ORIGINAL ARTICLE

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Repeated freeze–thaw cycles induce embolism in drought stressed conifers (Norway spruce, stone pine)

Abstract Freezing and thawing lead to xylem embolism when gas bubbles caused by ice formation expand during the thaw process. However, previous experimental studies indicated that conifers are resistant to freezing-induced embolism, unless xylem pressure becomes very negative during the freezing. In this study, we show that conifers experienced freezing-induced embolism when exposed to repeated freeze–thaw cycles and simultaneously to drought. Simulating conditions at the alpine timberline (128 days with freeze–thaw events and thawing rates of up to 9.5 K h^{-1} in the xylem of exposed twigs during winter), young trees of Norway spruce [*Picea abies* (L.) Karst.] and stone pine (*Pinus cembra* L.) were exposed to 50 and 100 freeze–thaw cycles. This treatment caused a significant increase in embolism rates in drought-stressed samples. Upon 100 freeze–thaw cycles, vulnerability thresholds (50% loss of conductivity) were shifted 1.8 MPa (Norway spruce) and 0.8 MPa (stone pine) towards less negative water potentials. The results demonstrate that freeze–thaw cycles are a possible reason for winter-embolism in conifers observed in several field studies. Freezing-induced embolism may contribute to the altitudinal limits of conifers.

Keywords Cavitation · Conifer · Embolism · Freeze–thaw · Winter stress · Xylem temperature

Symbol ψ xylem water potential

Introduction

Freeze–thaw events can induce embolism in plant water transport systems. When water within xylem freezes, gas bubbles accumulate in the conduits, because air is not soluble in ice. During thawing, enclosed gas bubbles either dissolve again or expand, thereby leading to a blockage of conducting elements. Whether a bubble dissolves or expands depends on its internal pressure, which is a function of the xylem sap's surface tension, the xylem water potential (ψ) and the bubble radius (e.g. Ewers 1985; Sperry and Sullivan 1992; Sperry et al. 1994; Tyree et al. 1994; Davis et al. 1999; Hacke and Sperry 2001; Sperry and Robson 2001).

The induction of embolism upon freeze–thaw cycles therefore mainly depends on two factors: ψ and conduit diameter. The risk of embolism increases with the size of conduits (e.g. Davis et al. 1999) as larger elements contain larger amounts of dissolved gas. Sperry and Robson (2001) concluded from experimental data that conduits with diameters larger than 30 μm are vulnerable to freeze–thaw-induced embolism: in many ring and diffuse porous species, freeze–thaw-induced embolism could be proven, such as in *Alnus* (Sperry et al. 1994), *Betula* (Sperry and Sullivan 1992; Sperry et al. 1994; Utsumi et al. 1998; Zhu et al. 2001), *Fagus* (Lemoine et al. 1999), *Populus* (Just and Sauter 1991; Sperry and Sullivan 1992; Sperry et al. 1994), *Quercus* (Cochard and Tyree 1990; Lo Gullo and Salleo 1993; Sperry et al. 1994; Nardini et al. 2000), *Rhododendron* (Lipp and Nilsen 1997) or *Salix* (Utsumi et al. 1998). In contrast, conifers are hardly vulnerable to freeze–thaw-induced embolism due to their narrow tracheids: single freeze–thaw cycles did not impair water transport in young *Thuja occidentalis*, *Picea glauca* and *Pinus sylvestris* (Sucoff 1969) or *Tsuga canadensis* (Hammel 1967). Also, Sperry et al. (1994) reported that one freeze–thaw cycle does not induce embolism in drought-stressed *Picea glauca*, *Larix laricina* and *Abies lasiocarpa* (see also Davis et al. 1999). In *Abies lasiocarpa*, even 11 freeze–thaw cycles were not sufficient to cause embolism (Sperry and Sullivan 1992). Feild and Brodribb (2001) exposed *Diselma archeri*, *Podocarpus lawrencei* and *Phyllocladus aspleniifolius* branches to one freeze–thaw cycle and did not observe any embolism either. The only experimental evidence for embolism induction by freeze–thaw in conifers comes

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from *Juniperus scopulorum*, which showed small but significant effects at low ψ (Sperry and Sullivan 1992).

Although experimental findings indicate conifers to be highly resistant to freeze–thaw-induced embolism, field studies showed winter-embolism in several conifers: Sperry and Sullivan (1992) and Sperry et al. (1994) found conductivity losses during winter in *Picea glauca*, *Larix laricina* and *Abies lasiocarpa*. Sparks et al. (2001) showed winter-embolism in *Pinus contorta* to be correlated with cumulative freeze–thaw events as well as with ψ. A study at the European Central Alps timberline (Mayr et al. 2002) revealed frost-drought ("Frosttrocknis") causing extremely low xylem ψ to be the main reason for winter-embolism in *Picea abies*—nevertheless, data also indicate a possible role of freeze–thaw cycles, which occur frequently at the timberline ecotone (Groß et al. 1991). Furthermore, in the harsh winter 2000/2001 a significant correlation between the number of freeze–thaw cycles and embolism rates was observed (Mayr et al. 2003).

The present study should explain this discrepancy between the experimentally demonstrated resistance of conifers and observed indications for freeze–thaw-induced embolism in field studies. According to findings at the timberline (Mayr et al. 2002, 2003), freeze–thaw events were hypothesised to induce embolism in conifers when trees are (i) under drought stress and (ii) exposed to numerous freeze–thaw cycles.

Repeated freeze–thaw cycles were experimentally simulated according to xylem temperature data from the Alpine timberline. Young Norway spruce (*Picea abies*) and stone pine (*Pinus cembra*) trees dehydrated to various extents were exposed to 50 and 100 temperature cycles and compared with trees that were solely drought-stressed. Vulnerability analysis should allow the extent of freeze–thaw-induced embolism in conifer xylem to be estimated.

Materials and methods

Plant material

Experimental studies were carried out on potted, 5-year old specimens of Norway spruce [*Picea abies* (L.) Karst.] and stone pine (*Pinus cembra* L.). Trees were planted in the Botanical Garden, University of Innsbruck, 600 m, until used for freeze–thaw experiments (November 2001 to March 2002).

Temperature course at the Alpine timberline

Freeze–thaw experiments were based on temperature data (stored by datalogger Grant SQ 1045; Grant Instruments, Cambridge, UK) from a 3-m-high Norway spruce tree growing at 2,100 m at Praxmar, Tyrol, Central Alps. Thermocouples were inserted in the stem at 80 cm and 210 cm height and in a sun-exposed branch (junction at 225 cm height) at 10, 30, 45 and 70 cm distance from the stem.

Between October 2001 and May 2002, the most distal sensor recorded 128 days with freeze–thaw events, when days with a minimum xylem temperature below −1°C and a maximum above
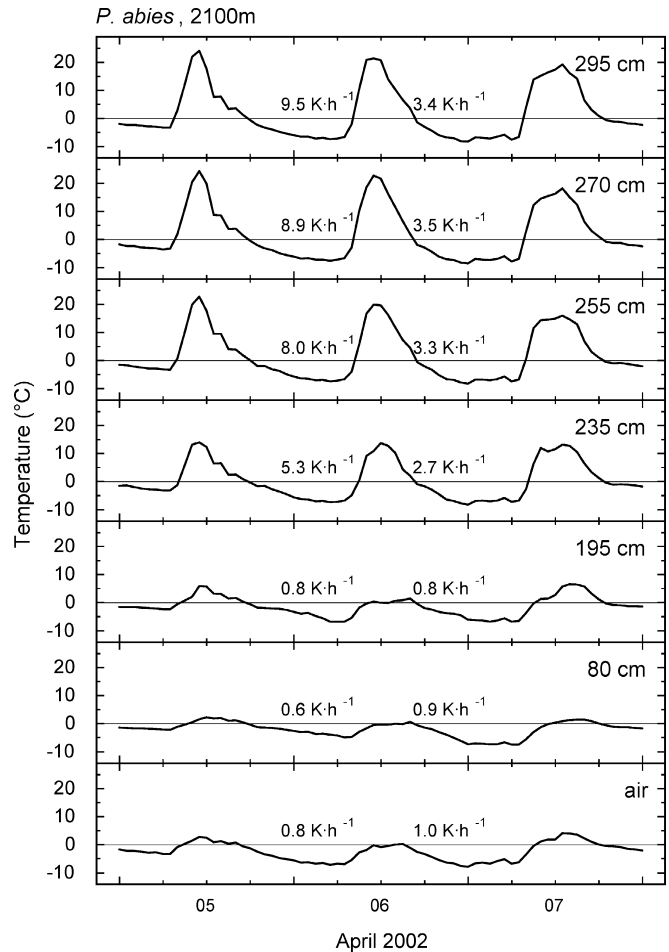
Fig. 1 Air and xylem temperatures in a Norway spruce (*Picea abies*) tree at the alpine timberline (2,100 m) from 5 April to 7 April 2002. Xylem sensors are referred to their cumulative axis length from the soil (e.g. 225 cm stem axes plus 70 cm branch axis length is sensor 295 cm). Two thermocouples were inserted in the stem at 80 cm and 195 cm above ground, the upper four sensors in a branch situated at 225 cm. Freezing and thawing rates are indicated for 6 April

1°C were counted (110 days with full freeze–thaw cycles, 18 days with freezing or thawing solely). Defining −2/+2 °C, −3/+3 °C and −4/+4 °C as daily minimum/maximum thresholds still led to 100, 70 and 45 days, respectively, with freeze–thaw. Mean freezing rate at the distal sensor was 1.8 K h^{-1} (maximum 5.3 K h^{-1}), mean thawing rate 4.1 K h^{-1} (maximum 9.5 K h^{-1}). Figure 1 shows the temperature course of all sensors from 5 April to 7 April, when the most extreme thawing rates were observed. Freezing and thawing rates significantly decreased in sensors situated closer to the stem.

Freeze–thaw experiments

In experimentally simulated freeze–thaw cycles, thawing rates (which may influence the extent of embolism formation; Langan et al. 1997; Feild and Brodribb 2001) were chosen that were similar to mean rates measured in exposed twig sections at the timberline. Freezing rates (to save time) were about 2.5 times higher than in the field—this should result in even lower effects upon freeze–thaw cycles as formation of large bubbles is limited by freezing velocities (Robson and Petty 1987; Sperry and Robson 2001).

Potted trees were dried in a greenhouse (temperature about 10 °C) to various extents. After drying (down to minimal −5 MPa) a first set of trees was directly used for determination of embolism rates. Control trees of each species (seven Norway spruce trees at −2.6 to −3.0 MPa, six stone pine trees at −3.2 to −3.4 MPa) were wrapped up in a plastic bag to avoid further water losses and kept at 10 °C for 25 days without freeze–thaw cycles.

A second set of trees, after dehydration was wrapped up in a plastic bag and exposed to 50 or 100 freeze–thaw cycles in a climate chamber (Ecophyt VEPHL 1350; Heraeus Vötsch, Barlingen, Germany), which cycled between −9.5 °C and +6.5 °C air temperature, 4 times each day. The climate chamber was illuminated (70 µmol photons m^{-2} s^{-1}) daily from 0800 to 1700 hours. Soil and stem xylem temperatures of representative trees were recorded with thermocouples: mean freezing and thawing rate was 4.2 K h^{-1}, xylem temperature cycled between −6.6 °C and +6.4 °C. Freezing exotherms were observed at −2 to −4 °C. After 12.5 days (50 temperature cycles) or 25 days (100 cycles) trees were unwrapped, and ψ and conductivity for vulnerability analysis were determined.

Water potential (ψ)

ψ was measured with a pressure chamber (Model 1000 Pressure Chamber; PMS Instrument Company, Corvallis, USA) using end segments (length < 5 cm) of twigs.

Measurements of embolism rates

Conductivity of xylem samples was measured with a modified Sperry apparatus (Sperry et al. 1988; Chiu and Ewers 1993; Vogt 2001) described in Mayr et al. (2002), whereby embolism rates are quantified by determination of the increase in hydraulic conductivity after removal of enclosed air by repeated high-pressure flushing. Xylem segments of the basal stem part (up to three samples per tree) were prepared (about 2.5 cm length and up to 5 mm in diameter) immersed in distilled water. After removal of the bark, samples were sealed in silicone tubes for measurement of hydraulic conductivity. Measurement pressure was set to 4 kPa (controlled via a 0.4-m glass capillary). Flow rate was determined with a PC-connected balance (Sartorius BP61S, 0.0001 g precision; Sartorius, Göttingen, Germany) by weight registration every 10 s; flow rate was calculated by linear regression over 200 s. Flushing (0.13 MPa, 20 min) and conductivity measurements were done with distilled, filtered (0.22 µm) and degassed water containing 0.005% (v/v) "Micropur" (Katadyn Products, Wallisellen, Switzerland) to prevent microbial growth (Sperry et al. 1988). Flushing was repeated until measurements showed no further increase in conductivity. Percentage loss of conductivity was calculated from the ratio of initial to maximal conductivity.

Vulnerability curves

Vulnerability curves were obtained by plotting percent loss of hydraulic conductivity vs. ψ. Curves were fitted with the exponential sigmoidal equation (Eq. 1) given in Pammenter and Vander Willigen (1998):

$$PLC = 100/(1 + \exp(a(\Psi - b))) \tag{1}$$

where PLC is the per cent loss of conductivity, Ψ is the corresponding water potential and a and b are constants. Constant b corresponds to 50% loss of conductivity (P50).

Anatomical investigations

Anatomical measurements were done on samples used for vulnerability analysis. Tracheid diameters (assuming rectangular cross-sections) were calculated from cross-sectional areas, which were determined microscopically (Olympus BX50; Olympus Austria Corporation, Vienna, Austria; 200-fold magnification) with an automated image analysis system (Optimas 6.0; Optimas Corporation, Washington, USA). Mean hydraulic diameters were calculated as described in Kolb and Sperry (1999).

Number of samples; statistics

Vulnerability was analysed on 29 (0 temperature cycles), 19 (50 cycles) and 34 (100 cycles) Norway spruce trees as well as on 15 (0 cycles), 10 (50 cycles) and 14 (100 cycles) stone pine trees. ψ determination was done on at least three parallel samples of each tree. Anatomical measurements were carried out on cross-sections of 8 (Norway spruce) and 4 (stone pine) stems.

Values are given as mean ± standard error. Differences were tested at 5% probability level with the Student's t-test after checking for normal distribution and variance of the data or with the Mann-Whitney-Test (tracheid diameter distributions).

Results

Vulnerability to drought-induced embolism

Norway spruce was significantly more resistant to drought-induced embolism than stone pine (P50 at −4.07 MPa and at −3.3 MPa, respectively; Figs. 2, 3). The latter also exhibited a significantly wider range over which conductivity was lost (parameter a; Table 1, 0 cycles).

Control trees were dehydrated to ψ higher (Norway spruce, Fig. 2) or lower (stone pine, Fig. 3) than the upper vulnerability threshold—controls showed no changes in embolism rate when stored for 25 days before conductivity measurements. This proves that the time required for freeze–thaw cycles does not cause any systematic error.

Freeze–thaw experiments

ψ increased during incubation in the climate chamber (mean +0.3 MPa), probably because of re-saturation from the soil within the pot and from water releases due to embolism (Sucoff 1969; Sperry and Sullivan 1992).

Table 1 Vulnerability curve parameters according to Pammenter and Vander Willigen (1998) of Norway spruce (*Picea abies*) and stonepine (*Pinus cembra*) exposed to 0, 50 and 100 temperature cycles. Parameter b (50% loss of conductivity, P50) and the upper vulnerability threshold (10% loss of conductivity, P10) are given in MPa. Within species, values in each column not followed by thesame letter differ significantly at $P \leq 0.05$ (Student's t-test). Mean ± SE

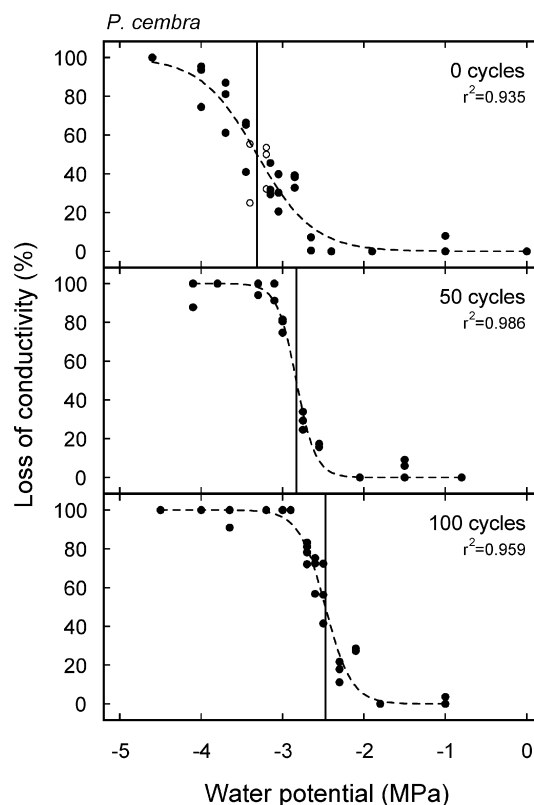|	Temperature cycles	Parameter a (curve slope)	Parameter b (P50)	Upper threshold (P10)
P. abies	0	5.54 ± 0.35[a]	−4.07 ± 0.01[a]	−3.68 ± 0.04[a]
	50	2.02 ± 0.26[b]	−3.43 ± 0.06[b]	−2.34 ± 0.20[a]
	100	1.95 ± 0.13[b]	−2.29 ± 0.04[c]	−1.17 ± 0.12[b]
P. cembra	0	2.90 ± 0.30[a]	−3.30 ± 0.04[a]	−2.56 ± 0.12[a]
	50	8.43 ± 0.70[b]	−2.83 ± 0.01[b]	−2.57 ± 0.03[a]
	100	6.19 ± 0.68[c]	−2.47 ± 0.02[c]	−2.12 ± 0.06[b]
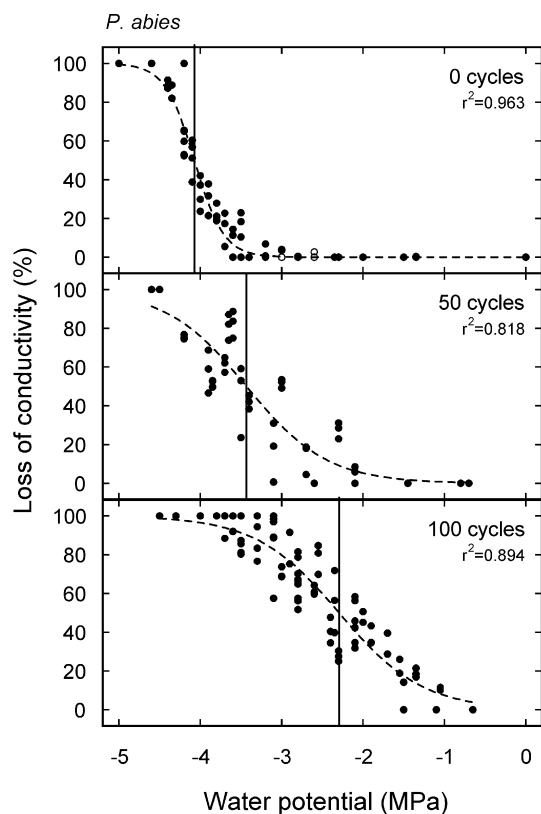
Fig. 2 Vulnerability curves (loss of conductivity in stem vs. ψ) of 5-year-old Norway spruce trees exposed to 0, 50 and 100 freeze–thaw cycles. *Open symbols* indicate control trees that were stored at 10 °C for 25 days after dehydration (without freeze–thaw cycles; plastic bag avoided further water losses) until conductivity measurements. Curves were fitted by the equation described in Pammenter and Vander Willigen (1998). P50 is indicated by vertical lines

Fig. 3 Vulnerability curves (loss of conductivity in stem vs. ψ) of 5-year old stone pine (*Pinus cembra*) trees exposed to 0, 50 and 100 freeze–thaw cycles. *Open symbols* indicate control trees that were stored at 10 °C for 25 days after dehydration (without freeze–thaw cycles; plastic bag avoided further water losses) until conductivity measurements. Curves were fitted by the equation described in Pammenter and Vander Willigen (1998). P50 is indicated by vertical lines

ψ for vulnerability curves was always measured before exposure to freeze–thaw cycles.

In both investigated species, increasing the number of freeze–thaw cycles caused significantly increased embolism rates in drought-stressed trees (Figs. 2, 3). After 100 freeze–thaw cycles, 50% loss of conductivity was reached at a ψ value about 1.8 MPa (Norway spruce) and 0.8 MPa (stone pine) higher than that of trees that had only been drought-stressed (Table 1). In Norway spruce a significant decrease in the curve slope (parameter a) after repeated freeze–thaw cycles was observed, whereas stone pine exhibited significantly steeper curves (Table 1). In consequence, after 100 temperature cycles the upper vulnerability threshold (P10, 10% loss of conductivity) was shifted 2.51 MPa in Norway spruce but only 0.44 MPa in stone pine compared with samples that had only been drought-treated.

Anatomy

Stone pine exhibited significantly smaller tracheid diameters (mean: 6.13 ± 0.05 μm, maximum: 15.4 μm) than Norway spruce (mean: 6.85 ± 0.03 μm, maximum:

16.9 μm) (Fig. 4). The mean hydraulic diameter was 9.87 μm in stone pine and 10.73 μm in Norway spruce.

Discussion

The presented experiments clearly prove that freeze–thaw can induce embolism in conifers but—according to our hypothesis formulated in the Introduction—(i) repeated freeze–thaw cycles and (ii) the combination with drought stress are required. Trees at moderate ψ were hardly affected, which led to significant and, with the number of freeze–thaw cycles, increasing horizontal shifts of vulnerability curves (Figs. 2, 3): P50 was shifted up to 1.8 MPa (Norway spruce, 100 temperature cycles) to less negative ψ. Similar curve shifts were previously shown for angiosperms upon single freeze–thaw events (Davis et al. 1999).

Based on these findings, the discrepancies between earlier experimental and field investigations can now be explained: The lack of effects upon freeze–thaw in experiments published by Sucoff (1969), Sperry and Sullivan (1992), Sperry et al. (1994), Davis et al. (1999) and Feild and Brodribb (2001) is due to the low number
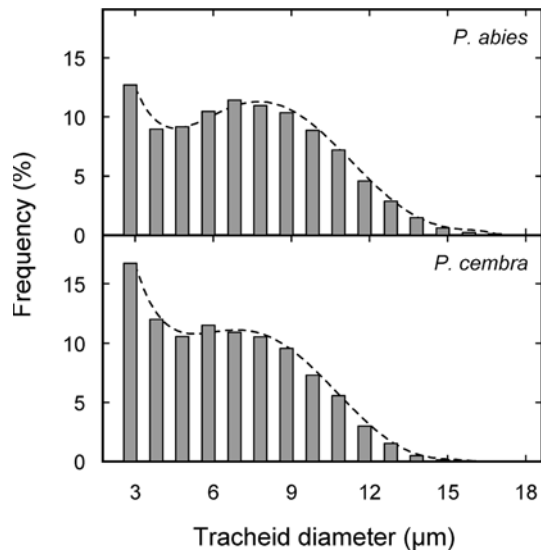
Fig. 4 Frequency distribution of tracheid diameters in the stems of 5-year-old Norway spruce and stone pine trees (1-μm classes, first class 0–3 μm). Curves were fitted by quintic regressions. Frequency distributions differ significantly at $P \leq 0.05$

of freeze–thaw cycles (in most cases 1 cycle). Embolism rates caused by 11 temperature cycles (Sperry and Sullivan 1992) were possibly masked by the relatively broad variance of conifer vulnerability curves. On the other hand, observed winter-embolism in field studies (Sperry and Sullivan 1992; Sperry et al. 1994; Sparks et al. 2001) was probably caused by repeated freeze–thaw cycles in combination with ψ near the upper vulnerability threshold. Also, Sparks et al. (2001) suggested that the high number of freezing events during winter was responsible for embolism in *Pinus contorta*—ψ was found to be low but 1.6 MPa above the drought-related vulnerability threshold. Mayr et al. (2002, 2003) compared vulnerability to drought-induced embolism of Norway spruce with embolism vs. ψ relations observed at the timberline during winter: strikingly similar to the vulnerability curves of our freeze–thaw experiments, a horizontal curve shift of 2.4 MPa was observed for field values. We therefore conclude that embolism rates observed in the mentioned field studies were caused by repeated freeze–thaw cycles in combination with drought stress. In contrast, Sperry et al. (1994) observed a correlation between embolism rates and the number of freeze–thaw events in angiosperms, but not in conifers—maybe refilling (e.g. Borghetti et al. 1991; Sobrado et al. 1992; Edwards et al. 1994; Mayr et al. 2003) was partially responsible for decreasing embolism rates already during winter.

Although Norway spruce and stone pine showed overall similar responses in the presented freeze–thaw experiments there are several differences worth discussing: Young Norway spruce trees exhibited a higher resistance to drought-induced vulnerability than stone pine (Figs. 2, 3, 0 cycles), similar to the properties of twigs of adult trees (Mayr et al. 2003). The effect of

freeze–thaw cycles on embolism rates and on the shift of the vulnerability curve was higher in Norway spruce than in stone pine. This lower resistance to freeze–thaw-induced embolism corresponds to the bigger tracheid diameters of Norway spruce (Fig. 4). Wider tracheids (inter-specific comparison) were reported to be closely related to higher freeze–thaw susceptibility (Davis et al. 1999; Hacke and Sperry 2001; Sperry and Robson 2001), but not to drought-related vulnerability. Stone pine was found to be especially resistant to freeze–thaw-induced embolism at its upper vulnerability threshold, leading to a significant increase of parameter a of the vulnerability curve (curve slope, Table 1). This is of interest from an ecophysiological point of view: stone pine—despite its lower resistance to drought—showed a "safe" range down to −2 MPa even after 100 temperature cycles, whereas Norway spruce had already started to embolise near −1 MPa. This may help to explain why in field studies winter-embolism was observed in Norway spruce but not in stone pine (Mayr et al. 2003).

What happens in the xylem when trees are exposed to consecutive freeze–thaw cycles? Theoretically (e.g. Sperry et al. 1994; Sperry and Robson 2001), conditions for re-dissolving of bubbles in tracheids should not change from one thawing process to the other. Embolism would even lead to improved ψ in adjacent tracheids and therefore support re-dissolving of bubbles during consecutive freeze–thaw cycles. How could experiments reveal increased embolism rates after 50 and 100 freeze–thaw cycles? Why did Norway spruce loose up to 1% conductivity (at −3.5 MPa, calculated from Fig. 2) during each freeze–thaw cycle? This may be considered on a probability level: with every freeze–thaw cycle there is a distinct probability that a tracheid will cavitate even when most of the gas bubbles formed during freezing will dissolve again (Sperry et al. 1994). During repeated temperature cycles the number of embolised tracheids would rise continuously. The probability of cavitation may thereby depend not only on ψ and tracheid sizes but also on freezing rates (bubble formation; Robson and Petty 1987; Sperry and Robson 2001) and thawing rates (Langan et al. 1997; Feild and Brodribb 2001). Sucoff (1969) proposed that the number of tracheids cavitating each freeze cycle would be proportional to the amount of water migrating out of the wood during freezing. We suppose that already embolised tracheids might also promote the cavitation of neighbouring tracheids during consecutive freeze–thaw cycles. It is unclear if tracheids are totally separated because of pit closure during freezing (Hammel 1967) as unaspirated pits were also found in frozen conifer xylem (Robson et al. 1988). Future experiments will enable a detailed analysis of these aspects.

Conifer xylem was demonstrated to be limited in its resistance to freeze–thaw-induced embolism—exposure to numerous temperature cycles combined with drought stress impairs the tree's water transport system. As these conditions are found in extreme habitats of conifers,

freeze–thaw-induced embolism should be a substantial stress factor to which these trees are adapted. In consequence, the resistance properties of their xylem may contribute to the altitudinal and latitudinal limits of conifers.

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