Freeze-Thaw Stress: Effects of Temperature on Hydraulic Conductivity and Ultrasonic Activity in Ten Woody Angiosperms

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Freeze-thaw events can affect plant hydraulics by inducing embolism. This study analyzed the effect of temperature during the freezing process on hydraulic conductivity and ultrasonic emissions (UE). Stems of 10 angiosperms were dehydrated to a water potential at 12% percentage loss of hydraulic conductivity (PLC) and exposed to freeze-thaw cycles. The minimal temperature of the freezing process on hydraulic conductivity and ultrasonic emissions (UE). Stems of 10 angiosperms were dehydrated to a water potential at 12% percentage loss of hydraulic conductivity (PLC) and exposed to freeze-thaw cycles. The minimal temperature of the freezing process correlated positively with induced PLC, whereby species with wider conduits (hydraulic diameter) showed higher freeze-thaw-induced PLC. Ultrasonic activity started with the onset of freezing and increased with decreasing subzero temperatures, whereas no UE were recorded during thawing. The temperature at which 50% of UE were reached varied between −9.1°C and −31.0°C across species. These findings indicate that temperatures during freezing are of relevance for bubble formation and air seeding. We suggest that species-specific cavitation thresholds are reached during freezing due to the temperature-dependent decrease of water potential in the ice, while bubble expansion and the resulting PLC occur during thawing. UE analysis can be used to monitor the cavitation process and estimate freeze-thaw-induced PLC.

Xylem embolism is a limiting factor for plant survival and distribution (Choat et al., 2012; Charrier et al., 2013). Two major factors can induce embolism in the xylem of plants: drought and freeze stress. Freeze-thaw-induced embolism is caused by bubbles formed during freezing that then expand on thawing (Lemoine et al., 1999; Häcke and Sperry, 2001; Cruziat et al., 2002; Tyree and Zimmermann, 2002). As wider conduits contain more gas and form larger bubbles, which expand at less negative tension, conduit diameter and xylem sap tension are critical for the formation of freeze-thaw-induced embolism (Davis et al., 1999; Pittermann and Sperry, 2003). Accordingly, Mayr and Sperry (2010) observed a loss of conductivity only when samples were under critical tension during thawing. Under drought stress, tension in the xylem sap increases the sensitivity to embolism generated by successive freeze-thaw cycles (Mayr et al., 2003, 2007).

Ultrasonic emissions (UE) analysis can be used to detect cavitation events in wood. It is unclear how well related UE are to cavitation events, as they are extracted from continuous acoustic emissions and depend on set definitions. However, UE analysis has been proven effective for monitoring drought-induced embolism in the laboratory (Pena and Grace, 1986; Salleo and Lo Gullo, 1986; Borghetti et al., 1993; Salleo et al., 2000) as well as in field experiments (Ikeda and Ohtsu, 1992; Jackson et al., 1995; Jackson and Grace, 1996; Hölttä et al., 2005; Ogaya and Penuelas, 2007). In a cavitating conduit, signals are probably produced by the disruption of the water column and subsequent tension relaxation of cell walls.

UE have also been detected during freezing events, but the origin of these signals was less clear. In some cases, UE were observed during thawing, which are thus probably related to embolism formation according to the classic thaw-expansion hypothesis (Mayr and Sperry, 2010); however, all species studied have produced UE on freezing, which cannot yet be explained (Raschi et al., 1989; Kikuta and Richter, 2003; Mayr et al., 2007; Mayr and Sperry, 2010; Mayr and Zublasing, 2010). The low solubility of gases in ice prompted the idea that air bubbles expelled from the ice structure produce UE near the ice-liquid interface (Sevanto et al., 2012). As the water potential of ice is strongly temperature dependent, the minimum temperature during freezing might be a relevant factor. Numerous studies have analyzed UE patterns during freeze-thaw cycles in conifers.

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induced PLC (between the minimal temperature and freeze-thaw-slightly in Across all species, significant decreases in PLC after dehydration to a water potential at 12% PLC (control) and exposure to one freeze-thaw cycle down to 10˚C, 20˚C, 30˚C, or 40˚C. Statistically, minimal temperature (θ) and Dh were both significantly correlated to PLC (P < 0.001), but their interaction was significantly stronger than their single effect in predicting freeze-thaw-induced PLC (θ × Dh, P = 0.036; θ, P = 0.97; Dh, P = 0.67 with r² = 0.672).

In J. regia, UE were generated between the onset of freezing in the wood (dotted line in Fig. 3A) and when minimum temperature was reached. No UE were recorded during the temperature plateau at −40°C or during thawing. Across replicates, the total number of UE after one freeze-thaw cycle was variable (i.e. from 50,000 to 1,000,000 in J. regia), but relative cumulated ultrasonic emissions (cumUE) during a freeze-thaw cycle showed little variability (Fig. 3A). The plot of cumUE versus temperature revealed a sigmoidal curve (r² = 0.989, P < 0.001), with an increase in ultrasonic activity until approximately −17°C and a decrease at lower temperatures (Fig. 3B). In November, there was a shift in the relationship between cumUE and temperature in J. regia. The temperature generating 50% of cumulated ultrasonic emissions (T50) decreased from −16.8°C to −21.5°C, and the curve slope decreased.

Minimal temperatures had a differential effect on UE generation across species (Fig. 4). In J. regia, UE were generated during four successive cycles with decreasing minimal temperatures, with half of all UE recorded during the −30°C cycle. In F. sylvatica, most UE were also generated during the −30°C cycle (82% of total UE), whereas in Sorbus aucuparia, most UE were generated during the −40°C cycle (90% of total UE). In all species, UE were only recorded when temperatures fell below those reached in previous cycles (Fig. 4).

All species produced a similar sigmoid pattern of cumUE versus temperature (r² > 0.928 and r² > 0.975 in nine species), but temperature thresholds differed across species: temperatures inducing 50% of cumUE ranged from −9.2°C in B. pendula to −31.0°C in Sorbus aucuparia (Table I). Across species, cumUE at −10°C,
−20°C, and −30°C were significantly correlated to the relative PLC (\(r^2 = 0.653, P < 0.001\); Fig. 5).

Across species, \(T_{50}\) was significantly correlated to water potential inducing 50% PLC (\(r^2 = 0.517, P = 0.010\); Fig. 6). In drought-sensitive species (Table II), \(T_{50}\) was less negative (e.g. −9.2°C in *B. pendula* and −14.6°C in *Salix alba*) than in drought-resistant species (e.g. −31.0°C in *Sorbus aucuparia* and −24.6°C in *P. cerasifera*).

**DISCUSSION**

In the studied angiosperm species, freeze-thaw-induced PLC increased with decreasing minimal temperature (Figs. 1 and 2A), as also demonstrated by Pockman and Sperry (1997) and Ball et al. (2006) on angiosperms, but in contrast to Mayr and Sperry (2010) on a conifer. Freeze-thaw-induced PLC was also positively correlated with \(D_p\). A similar relationship was previously described in laboratory experiments on conifers (Davis et al., 1999; Pittermann and Sperry, 2003, 2006) and angiosperms (Stuart et al., 2007; Choat et al., 2011) and in field studies (Charrier et al., 2013; Schreiber et al., 2013).

Temperatures also influenced ultrasonic activity during the freezing process, as cumUE increased with falling temperatures (Fig. 3). As in conifers (Mayr et al., 2007; Mayr and Zublasing, 2010) and other angiosperms...
parenchyma cells have been cited as a potential source of UE during freezing (Weiser and Wallner, 1988; Ristic and Ashworth, 1993). The ratio of signals from living cells to signals from conduits is unclear, but the process could explain why angiosperm wood, which contains more living cells, generated far more UE (hundreds of thousands) than conifer wood (several hundreds). Planned studies of the intrinsic parameters of acoustic signals (e.g. amplitude and energy) may bring insights into the sources of UE, as done for drought stress, from xylem (approximately 25%) and symptomatic cavitation (approximately 75% in Wolkerstorfer et al., 2012).

Freeze-thaw-induced PLC and UE were positively correlated, as both were dependent on temperature during the freezing process (Fig. 5). Another probable source of UE during freezing is the formation of bubbles during the freezing process (Fig. 5). Another probable source of UE during freezing is the formation of bubbles (Weiser and Wallner, 1988; Ristic and Ashworth, 1993). The ratio of signals from living cells to signals from conduits is unclear, but the process could explain why angiosperm wood, which contains more living cells, generated far more UE (hundreds of thousands) than conifer wood (several hundreds). Planned studies of the intrinsic parameters of acoustic signals (e.g. amplitude and energy) may bring insights into the sources of UE, as done for drought stress, from xylem (approximately 25%) and symptomatic cavitation (approximately 75% in Wolkerstorfer et al., 2012).

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a bubble, releasing tension in conduits and probably emitting UE (Davitt et al., 2010). Many more UE are detected during freezing than during drying, especially in species with wider conduits (Kikuta and Richter, 2003). Anatomic studies did not detect bubbles in frozen conduits using the cryo-scanning electron microscopy technique (Utsumi et al., 1998; Ball et al., 2006; Cobb et al., 2007), but this technique potentially induces artifacts (CoChard et al., 2000), as rapid freezing velocities (i.e. faster than 75 μm s⁻¹ as induced by liquid nitrogen) entrap the gas into ice without forming bubbles (Sperry and Robson, 2001) or induce bubble sizes that fall below the resolution of the microscope system (Sevanto et al., 2012). Nevertheless, at a slower freezing rate, bubbles of approximately 2 μm in diameter were observed in the center of lumens (Robson et al., 1988).

The water potential at the ice-liquid interface decreases with decreasing temperature as the potential of ice changes at a ratio of approximately −1 MPa K⁻¹ (Hansen and Beck, 1988; Cavender-Bares, 2005). Another factor to consider is that solutes (e.g. salts and carbohydrates) have a lower solubility in ice than in liquid water and are thus concentrated in the remaining liquid volumes near the ice-liquid interface (Sevanto et al., 2012). The enormous effect of temperature on the water potential may cause bubbles to expand or air seeding from adjacent, already air-filled conduits (Sperry and Tyree 1990; Cochard et al., 1992). Samples already at critical tension (e.g. water potential generating 12% loss of conductivity, as in the samples used here) are probably most affected and thus generate many UE (Mayr and Zublasing, 2010). Air seeding is the underlying mechanism of drought-induced embolism (Hacke and Sperry, 2001), which may explain the observed link between the temperature dependence of UE activity and the vulnerability to drought-induced embolism (Fig. 6). This correlation indicates that air seeding plays a role in drought-induced as well as freeze-thaw-induced embolism. Pit membrane porosity is a crucial factor for air seeding (Lens et al., 2011), and future studies should focus on possible links between pit structures and vulnerability to freezing-induced embolism. Furthermore, the temperature of ice nucleation in the sap is lower and the cavitation threshold is higher in species with small conduits than in species with wider conduits, which also tends to link both mechanisms (Lintunen et al., 2013). It is also possible that minimal temperature may not affect PLC in some species (B. pendula, P. cerasifera [Fig. 1], and P. contorta [Mayr and Sperry, 2010]) because of their xylem anatomy. Small plasmodesmatal pores in pits could prevent air seeding (Jansen et al., 2009), and scalariform plates could stop air passing through perforations (Tyree and Zimmermann, 2002). Both minimal temperature and anatomy thus play important roles in freeze-thaw-induced PLC (Fig. 2).

Bubbles and air-water menisci in pits are blocked by the arriving ice front and can only expand at thawing (Cruiziat et al., 2002), depending on the water potential in the surrounding sap and the bubble radius (Ewers, 1985; Davis et al., 1999; Pittermann and Sperry, 2003). Wider conduits contain more dissolved gases and, consequently, more bubbles after freezing. At thawing, coalesced bubbles may more easily reach the critical radius (Sperry and Sullivan, 1992; Davis et al., 1999; Pittermann and Sperry, 2003). The proposed mechanism is schematized in Figure 7.

Figure 7. Proposed mechanism of freeze-thaw-induced embolism. In moderately dehydrated samples, some vessels are air filled (white) before freezing (A). During freezing, ice (light gray) propagates through vessels (arrows), air nucleates near the ice-water interface, and a local, low water potential (Ψ < 0) induces air seeding from air-filled vessels. This leads to relaxation of the tension in cell walls and the emission of ultrasonic waves (B). Bubbles of different size (white circles) are entrapped by the arriving ice front (C) but can coalesce and dilate during thawing when the tension is low enough (Ψ < 0; D), thus inducing embolism (E).
CONCLUSION

Subzero temperatures play an important role in the formation of freeze-thaw-induced embolism and ultrasonic activity during freezing. Damage to living cells may be a source of UE. However, the temperature-dependent decrease in water potential likely influences bubble formation and air seeding in xylem conduits during the freezing process, with the thresholds for these processes being dependent on anatomical structures. UE can be used to monitor the cavitation process during freezing and to estimate the resulting loss of conductivity detected after complete freeze-thaw cycles.

MATERIALS AND METHODS

Plants

Ten European tree species were chosen that differed in anatomy and resistance to drought-induced embolism (Table II). Branches, 1 m in length, were harvested during the growing season (from July to September) from trees at natural sites in Austria (Innsbruck) or France (Clermont-Ferrand). In addition, Juglans regia was also harvested after leaf fall (end of November). Samples were wrapped in plastic bags, and the base was kept in water. The basal diameter of twigs was around 2 cm. On the laboratory bench, branches were dehydrated to a water potential generating 12% loss of conductivity (around 2 cm). On the laboratory bench, branches were dehydrated to a water potential of sample twigs were measured using a Scholander pressure chamber (model 1000 pressure chamber; PMS Instrument). Samples were cut to 50 cm, and side branches were removed. Samples were then tightly wrapped in Parafilm (Alcan) to prevent further dehydration.

Freeze-Thaw Treatments

Samples were exposed to freeze-thaw treatments in a temperature-controlled chamber (MK999 [Clermont-Ferrand] or MK53 [Innsbruck]; Binder). The following experiments were performed. (1) All species were exposed to one freeze-thaw cycle down to −20°C with thawing to +5°C. Temperature changed at 5 K h⁻¹ but remained constant at every 5 K step (on freezing and thawing). Air temperature was held constant for 4 h at minimal temperature before thawing. (2) Fagus sylvatica, J. regia, and Sorbus aucuparia were exposed to four successive freeze-thaw cycles to −10°C, −20°C, −30°C, and −40°C with thawing to +5°C between cycles. Temperature changed at 5 K h⁻¹ and was held constant for 1 h at minimal and maximal temperatures.

Detection of UE

UE acquisition was performed on an eight-channel SAMOS (Euro-Physical Acoustics) or a PCI-8 system ( Physical Acoustics) equipped with 150-kHz (v/v), 70% (v/v), and 100% (v/v) ethanol. Cross sections were then observed for 3 to 5 min. Stained tissues were successively washed with water and 50% acetic acid (1% [v/v]) and stained with safranin (1% [v/v]). Samples, 7 cm in length, were submerged in water and cut with a scalpel to prevent air entry into vessels (n = 5–10 per species and temperature). Conductance (k) was measured using a solution of KCl (0.01 mol L⁻¹) and CaCl₂ (0.001 mol L⁻¹) at low pressure (3.50 kPa). After perfusing the same solution at high pressure (140 kPa) to remove embolism, conductance was remeasured. Measurements and flushing were repeated twice until maximal conductance (kmax) was reached. PLC was then calculated as:

\[
\text{PLC} = \frac{(k_{\text{max}} - k)/k_{\text{max}}}{C_0}
\]

For correlations analysis, different hydraulic parameters were calculated.

\[
\text{PLC}_{\text{FT}} = \text{PLC} - \text{PLC}_{\text{control}}
\]

where PLC_FT is the freeze-thaw-induced PLC and PLC_control is the PLC without the freeze-thaw cycle.

\[
\text{rPLC} = \frac{\text{PLC} - \text{PLC}_{\text{control}}}{\text{PLC}_{\text{max}} - \text{PLC}_{\text{control}}}
\]

where rPLC is the relative PLC and PLC max is the maximal PLC measured after one freeze-thaw cycle among all tested temperatures.

Anatomy

Samples were progressively included in polyethylene glycol (Carbowax; Dow) and then cut into 19-μm-thick slices using a cryomicrotome (Reichert). Cells were lysed using sodium hypochlorite (5 g L⁻¹) for 15 to 30 min. Samples were rinsed with acetic acid (1% [v/v]) and stained with safranin (1% [v/v]) for 3 to 5 min. Stained tissues were successively washed with water and 50% (v/v), 70% (v/v), and 100% (v/v) ethanol. Cross sections were then observed with a microscope (400x), and images were analyzed using ImageJ software (1.45; National Institutes of Health). Three different 1 × 1-mm images were analyzed per species. Diameters of vessels were measured using the analyze particles function, and mean Dv was calculated using the Hagen-Poiseuille equation, which states that water flow in a capillary is proportional to the fourth power of the radius:

\[
D_v = \frac{8 \times \text{flow}}{\pi \times \text{pressure}}
\]

where Dv is the individual vessel diameter.

Statistical Analysis

After testing for Gaussian distribution, we calculated linear regression and P values using R software (R Development Core Team, 2005). Multiple linear regression was calculated by r² minimization using the lm function in the R software.

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LITERATURE CITED


comparison of four species at different sites and altitudes. Ann For Sci 69: 325–333
Pittermann J, Sperry JS (2003) Tracheid diameter is the key trait determining the extent of freezing-induced embolism in conifers. Tree Physiol 23: 907–914
Pittermann J, Sperry JS (2003) Tracheid diameter is the key trait determining the extent of freezing-induced embolism in conifers. Tree Physiol 23: 907–914