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Technical Note

Changes in ultrasound velocity and attenuation indicate freezing of xylem sap

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ABSTRACT

Freezing is a limiting factor for plant life, as it can cause damage of living tissues and embolism formation in conduits. Ice formation in plant tissues is usually detected by exotherm analysis. In this study, a new method based on changes in ultrasonic properties of wood was used to monitor xylem freezing. Ultrasound propagation velocities and attenuation were measured with an ultrasonic emission analysis system in branches of three conifer and three angiosperm tree species by signal induction *via* auto sensor test (AST) or lead break (LB: Hsu–Nielsen source). In all species under study, ultrasound velocity was 1.2–3.6-times higher in frozen xylem (−10 °C) compared to samples at 10 °C. In *Picea abies*, velocities of AST signals increased from 2193 to 3085 m s^{-1} and in *Fagus sylvatica* from 2369 to 3009 m s^{-1}, while signal attenuation decreased in both species. The crystalline structure of ice with slower molecular movements and strong hydrogen bonding caused the faster propagation and reduced attenuation of acoustic waves after xylem freezing. Xylem anatomy also influenced acoustic properties as demonstrated by inter-species differences in temperature responses. The analysis of ultrasonic properties provides a new method for the detection of ice in the xylem of trees and may be used to monitor stress intensities and estimate physiological effects *in situ*.

1. Introduction

Freezing is a major limiting factor for plant life in several environments, such as temperate and boreal biomes or alpine areas. In the xylem of woody plants, freezing can lead to damage by different mechanisms. Due to the volume increase of freezing sap, mechanical constraints can exceed cell wall rigidity and provoke frost cracks (Ishida, 1963; Cinotti, 1991). Ice formation also causes dehydration and osmotic stress affecting the cytoplasm and membranes of living cells (Steponkus, 1981; Ruelland et al., 2009). Extreme temperatures below −30 °C can provoke intracellular ice formation even in deep supercooling species (Fujikawa and Kuroda, 2000), which is always lethal for cells (Wolfe and Bryant, 2001). Plants also suffer from freeze–thaw cycles, when embolism is induced in xylem conduits (Sperry and Sullivan, 1992; Tyree et al., 1994; Hacke et al., 2001; Mayr et al., 2007). Within a plant, freezing susceptibility differs between tissues so that dynamics of ice formation play an important role in development and extent of damages. Similarly, the pattern of ice in the xylem determines hydraulic blockages. Monitoring freezing and temporal and spatial dynamics of ice formation in plants are thus a prerequisite to evaluate stress intensities and their physiological effects *in situ*.

Freezing in plant tissues can be monitored by high resolution temperature measurements, which enable the detection of exotherms. When liquid water turns into ice, the molecular structure changes a crystalline formation. This causes a release of latent heat energy (L = 334 J g^{-1}), which can be recorded as an exotherm (Muldrew et al., 2004). When apoplastic water freezes, a first high temperature exotherm is observed. When the temperature decreases further, a second exotherm corresponding to intracellular ice nucleation appears. The cell sap freezes at lower temperatures because the higher solute concentration causes a deeper freezing point (Kasuga et al., 2007). Numerous studies based on exotherm analysis enabled insights into the freezing process and frost resistance (*e.g.* Burke et al., 1976; Kuroda et al., 1999), but *in situ*, the detection of exotherms is difficult due to rapid dispersion of released heat, especially under turbulent atmospheric

Abbreviations: AST, automated sensor test; LB, lead break, refers to Hsu–Nielsen simulation test.

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conditions. The first attempts were performed by Ashworth et al. (1985) on peach trees: differential thermal analysis enabled the detection of temperature differences at high resolution (Fujikawa et al., 1994; Pramsohler et al., 2012). Exotherms can be visualised also *via* infra-red thermography (Wisniewski et al., 1997). Up to now, this technique has only been used in laboratory experiments (*e.g.* Hacker and Neuner, 2008) and it allows temperature analysis only at the sample surface. In a few studies, time domain reflectrometry was used to detect ice fractions in stems (Sparks et al., 2000). Sparks et al. (2001) found portions of liquid water in *Pinus contorta* stems even in deep winter. Effect of freeze–thaw stress can also be monitored *via* acoustic emission analysis. In several studies (Kikuta and Richter, 2003; Mayr et al., 2007; Mayr and Zublasing, 2010; Mayr and Sperry, 2010) acoustic emissions were registered during freezing and found to be correlated with the loss of hydraulic conductivity in the xylem. Up to now, acoustic emission analysis on plants predominantly focused on the number and dynamics of signals induced by drought (Tyree and Dixon, 1983; Salleo and Lo Gullo, 1986) or freeze–thaw events (Raschi et al., 1989). The signal quality was hardly considered (Mayr and Rosner, 2011) although it may give important additional information.

Sound waves are mechanical vibrations creating periodic disturbances as a succession of compression/rarefaction. The ability of a solid material to allow sound propagation depends on its elasticity and density (Lampriere, 2001):

$$C_l = \sqrt{\frac{1-\upsilon}{2(1+\upsilon)(1-2\upsilon)}} \times \sqrt{\frac{E}{\rho}} \quad (1)$$

where C_l is the propagation velocity of sound waves in longitudinal direction, υ is the Poisson's ratio, ρ is the density of the material and E is the Young's modulus.

In a heterogeneous material like wood, with solid, liquid and even gases, sound propagation is more complex. It depends on density, elasticity, adiabatic compressibility coefficient (for liquids) and, because it is an orthotropic material, direction of the propagation (Bucur, 2006). Inter-species variability in sound propagation velocities is directly related to Young's modulus and density (Kretschmann, 2010).

Temperature also influences sound propagation velocity because of its specific effect on the elasticity and density of the medium. In liquids and gases, sound velocity is proportional to temperature, whereas in solids, increasing temperatures induce a decrease in Young's modulus and, in consequence, in velocity. During the transition of water to ice, the density of the medium decreases and Young's modulus increases. In consequence, sound propagation velocity increases from *ca.* 1480 m s^{-1} in water (Bilaniuk and Wong, 1993) to *ca.* 3900 m s^{-1}, in ice (Smith and Kishoni, 1986).

We hypothesised that the ultrasound propagation velocity and other qualitative parameters (*e.g.* amplitude, attenuation) of ultrasonic signals might be used as an indicator for the phase of water in the xylem. In this study, we tested the effect of freezing on ultrasound velocity, amplitude and attenuation in stems of several tree species. We induced artificial ultrasonic signals and expected that (i) ultrasound propagation velocities in frozen samples were higher and the attenuation lower than in unfrozen samples and that (ii) differences among species were small as the sound propagation predominantly occurs *via* the water column.

2. Material and methods

2.1. Plants

Branches were sampled from mature trees growing near the Department of Botany in Innsbruck, Austria. Measurements were performed on three angiosperm (*Acer pseudoplatanus* L. (n = 3), *Fagus sylvatica* L. (n = 8) and *Juglans regia* L. (n = 3)) and three conifer species (*Larix decidua* Mill. (n = 3), *Picea abies* (L.) Karst. (n = 8) and *Pinus sylvestris* L. (n = 3)). Branches were approximately 1–2 cm in basal diameter and 60 cm in length. From branches, 30 cm long segments of the main stem were used. The mean vessel length of all species was shorter than 30 cm and thus shorter than the sample length. Side branches (if present) were removed as the space in the temperature chamber was limited. Segments were cut out of the stem under water and flushed with water (*a.d.*) at 0.16 MPa for 10 min to release tension and remove xylem embolism (enclosed air might affect the freezing process by local insulation and slow down sound propagation). Samples were wrapped into parafilm (Alcan, Montreal, Canada) to prevent dehydration.

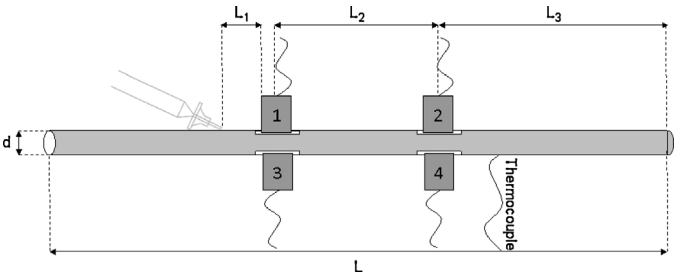

Fig. 1. Scheme of sample dimensions, sensor and lead break positions (pencil in grey): total length L = 30 cm; diameter d *ca.* 1 cm; distance to lead break L_1 = 2 cm (conifers) or 4 cm (angiosperms); distance between sensors L_2 = 4 cm (conifers) and 8 cm (angiosperms); minimal distance to the end $L_3 \geq 5$ cm. Bark is in grey and wood in white. In experiment 1, all four sensors were present (radial velocity was calculated between 1 and 3 or 2 and 4; axial between 1 and 2 or 3 and 4). In experiment 2 and 4 only sensor 1 and 2 were present. In experiment 3, only one sensor was present and different distances to LB source were used (L_1: 1–25 cm).

2.2. Freeze–thaw cycles

Freeze–thaw cycles were performed in a temperature test chamber (MK53, Binder GmbH, Tuttlingen, Germany). Within a frost cycle, temperature decreased from +10 to −10 °C at 5 K h^{-1} and stayed one hour at minimal temperature before thawing at 5 K h^{-1}. Xylem and air temperature were monitored using copper-constantan thermocouples (one per sample, on one end, 2 cm from an acoustic sensor) connected to a datalogger (CR10X, Campbell Scientific Ltd., England). To measure temperature effects in ice, some experiments were performed down to −20 °C at identical freezing and thawing rates in *Fagus* and *Picea*.

2.3. Ultrasound propagation velocity

Ultrasonic measurements were performed with a PCI-2-based system (PAC125, 18-bitA/D, 3 kHz–3 MHz) and 150 kHz resonance sensors (R15) connected to a preamplifier set to 40 dB (all components: Physical Acoustics Deutschland, Wolfegg, Germany). The threshold was set to 45 dB_{EA} (0 dB_{EA} = 1 μV; *e.g.* Mayr et al., 2007; Mayr and Rosner, 2011). Registration and analysis of ultrasonic events were done with AEwin software (Mistras Holdings Corp., Princeton, USA). About 1 cm^2 of bark (and parafilm) were removed from the samples and the xylem was covered with silicone grease (to ensure acoustic coupling and prevent dehydration) before attaching the sensors with clamps. Sensors were placed at a distance of 8 cm on angiosperm stems and of 4 cm on conifers because of higher attenuation in coniferous species (Fig. 1).

Ultrasound velocity was calculated from the distance between sensors divided by the time difference of corresponding hits. Temporal resolution of the acquisition system is *ca.* 0.25 μs, which gives high accuracy for velocity measurements. Ultrasonic signals were