

## Ice nucleation and frost resistance of *Pinus canariensis* seedlings bearing needles in three different developmental states

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**Abstract** – Frost resistance and ice formation in different developmental states of needles of *P. canariensis* seedlings were assessed. Regrowth after frost damage was used to determine the overall frost survival capacity. Two distinct freezing exotherms (E1, E2) were registered. E1 was between  $-1.7$  and  $-2.0$  °C. Initial frost damage (LT<sub>10</sub>) was  $1.5$ – $2.7$  °C below E1. E2 was between  $-5.6$  and  $-6.0$  °C, and either corresponded with LT<sub>50</sub> or occurred in between LT<sub>10</sub> and LT<sub>50</sub>. Current year needles were less frost resistant than 1-year-old needles. The overall recuperation capacity of seedlings revealed that frost survival may be underestimated when only needle damage is assessed. Freezing of seedlings with or without roots had no effect on the frost resistance of needles but recuperation capacity was significantly affected. Seedlings survived  $-10$  °C during summer indicating that they withstand the lowest naturally occurring frosts in Tenerife.

**extracellular ice formation / freezing exotherm / *Pinus canariensis* / regrowth / subzero temperatures**

**Résumé** – Nucléation de glace et résistance au froid de semis de *Pinus canariensis* portant des aiguilles à trois différents stades de développement. La résistance au froid et la formation de glace dans des aiguilles à différents stades de développement ont été déterminées chez des semis de *Pinus canariensis*. La repousse après les dommages du froid a été utilisée pour déterminer l'ensemble de la capacité de résistance au froid. Deux exothermes de congélation (E1, E2) ont été enregistrés. E1 était entre  $-1,7$  et  $-2,0$  °C. Les premiers dommages du froid (LT<sub>10</sub>) ont été constatés entre  $1,5$  et  $2,7$  °C sous E1. E2 était entre  $-5,6$  et  $-6,0$  °C et soit correspondait à LT<sub>50</sub> ou arrivait entre LT<sub>10</sub> et LT<sub>50</sub>. Les aiguilles de l'année en cours ont été moins résistantes au froid que les aiguilles âgées de 1 an. La capacité de récupération globale des semis a révélé que la survie au froid pouvait être sous estimée quand on détermine seulement les dommages subis par les feuilles. La congélation des semis avec ou sans racines n'a pas eu d'effet sur la résistance au froid des aiguilles, mais la capacité de récupération a été significativement affectée. Les semis ont survécu à  $-10$  °C pendant l'été indiquant qu'ils étaient capables de résister aux basses températures qui se produisent à Ténérife.

**formation de glace extra cellulaire / exotherme de gel / *Pinus canariensis* / repousse / températures en dessous de zéro**

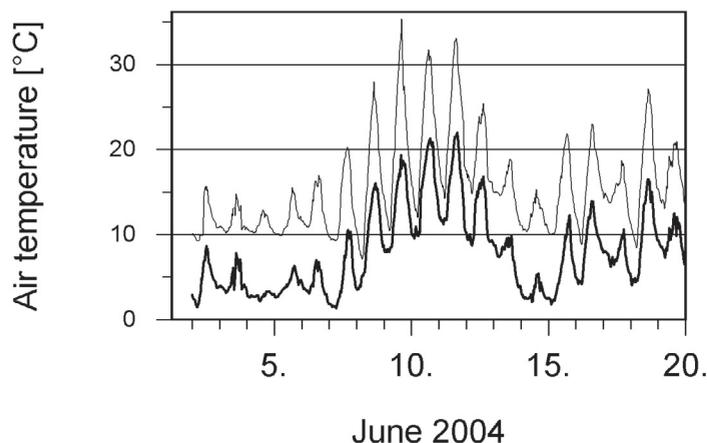
**Abbreviations:** LT<sub>10</sub>: Temperature at 10% frost damage; LT<sub>50</sub>: Temperature at 50% frost damage; LT<sub>100</sub>: Temperature at 100% frost damage; E1: High temperature freezing exotherm; E2: Low temperature freezing exotherm; ΦPSII: Photochemical Efficiency of Photosystem II.

### 1. INTRODUCTION

Freezing stress is one of the most important environmental constraints limiting plant distribution [26]. During sprouting conifers are particularly susceptible to frost damage as their comparatively low frost resistance coincides with sub-zero temperatures [27, 28]. Frost damage to expanding leaves and shoots of conifers has been repeatedly observed in the timberline ecotone of the European Alps [11, 27]. Even *Pinus cembra*, which is one of the most frost resistant conifers (maximum frost resistance  $< -90$  °C (see [3]); USDA climatic zone 1 ( $< -45.6$ )) may get damaged during sprouting. In this

species initial frost damage at  $-4.8$  °C which is surprising in an evolutionary sense. Ultra-structural changes during sprouting and cell elongation may result in insufficient frost hardening [27]. Seedlings of European timberline conifers have a mature, fixed growth pattern. Between the formation of shoot and needle primordia in summer there is a considerable time-lapse before sprouting in the following spring. During winter the primordia are protected in buds covered by scales and resinous material. In contrast, seedlings of several Mediterranean pines exhibit a juvenile, free growth pattern where stem units elongate shortly after their formation throughout the year [5, 13]. In seedlings of *Pinus canariensis* C. Sm ex D.C., the free growth habit can persist for 2 to 7 years [12]. Due to this juvenile, free growth pattern, seedlings and young plants continuously bear

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**Figure 1.** Air temperature ( $^{\circ}\text{C}$ ; half an hour mean values; 2 m above ground) recorded during June 2004 (thin line) in the Botanical garden of the university of Innsbruck (600 m a.s.l.) and (thick line) at 1950 m a.s.l. on Mt. Patscherkofel (Klimahaus).

sprouting shoots that are potentially very frost susceptible. In this study we aimed to assess frost resistance of needles in seedlings of *P. canariensis* in different stages of development: currently expanding, fully expanded, and 1 year old needles.

Some seeds of *P. canariensis* may germinate in autumn [16] and particularly suffer from frosts during winter. In reforestation, seedlings are usually planted in autumn to avoid drought, but this may render the seedlings susceptible to autumn frosts. While mature needles of *P. canariensis* from adult individuals have quite a low frost resistance compared to other pines (USDA Zone 9 ( $-1.1$  to  $-6.6$   $^{\circ}\text{C}$ )) they tolerate frosts as low as  $-10$   $^{\circ}\text{C}$  ( $\text{LT}_{10}$ ; [24]), little is known about the frost resistance of seedlings. In a reforestation project (Aconcagua, Chile) *P. canariensis* seedlings were found to survive two consecutive winters with temperatures down to  $-12$   $^{\circ}\text{C}$  without frost damage – seedling age and the developmental stages of needles appeared to influence frost survival [6]. Frost survival of seedlings can be considered as a crucial point for the establishment and the distribution of a species. Their frost resistance, however, can deviate distinctly from adults: seedlings of the same species have shown to be more [1, 2, 18], similar [8] or even less frost resistant [14, 19, 25] than adult trees. We therefore aimed to assess the overall frost survival capacity of *P. canariensis* seedlings. The combination of recent methodological approaches in testing frost resistance, including chlorophyll fluorescence, measurement of ice nucleation [27, 28] and electrolyte leakage should allow insights into the mechanism of frost resistance and susceptibility of PSII of *P. canariensis* needles to extracellular ice. The recuperation and survival capacity after frost damage of variable degrees of severity was assessed in whole plant regrowth tests.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

*Pinus canariensis* seedlings (Tenerife, Canary Islands, Spain; provenance FS-27/01/38/004) were grown in commercial containers

(ForesPot 400<sup>®</sup>) within a mixture (3:1) of Peat (Floratorf<sup>®</sup>) and Vermiculite (Europerlita S.A., Spain). 4g/L of low release fertilizer (Osmocote Plus: 16/ 8/12; N/P/K; Scotts, The Netherlands) was added to the substrate. Fifteen months old seedlings were sent to Austria by plane in February 2004 and grown there for three more months in the greenhouse of the Botanical Garden of the University of Innsbruck (natural daylength; day/night air temperature fluctuation: 25/10  $^{\circ}\text{C}$ ). Until the beginning of the experiments the seedlings had developed three kinds of needles: Last year's needles (1-year-old), fully expanded needles developed in the current year (new, fully expanded) and needles currently expanding (new, expanding).

### 2.2. Frost hardening treatment

The frost hardening potential of *P. canariensis* seedlings during sprouting was evaluated by using a frost hardening treatment. Half of the seedlings was transferred to the timberline (Klimahaus Research Station, 1950 m a.s.l.) on the north-west facing slope of Mt. Patscherkofel ( $47^{\circ} 14' \text{ N}$ ,  $11^{\circ} 30' \text{ E}$ ) near Innsbruck, Austria. There, seedlings were exposed to the prevailing subalpine environmental conditions (Fig. 1). From the elevational difference (1350 m) we expected a drop in air temperature of 10  $^{\circ}\text{C}$ .

### 2.3. Freezing treatment

Controlled freezing of potted *P. canariensis* seedlings was conducted in two ways. In the first experiment we used a recently developed field portable freezing system (MCC-6, BK-Elektronik, Natters, Austria; <http://www.bk-elektronik.com>) that consists of six freezing chambers, each of them to be programmed independently by a control unit. Each freezing chamber (interior diameter  $11 \times 11 \times 15$  cm) permits the insertion of shoots that remained attached to the plant [27], while the roots remained outside and untreated. In the second experiment frost treatments were conducted inside computer controlled commercial freezers (Huber, Innsbruck, Austria) where the whole potted, plants including their roots, were exposed. In both methods, controlled freezing programs followed a constant cooling and thawing rate of 2  $^{\circ}\text{C h}^{-1}$  and a 4-h- exposure to six different target freezing temperatures [27]. Deviations from the pre-programmed set-point

temperatures were less than  $\pm 0.2$  °C. The exposure temperatures were selected so the highest temperature did no damage and the lowest killed all the leaves ( $LT_{100}$ ). The difference between adjacent target temperatures was less than 1.5 °C.

## 2.4. Viability assay

Frost damage was assessed 2 days after the end of the frost treatment by electrolyte leakage. Similar portions of needles were put in 3 mL of deionized water for 24 h and then electrolyte leakage was measured with a conductivity meter (HDSL13, Delta Ohm, Padova, Italy). Relative conductivity was used as a measure of frost damage (%) and determined after the method described by Neuner and Buchner (1999). Percentage frost damage was then plotted against treatment leaf temperatures. A classic logistic function was fitted to the data using P-Fit software (Biosoft, Durham, USA).  $LT_{50}$ -values, i.e., the temperature at 50% frost damage, can be read directly from the curve fitting protocol.  $LT_{10}$ , the temperature at 10% frost damage, was determined graphically from the calculated and plotted logistic curve.  $LT_{100}$  is the highest temperature causing 100% tissue death.

## 2.5. Assessment of the recuperation capacity

The assessment of the recuperation capacity was conducted one month after the freezing treatment. During recovery plants were cultivated in the greenhouse under natural daylength, controlled temperature conditions (day/night air temperature fluctuation: 25/10 °C) and were regularly watered.

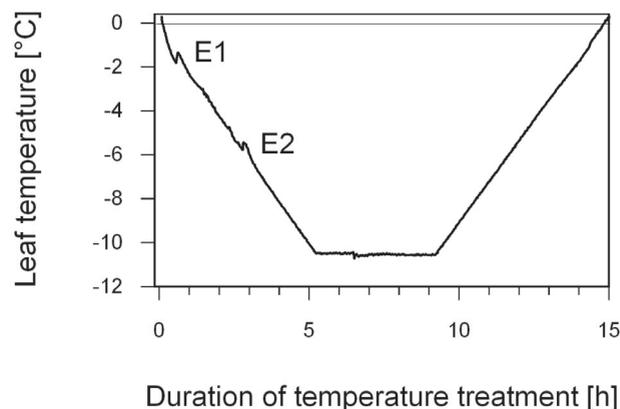
The recuperation capacity was determined using a numerical classification system: (0) when all kinds of needles were killed and no regrowth was observed; (1) when regrowth occurred although current-year needles were frost killed; (2) when despite frost damage to current-year needles regrowth occurred from resting stem buds and finally (3) was assigned when plants were undamaged.

## 2.6. Ice nucleation temperatures

During the cooling phase of the freezing tests (cooling rate: 2 °C h<sup>-1</sup>) ice nucleation temperatures were recorded with type T copper constantan fine-wire thermocouples (welding spot diameter: 0.127 mm). Temperatures were measured every 12 s with a CR10X Micrologger (Campbell Scientific Instruments, Logan, USA). Thermocouples were fixed to the leaves with lightweight, thermally insulated leaf clips. Ice nucleation temperatures were determined graphically from the temperature record (Fig. 2). Usually two distinct freezing exotherms were recorded, a high temperature exotherm (E1) and a low temperature exotherm (E2).

## 2.7. Effects of frost on PSII photochemical efficiency

Photochemical efficiency of PSII ( $F_v/F_m$ ) in all three types of needles ( $N = 10$ ) was measured 24 h after the frost treatment using a portable chlorophyll fluorometer (Mini-PAM, Walz, Effeltrich, Germany). Basic fluorescence,  $F_0$ , was determined after sufficient dark adaptation. Maximum fluorescence ( $F_m$ ) was measured during a 0.8 s saturating flash at 6000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .  $F_v/F_m$  was then calculated as  $(F_m - F_0)/F_m$ .



**Figure 2.** Two distinct freezing exotherms, E1, corresponding with extracellular ice formation and E2, released during intracellular freezing, were recorded on 1 year old needles of potted seedlings of *P. canariensis* during controlled freezing treatments (cooling and thawing rates: 2 °C h<sup>-1</sup>; exposure time: 4 h).

## 2.8. Statistical data analysis

Frost resistance and ice nucleation were determined on 1-year-old, fully expanded and expanding current-year needles of 36 seedlings. The significance of differences between mean values of frost resistance and ice nucleation temperatures was determined by analysis of variance (ANOVA) and the Tukey-b test ( $p < 0.01$ ) using 12.0 SPSS software (SPSS, Chicago, IL, USA).

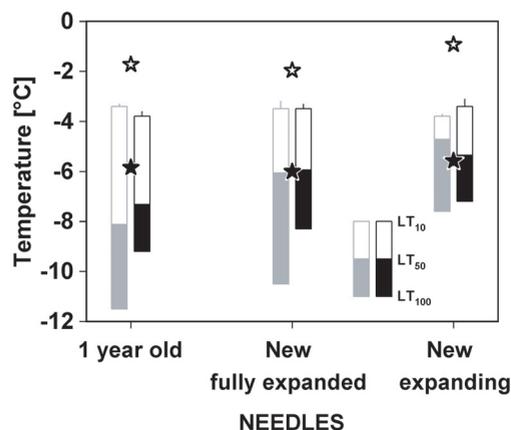
## 3. RESULTS

### 3.1. Freezing patterns and ice nucleation temperatures

Irrespective of needle age two distinct freezing exotherms were registered (Fig. 2). In currently expanding needles the first freezing event (E1) was recorded at significantly higher freezing temperatures (c.  $-0.9$  °C) than in needles at other developmental states. E1 occurred between  $-1.7$  and  $-2.0$  °C and E2 was on average 4.3 °C lower than E1 (Fig. 2). E2 ranged between  $-5.6$  °C and  $-6.0$  °C and was unaffected by needle age.

### 3.2. Relationship between freezing exotherms and frost damage

In *P. canariensis*, initial frost damage ( $LT_{10}$ ) occurred at temperatures between 1.5 and 2.7 °C colder than the formation of extracellular ice (E1; Fig. 3). Irrespective of the developmental state of the needles, extracellular ice formation was to some extent tolerated even during summer. In current-year needles, E2 coincided with 50% frost damage, in older needles, E2 occurred between  $LT_{10}$  and  $LT_{50}$ . The temperature range associated with frost damage ( $LT_{10}$ - $LT_{100}$ ) differed significantly between needle ages. While currently expanding



**Figure 3.** Frost resistance ( $LT_{10} \pm SE$ ,  $LT_{50}$  and  $LT_{100}$ ) of 1 year old, fully new expanded and new expanding needles of *P. canariensis* seedlings measured before (grey bars) and after (black bars) a frost hardening treatment under natural subalpine environmental conditions. E1 (open star) was recorded slightly above initial frost damage ( $LT_{10}$ ) and recorded at mean at 4.3 °C higher freezing temperatures than E2 (black star).

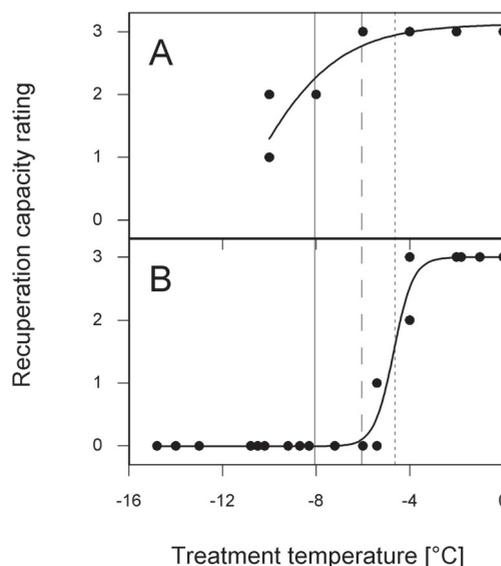
leaves showed a rapid increase in frost damage ( $LT_{10}$ - $LT_{100}$  4 °C), it was twice as slow for the older needles ( $LT_{10}$ - $LT_{100}$  up to 8 °C). The frost hardening treatment conducted under natural subalpine environmental conditions had no significant effect on frost resistance ( $LT_{10}$ ,  $LT_{50}$ ) of *P. canariensis* needles.

### 3.3. Recuperation capacity after frost damage

The comparison of frost resistance of *P. canariensis* needles with the recuperation capacity of seedlings (Fig. 4A) reveals that frost survival is underestimated when only needle damage is assessed. Even after complete loss of needles, sprouting from resting buds on the remaining intact shoot made regrowth possible. Figure 4B shows the recuperation capacity of seedlings that were frost treated as a whole, including roots. Despite the contrasting frost treatment, with or without roots, the same frost resistance of needles was recorded. However, recuperation capacity of seedlings was significantly affected as they would not survive a frost of -6 °C while without root freezing they survived exposure to freezing temperatures down to -10 °C.

### 3.4. Frost susceptibility of PSII

Frost susceptibility of PSII of 1 year old and currently expanding needles is shown in Figure 5. No significant differences in  $F_v/F_m$  were found in control values between needles of different age, being within the range obtained for *Pinus canariensis* [9, 17, 23]. While extracellular ice formation had hardly an effect on PSII, a significant reduction of photosystem II efficiency was observed with the onset of frost damage. In



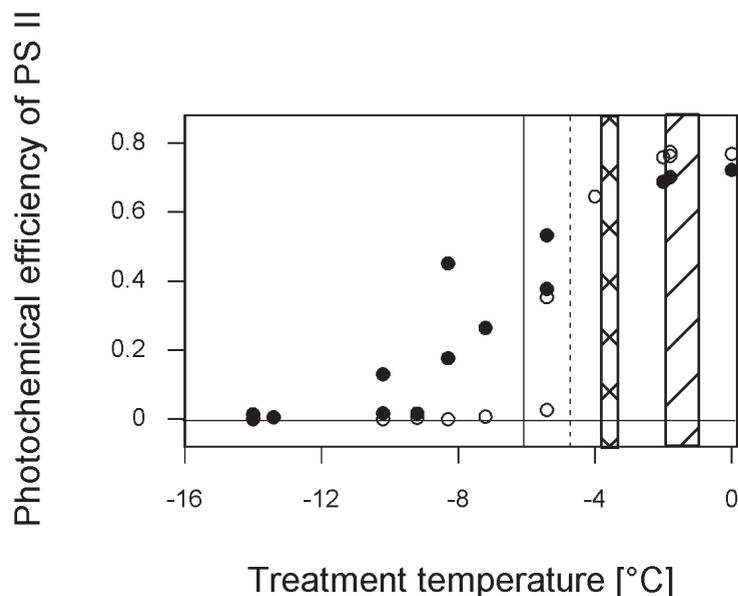
**Figure 4.** Recuperation capacity of *P. canariensis* seedlings after a frost treatment of (a) only the above ground parts and (b) of the whole seedlings including roots. Recuperation capacity was assessed one month after the frost treatment. Vertical lines show  $LT_{50}$  of 1 year old (solid), fully expanded new (dashed) and currently expanding needles (dotted). 0: no regrowth, 1: regrowth occurred although current-year needles were frost killed, 2: regrowth from resting stem buds and finally, 3: plants undamaged.

current-year needles  $F_v/F_m$  reached zero around -6 °C, coincident with E2 and the point of frost damage. 1-year-old needles of seedlings showed a similar depression around -10 °C which was close to their  $LT_{100}$ .

## 4. DISCUSSION

Frost resistance of currently expanding needles of *P. canariensis* seedlings was only slightly less resistant (1 °C) than that of European timberline conifers on the basis of initial frost damage (Tab. I).  $LT_{50}$ , however, was similar to that of *Picea abies*, whereas *P. cembra* and *Larix decidua* were, respectively, 1 °C and 3 °C more frost resistant. The 1-year-old needles of *P. canariensis* seem to be significantly less frost resistant than those of continental timberline conifers such as comparatively *P. cembra* which remained undamaged at temperatures as low as -12 °C in the same season (Taschler D., pers. comm.).

The similarity in frost resistance of expanding current-year needles of the Canary Island pine and subalpine evergreen timberline conifers of continental Europe suggest that frost hardening during sprouting and cell elongation is suppressed. Under the experimental conditions of our frost hardening treatment we did not observe any increase in frost resistance, supporting the above suggestion. However, this could also be due to seasonal timing as the rate of frost hardening of woody plants in spring is known to proceed slowly [15]. Temperature



**Figure 5.** Photochemical efficiency of PS II ( $F_v/F_m$ ) measured 24 h after the frost treatment on 1 year old (black circle) and currently expanding needles (open circle) of *P. canariensis* seedlings. Extracellular ice formation (Hatched box),  $LT_{10}$  (cross hatched box),  $LT_{50}$  of currently expanding needles (dotted line) and  $LT_{50}$  of 1 year old needles. (solid line).

**Table I.** Comparison of minimum and maximum values of frost resistance ( $LT_i$ ,  $LT_{10}$  and  $LT_{50}$ ; °C) of expanding needles of *P. canariensis* seedlings with to that of needles of three timberline conifers of the European Alps: *Pinus cembra*, *Picea abies* and *Larix decidua*.

Species	$LT_{10}$ (min/max)	$LT_{50}$ (min/max)
<i>P. canariensis</i>	-3.4/-3.8	-4.7/-6.1
<i>P. abies</i> *	-4.4/-5.6	-4.8/-6.1
<i>P. cembra</i> *	-4.8/-6.5	-5.5/-7.0
<i>L. decidua</i> *	-6.3/-6.8	-7.8/-10.9

\* From Taschler et al. [27].

conditions in the frost hardening treatment may significantly influence the resultant frost hardening response. Not only minimum temperatures (optimal < 5 °C – [26]) but also daytime leaf temperature maxima may significantly retard frost hardening [20]. Our frost hardening treatment under natural sub-alpine environmental conditions maybe have been insufficient as it included some warm days with minimum air temperatures higher than 5 °C and maxima above 20 °C on at least two days.

In needles of *P. canariensis*, extracellular ice formation (E1) was recorded between -0.9 and -2.0 °C. This corresponds well with observations of adult European timberline conifers in field freezing experiments [27,28] and observations in other plant species under field conditions [22]. In *P. canariensis* seedlings, ice formed initially at -0.9 °C in the currently expanding needles. Spreading of extracellular ice to older needles was significantly retarded (5–30 min) compared to the ice spreading rates usually reported (4–40 mm.s<sup>-1</sup>; [22,26]).

Extracellular ice formation per se was non-injurious. This is not only a feature of freezing tolerant plants but is also observed in non-acclimated leaves such as barley and in leaves with no capacity for cold acclimation [22]. Initial frost damage

( $LT_{10}$ ) occurred 1.5 to 2.7 °C below E1 and must be considered as a consequence of extracellular ice and successional freeze dehydration of cells. E2 cannot explain initial frost damage. E2 very likely originated from intracellular freezing caused by a rupture of cell membranes [21] as E2 corresponded with  $LT_{50}$  or occurred as in 1-year-old needles between  $LT_{10}$  and  $LT_{50}$ . A similar variable relationship between E2 and frost damage ( $LT_{10}$ – $LT_{50}$ ) was also reported for European timberline conifers by Taschler et al. [27]. In some expansion stages needles of *P. abies* may be killed at temperatures even higher than E2 which however, was not observed for *P. canariensis*.

Species-specific differences in ontogenetic changes in frost resistance do obviously exist. In broad-leaved evergreen tree species, seedlings were found to be more frost susceptible than their adults [14, 19, 25], whereas *Nothofagus dombey* seedlings, that are pioneers in frost-prone areas, were more frost resistant than adult trees [18]. The same behaviour was observed in *Embothrium coccineum* and *Pittosporum eugenoides* seedlings being more resistant than adult trees [1, 2]. In *Pinus radiata*, the maximum frost resistance of both seedlings and mature trees was very similar [8]. We lack data for a direct comparison of frost resistance of seedlings and adults. There is only one report on frost resistance of *P. canariensis* [24]. 1-year-old mature needles of adult *P. canariensis* trees growing at different sites between 550 and 1950 m a.s.l. in Tenerife in November varied in their frost resistance between -9 °C and -14 °C ( $LT_{50}$ ), significantly exceeding the frost resistance of 1-year-old needles of seedlings obtained in our experiments, and strongly suggesting a lower frost resistance for *P. canariensis* seedlings.

At the upper distribution limit of *P. canariensis* in Tenerife (approximately 2250 m a.s.l.; [4, 7, 12]) absolute minimum air temperature during the winter ranges in average between -4 and -5 °C and absolute minimum air temperature reaches -15.0 °C (Spanish national institute of meteorology).

However, during radiation frosts subalpine plants can cool 3–8 °C below air temperature [10]. Thus, even in normal years, needles of *P. canariensis* seedlings may experience temperatures down to –13 °C. Therefore, seedlings and young plants with their juvenile free growth habit [12] may be exposed to frost damage in winter as the frost resistance of new needles was only –3.4 °C (LT<sub>10</sub>). Our regrowth experiments however, show that the overall recuperation capacity after frost damage is relatively high and therefore may contribute to the establishment of *P. canariensis* seedlings at high elevation field sites.

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