreezing temperatures, induce frost hardening independently. Since under natural conditions shortening of the photoperiod precedes the incidence of the first frost events, the hardening process is already in progress when the first frost occurs.

3.2 *Rates of frost hardening*

The rates of frost hardening were calculated from the frost hardiness data measured during autumn and early winter. Under natural conditions, frost hardening of Scots pine needles showed a two-phasic course with a slow initial increase after transition from long- to short-day, followed by a rapid phase after the incidence of frost. Short daily photoperiods and subfreezing temperatures, respectively, induce different rates of frost hardening. Under the influence of a short photoperiod, when ambient tem-

peratures were still above the freezing point, the needles started to harden with a rate of 0·30°C × day^{-1}. This is significantly less than hardening rates of around 0·9°C × day^{-1} which were observed after the first frost events (table 1). A significant difference in the rate of frost-triggered frost hardening could not be observed between trees under 9 h and 16 h daily photoperiod. However, the length of the photoperiod affected the ultimate degree of (extreme) frost tolerance that needles could produce under the influence of subfreezing temperatures. When cooled down to – 72°C needles of trees grown under SD conditions survived with less damage than those of trees kept under long photo-periods (figure 3).

3.3 *Dehardening*

Under natural conditions the trees maintained extreme frost hardiness of below – 42°C during the whole winter (figure 2), irrespective of transient changes of the environmental temperatures. But with the gradual increase of the ambient temperature during early spring the needles started to deharden with an average rate of 0·46°C × day^{-1}. Significant differences between trees subjected to a 9 h or a 16 h photoperiod, or growing under the natural day length were not observed. However, even during the period of dehardening, individual late frost events caused a transient increase in the degree of frost resistance of the needles.

Whereas the needles of the trees that were grown under natural day length or a constant 16 h daily photoperiod were completely dehardened until mid May, those maintained under the 9 h-photoperiod exhibited a second increase of the frost tolerance of the then previous-year needles during spring and early summer.

3.4 *Extension of day length with red and far red light*

Since the strong influence of the photoperiod on frost hardening suggests the involvement of a phytochrome as a photosensor, we investigated the influence of FR and R

Table 1. Rates of frost hardening of current-year needles of 4-year-old Scots pine (*P. sylvestris*) trees under outdoor climatic conditions.

Daily minimum temperature	Day length (h)	Rate of frost hardening (°C·day^{-1})
< 0°C	9	0·85[a] (n = 4)
	16	0·93[a] (n = 6)
> 0°C	9	0·30[b] (n = 4)

[a,b]Significant differences ($P < 0.05$) between regression coefficients.
Rates were calculated as the regression coefficients from the data shown in figure 2.

(+ FR) on the frost hardening process. FR had to be administered together with R to maintain a constant level of FR which otherwise would be degraded (Clough and Vierstra 1997). Frost-sensitive trees were transferred to climate chambers, where they were adapted for 2 weeks to a + 12°C/ + 6°C day-/night temperature regime and a 16 h-photoperiod with white light. Thereafter FR or R + FR were supplied for 30 min at the end of the white-light period. During that period a significant effect of the additional illumination on the frost tolerance of the needles could not be observed (figure 4). However, reduction of the white light period from 16 h to 9 h and expansion of the FR or R + FR-period from 30 min to 5 h resulted in the induction of frost hardening. After three months the needles that had received only FR in the day-extension had acquired extreme frost hardiness of below – 45°C, whereas the needles of the trees treated with R + FR were significantly less frost tolerant (– 28°C, figure 4).

3.5 *Influence of night-break treatment with R and FR on dehardening*

Considering these findings an effect of FR or R on dehardening of Scots pine trees could also be expected. When

during winter extremely frost tolerant Scots pine trees from the field ($LT_{50} < – 42°C$) were brought into climate chambers and subjected to moderate temperature conditions, dehardening became obvious after about one week (figure 5). A night-break treatment of 5 h with FR, however, resulted in an incomplete loss of frost hardiness of the trees and the needles maintained a moderate frost tolerance of below – 30°C. When the night-break treatment was performed with R + FR, the needles were significantly less frost-hardy (– 17°C).

When the night break treatment with FR was immediately followed by an equally long (5 h) treatment with R + FR, the effect of the FR was reversed and the degrees of frost tolerance of the needles were not significantly different from those which had been illuminated with R + FR only (figure 5). The reversibility of the physiological response to FR by R suggests the involvement of a phytochrome system in regulating the degree of frost tolerance.

However, also blue light has a marked effect on the frost tolerance of the needles. When a night break with FR was followed by a treatment with R + FR + blue light,
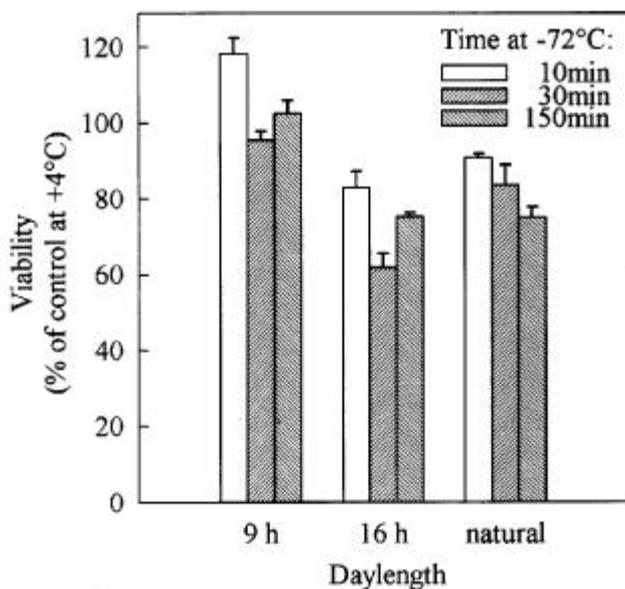
Figure 3. Viability of current-year needles of Scots pine after frost treatment at – 72°C for 10, 30, and 150 min. Naturally frost hardened needles were harvested on January 22, 1995 and cooled to – 42°C with a cooling rate of 2°C × h⁻¹. Subsequently the samples were stored over dry ice for the time interval as indicated and rewarmed with the same rate as for cooling to + 4°C. Viability after frost treatment was expressed as the percentage of 2,3,5-triphenyltetrazolium chloride (TTC) reduction as compared to untreated control needles (100%).
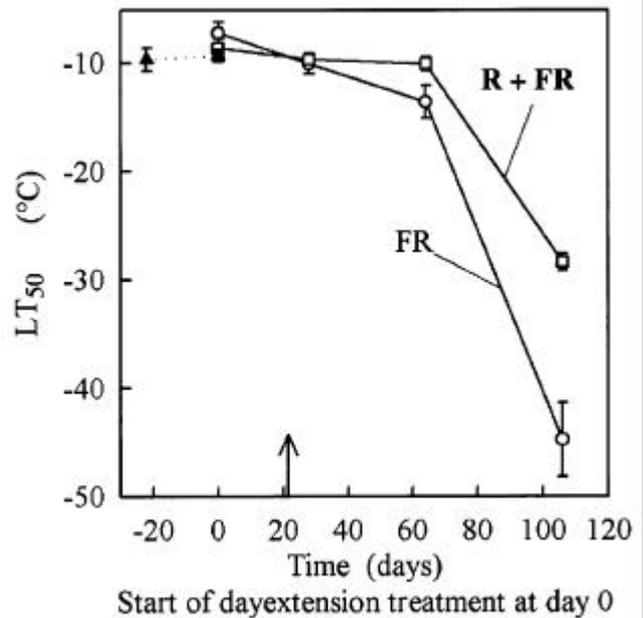
Figure 4. Effect of day extension with R + FR (□) or FR (O) on frost hardiness (LT_{50}) of current-year needles of 4-year-old Scots pine trees. Frost sensitive trees (▲) were transferred from the greenhouse into climate chambers 20 days before the onset of the day extension treatment and subjected to a + 12°C/ + 6°C day/night temperature regime and a daily white light period of 16 h. At the beginning of the FR or R + FR treatment the trees received a 16 h period of white light and a day extension of 30 min. After 3 weeks (arrow) the white light was reduced to 9 h and the period with FR or R + FR was extended to 5 h.

a complete loss of frost hardiness resulted. The needles were lethally damaged as soon as freezing of the cellular water took place.

Despite of the maintenance of the climatic or light conditions, and irrespective of the light quality to which the trees had been subjected during the night break treatment, the mature (i.e. the previous-year) needles, after dehardening for about 80 days, started a second hardening (figure 5). Obviously the immature needles were unable to permanently deharden in that stage of development. The rates of frost hardening were similar (between 0·27 and 0·35°C per day) in all sets of samples and a memory effect of the pre-treatment was not detectable. However, significant differences in the degree of frost hardiness were observed at the end of the experiment, depending on the degree of frost tolerance or sensitivity which the trees had achieved at the beginning of the endogenous frost hardening process.
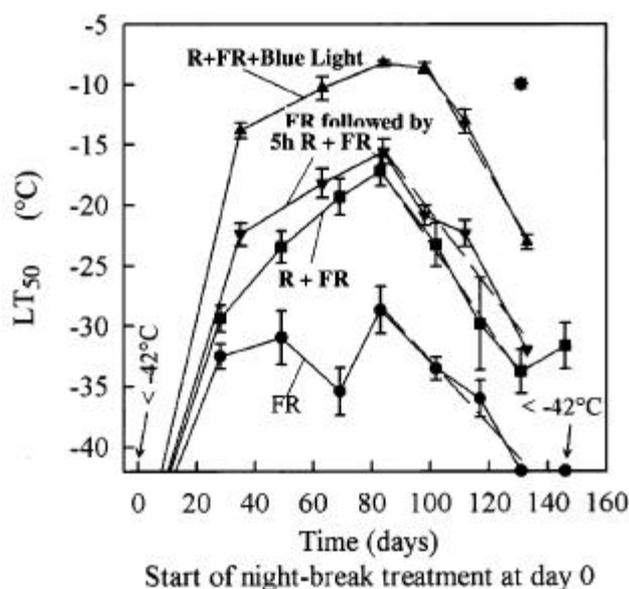

Figure 5. Effect of the light quality of night-break treatments on frost hardiness (LT_{50}) of current-year needles of 4-year-old Scots pine trees. At the beginning of the experiment (day 0) naturally frosthardened trees ($LT_{50} < -42$°C) were transferred from the field to the climate chamber and subjected to a + 12°C/ + 6°C day/night temperature program and a 9 h period with white light. During the dark period the trees received night-breaks (5 h) with R + FR (■), FR (●), R + FR + blue light (▲), and FR followed by 5 h with R + FR (▼), respectively. After bud break the developing needles were frost-sensitive (✳). The rates of frost-hardening calculated as the regression coefficients according to the regression lines were determined with 0·35 (■, $r^2 = 0.994$), 0·32 (●, $r^2 = 0.958$), 0·27 (▲, $r^2 = 0.970$), and 0·33 (▼, $r^2 = 0.989$) °C × day^{-1}; differences between the regression coefficients are not significant ($P > 0.2$).

3.6 *Influence of the temperature on frost hardening*

To investigate the influence of subzero temperatures on the frost hardening process the plants were kept under long day-conditions and four sets (each consisting of 6 plants) of trees were subjected to consecutive series of 2, 4, 6, and 8 frost events at – 4°C, each for 6 h during the 8 h dark periods. Five weeks prior to the first frost treatments the trees had been transferred from the field into climate chambers where they were stepwise adapted to the + 10°C/ + 6°C day-/night-temperature regime at a 16 h daily photoperiod.

One day after the end of the initial series of 2, 4, 6, and 8 frost nights a constant increase of the frost tolerance was observed, irrespective of the number of frost events to which the trees had been subjected (figure 6). Significant differences in the rates of hardening of the different sets of plants could not be found; all trees hardened at an average rate of 0·86°C × day^{-1}. After intervals of between 10 and 17 days at non-freezing temperatures half of each set of plants were subjected to a second identical series of frost events, and after another 15-days interval at non-freezing temperatures half of the second sample was subjected to a final set of two frost nights. Remarkable differences in the frost hardiness of the various treatments was observed with the lowest degree of frost tolerance of – 32°C of needles from those trees which had been subjected to 2 + 2 + 2 frost nights and the highest frost resistance of about – 42°C of the sample that had been treated with 8 + 8 + 2 frost nights. These results clearly demonstrate that a low number of frost events, presumably even one single frost event is effective in inducing the onset of the frost hardening process. Once it is induced the frost hardening process continues for at least 2 weeks before temperatures above the freezing point trigger dehardening. Thus, to achieve extreme frost hardiness recurrent frost events are necessary and a higher frequency of frost events results in a higher degree of frost resistance, although the rate of frost hardening remains more or less the same.

4. Discussion

4.1 *Induction of frost hardening by photoperiod and temperature*

Low temperature, the length of the daily photoperiod, and the quality of the incident light are the main environmental factors that influence the induction of frost hardening in Scots pine trees. Under natural conditions the development of frost tolerance takes place bi-phasically. The slow initial phase requires exposure to a photoperiod below 12 h, the second phase exposure to subfreezing temperatures (figure 2). Such biphasic course of frost har-

dening is not only specific of evergreen conifers (Christersson 1978; Silim and Lavender 1994), but has also been observed in broad-leaved trees (Howell and Weiser 1970; Tremblay and Lalonde 1987).

The experiments described in this work show, that both factors, short photoperiod and low temperature, induce the process of frost hardening independently, and that exposure to a short photoperiod is not a prerequisite of

frost hardening triggered by subfreezing temperatures (figure 2). Achievement of cold hardiness by exposing plants to either short photoperiods or low temperature has also been found in other woody plant species like *Picea abies* (Christersson 1978), *Taxus cuspidata* (Zehnder and Lanphear 1966) and *Cornus stolonifera* (Chen and Li 1978).

Because of significant differences in the rates of hardening under short-day and frost conditions (table 1), these
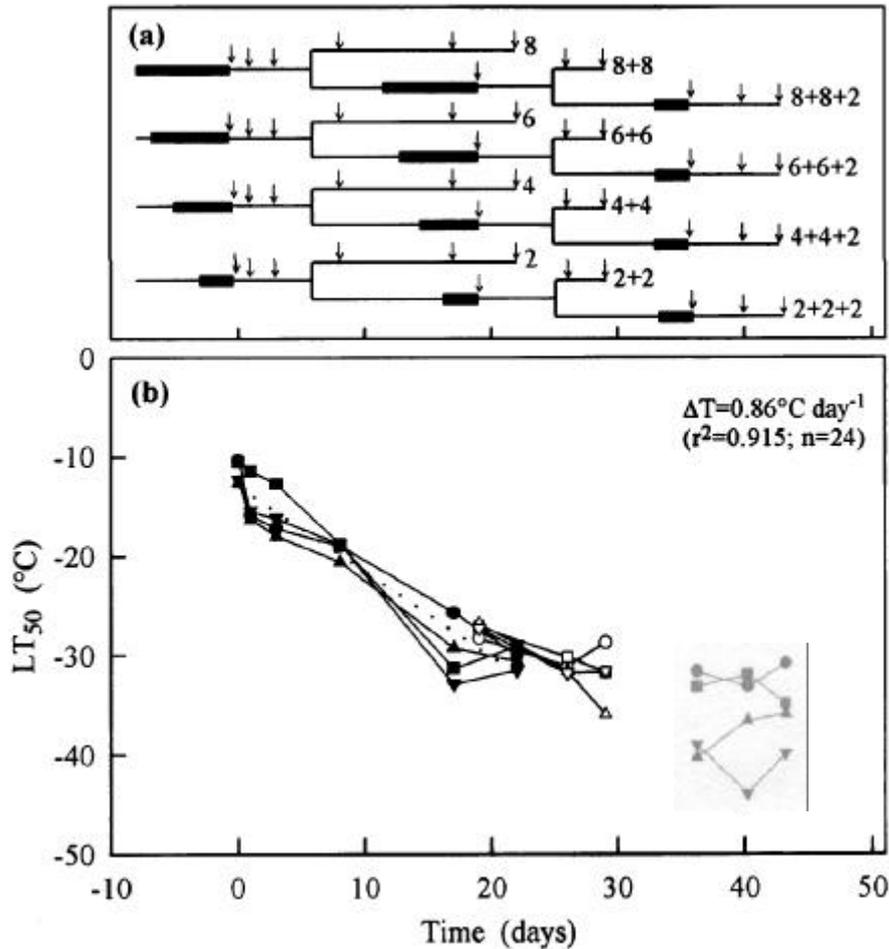

Figure 6. Influence of nocturnal frost on frost hardiness of current-year needles of 4-year-old Scots pine (*P. sylvestris*) trees. Frost sensitive trees were transferred from the field into climate chambers five weeks prior to the beginning of the experiment. During that period temperature was reduced in weekly intervals from a $+18°C/+12°C$ to a $+10°C/+6°C$ day-/night-temperature regime (18 h/6 h). (**a**) During the course of the experiment the trees received a daily 16 h photoperiod, at the day temperature of $+10°C$ (18 h) and were cooled during the night to $-4°C$ (6 h) (bold line), and $+6°C$ (thin line), respectively. The maximum numbers of frost nights each set of plants has received, and the dates of needle harvest ($\downarrow$) are indicated. (**b**) Frost hardiness (LT_{50}) of trees that have received a series of 2 ($\bullet$), 4 ($\blacksquare$), 6 ($\blacktriangle$), or 8 ($\blacktriangledown$) frost nights (closed symbols). Part of each set of plants received a second set of 2, 4, 6, or 8 frost nights (open symbols) and a third treatment of two frost nights (grey symbols) as indicated by the numbers in (**a**). The regression line (dotted) and the regression coefficient ($°C \times day^{-1}$) of the initial phase of frost hardening is indicated. Differences of the regression coefficients among the various treatments are not significant ($P > 0·9$).

environmental factors apparently induce frost hardening in different ways. Under natural conditions Scots pine trees achieve moderate frost hardiness under the influence of short photoperiods and extreme frost hardiness by the influence of subfreezing temperatures. However, the experiments with red- and far red light illumination showed that extreme frost hardiness can also be accomplished by light treatment alone.

The critical day length of 12 h for the induction of frost hardening under natural conditions is in agreement with findings in *Pinus radiata* (Greer and Warrington 1982) and *Pinus contorta* (Jonsson *et al* 1981). In several conifer species an inverse linear relationship between photoperiod and frost tolerance has been described (Jonsson *et al* 1981; Greer *et al* 1989; Bigras and D'Aoust 1993). Although the trees under natural conditions experience progressively decreasing photoperiods in autumn, our experiments in the field as well as under controlled, i.e. constant light periods clearly show that a constant short-day treatment is as effective as a decreasing photoperiod for the induction and maintenance of the frost hardening process. This has also been suggested for the needles of *P. abies* (Qamaruddin *et al* 1993).

4.2 *The dehardening process*

In *P. sylvestris* dehardening took place when in early spring day temperature exceeded + 10°C for about one week (figure 2), as is the case with a number of other evergreen tree species such as *T. cuspidata* (Zehnder and Lanphear 1966), *Pseudotsuga menziesii* (Van den Driesche 1969), *P. abies* (Aronsson 1975), and *P. radiata* (Greer and Stanley 1985). Dehardening appears to be less dependent on the photoperiod than hardening, since significant differences in its rates in long-day and short-day plants were not observed (figure 2). Overruling of the effect of the photoperiod by that of temperature in the dehardening process is also demonstrated by transient increases of frost tolerance upon incidence of late frost events (figure 2).

4.3 *The influence of light quality on frost hardiness*

The efficacy of FR and R, respectively, in manipulating the degree of frost resistance of Scots pine trees indicate the involvement of a phytochrome system (figure 4, 5). An increase in frost tolerance under the influence of FR has also been found in *P. banksiana* (Hoddinott and Scott 1996) and in a deciduous tree species (*Cornus stolonifera*, McKenzie *et al* 1974).

During dehardening triggered by temperature an interruption of a long night with FR as well as with R + FR resulted in the maintenance of frost hardiness to different extents. A night break with FR inhibited the dehardening

process more than illumination with R + FR (figure 5). Additional blue light during the R + FR night break on the other hand resulted in an accelerated and complete dehardening. An inhibiting effect of blue light on cold acclimation under long- but not under short-day conditions has been reported for *Salix pentandra* (Juntilla and Kaurin 1990). In Scots pine seedlings coaction of phytochrome and blue/UV-A light has been established for light control of hypocotyl growth and for the control of enzymes of the nitrogen metabolism (Fernbach and Mohr 1990; Elmlinger and Mohr 1994; Mohr 1994).

In our experiments frost hardening unexpectedly commenced again after a 2 to 3 months period of dehardening, even under a dehardening temperature regime and irrespective of the light quality the trees had been receiving during the night break treatment (figure 5). Frost hardiness increased linearly with the same rate as under natural and short photoperiods (table 1). Although the extraordinary length of the experiments may have triggered an unnatural performance of the needles, it can not be excluded that in mature needles endogenous stimuli contribute to the signals triggering the second frost hardening process in their life-time.

4.4 *Subfreezing temperatures as a signal inducing frost hardening*

To the best of our knowledge this is the first investigation on the number of short-term frost events which suffices to induce frost hardening in evergreen conifers. For *P. radiata* the efficacy of sequential non-lethal frost events in increasing the frost tolerance has been shown by Greer and Warrington (1982). However, in our experiments the number of consecutive frost events did not influence the rate of frost hardening (figure 6). A higher frequency of frost events appears to be necessary for the continuation of the frost hardening process, rather than for the achievement of an increased rate of frost hardening. Irrespective of short intermittent periods with moderate positive temperatures frost hardening continues as long as triggering frost events occur and therefore the final degree of frost resistance may be a function of the number and duration of repetitive frost events. In contrast to the signal transmitted by day length, perception of cold as a signal for frost hardening has not yet been unravelled.

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