Characteristics of ultrasonic acoustic emissions from walnut branches during freeze–thaw-induced embolism formation

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Received 10 July 2014; Revised 10 December 2014; Accepted 16 December 2014

Abstract

Ultrasonic acoustic emission (UAE) methods have been applied for the detection of freeze–thaw-induced embolism formation in water conduits of tree species. Until now, however, the exact source(s) of UAE has not been identified especially in angiosperm species, in which xylem tissues are composed of diverse types of cells. In this study, UAE was recorded from excised branches of walnut (Juglans regia cv. Franquette) during freeze–thaw cycles, and attempts were made to characterize UAEs generated by cavitation events leading to embolism formation according to their properties. During freeze–thaw cycles, a large number of UAEs were generated from the sample segments. However, the cumulative numbers of total UAE during freeze–thawing were not correlated with the percentage loss of hydraulic conductivity after thawing, suggesting that the sources of UAE were not only cavitation leading to embolism formation in vessels. Among the UAEs, cumulative numbers of UAEs with absolute energy >10.0 fJ strongly correlated with the increase in percentage loss of hydraulic conductivity. The high absolute energy of the UAEs might reflect the formation of large bubbles in the large lumen of vessels. Therefore, UAEs generated by cavitation events in vessels during freeze–thawing might be distinguished from other signals according to their magnitudes of absolute energy. On the other hand, the freezing of xylem parenchyma cells was followed by a certain number of UAEs. These results indicate the possibility that UAE methods can be applied to the detection of both freeze–thaw-induced embolism and supercooling breakdown in parenchyma cells in xylem.

Key words: Embolism formation, freezing stress, loss of hydraulic conductivity, ultrasonic acoustic emission, vessel, walnut, xylem parenchyma.

Introduction

Low temperature is a major environmental factor that restricts the geographical distribution and productivity of plants (Parker, 1963; Sakai and Larcher, 1987; Xin and Browse, 2000). In particular, subfreezing temperature causes a complex stress, generated not only by temperature stress itself but also by dehydration stress and mechanical stress due to ice crystal formation (Levitt, 1972; Yamazaki et al., 2008). To expand their distribution into cold areas and to survive under
severe freezing stress in winter, some plants have developed increased cold-hardiness through adaptive processes (Sakai and Larcher, 1987; Larcher, 1995).

To survive in cold areas, plants have improved the cold-hardiness of their living cells. However, the effects of freezing on living cells are not the only stresses on plants. Freeze–thaw cycles also induce embolism in water conduits, disturbing the water uptake of above-ground tissues and thus causing serious problems (Pratt et al., 2005). Continuity of the water column is essential to carry water from roots to leaves (the cohesion–tension hypothesis; Dixon, 1914). However, embolism generated by the formation of gas bubbles in water conduits breaks this continuity, resulting in failure of the water transport system. In the field, freeze–thaw-induced embolism is a common event and has been observed in many tree species during winter and early spring (Ewers, 1985; Sperry et al., 1988; Sperry and Sullivan, 1992; Lo Gullo and Salleo, 1993; Hacke and Sauter, 1995; Cochard et al., 1997; Améglio et al., 2002; McCulloh et al., 2011). The failure of the water transport system during winter might restrict the availability of water for the resumption of growth in spring. Therefore, it is thought that vulnerability to freeze–thaw-induced embolism is a key factor determining the geographical distribution of trees in cold areas (Langan et al., 1997; Pockman and Sperry, 1997; Mayr et al., 2003, 2006, 2014; Charrier et al., 2013).

Several methods have been employed for the assessment of embolism formation in water conduits of plants: (i) xylem hydraulic conductivity measurement before and after removing emboli (Vogt, 2001; Domce and Gartner, 2002; Mayr and Cochard, 2003); (ii) the use of dye to distinguish embolized conduits from conducting segments (Mayr and Cochard, 2003; Mayr et al., 2007; Hietz et al. 2008); and (iii) cryo-scanning electron microscopic observations of embolized vessels (Canny, 1997; Utsumi et al., 1998; Ball et al., 2006; Mayr et al., 2007). However, these methods are destructive and could induce artefacts (Wheeler et al., 2013). Therefore, information can only be obtained about the water status of the sample at one specific point in time. As non-destructive methods of directly observing cavitation events in living plants, (iv) magnetic resonance imaging (Holbrook et al., 2001); and (v) X-ray tomography (Charra-Vaskou et al., 2012a; Brodersen et al., 2013; Suuronen et al., 2013) are now available. However, both methods are utilized for small plants or small parts of plants in the laboratory. An alternative method of assessing embolism formation is (vi) recording ultrasonic acoustic emissions (UAEs). The cavitation events leading to embolism formation cause the sudden release of tension in the lumen of water conduits, resulting in UAEs with highest amplitudes in the range of 100–300 kHz (Tyree and Sperry, 1989). Therefore, UAEs allow the detection of cavitation events and estimation of the loss of hydraulic conductivity due to embolism formation. This method is also non-destructive, and therefore allows real-time assessment of embolism formation not only in the laboratory but also in the field. Acoustic emission analyses have been carried out to investigate the progression of embolism in water conduits under drought stress (Lo Gullo and Salleo, 1991; Ikeda and Ohtsu, 1992; Jackson and Grace, 1996; Jackson et al., 1999; Perkins et al., 2004; Hölttä et al., 2005; Rosner et al., 2006; Ogaya and Peñuelas, 2007; Johnson et al., 2009; Mayr and Rosner, 2011) and under freezing stress (Raschi et al., 1989; Kikuta and Richter, 2003; Mayr et al., 2007; Mayr and Sperry, 2010; Mayr and Zublasing, 2010; Charrier et al., 2014).

However, the cumulated number of UAEs is not always proportional to the loss of hydraulic conductivity in the xylem (Rosner et al., 2006; Mayr and Rosner, 2011). A possible reason is that a source(s) other than cavitation in water conduits produces UAEs. To distinguish the UAEs related to embolism formation from other signals, analyses of waveform characteristics have been progressed using coniferous trees (Rosner et al., 2006; Mayr and Rosner, 2011; Wolkerstorfer et al., 2012), and a correlation between the energy of UAEs and xylem lumen dimensions has been observed (Mayer and Rosner, 2011; Wolkerstorfer et al., 2012; Ponomarenko et al., 2014; Rockwell et al., 2014). Angiosperms have more complex secondary xylem tissues compared with coniferous trees in which tracheids assume the roles of both water transport and mechanical support, suggesting that further careful interpretation of UAEs is required for angiosperm species. Recently, the existence of correlations between relative cumulated UAEs during a freeze–thaw cycle and the relative percentage loss of hydraulic conductivity (PLC) after the freeze–thaw cycle, and between \( \psi_{S0} \) (the negative pressure required for 50% loss of water conductivity) and T50 (the subfreezing temperature generating 50% of cumulated UAEs), in angiosperm species was reported (Charrier et al., 2014). However, the study also indicated the presence of a seasonal shift in T50 during cold acclimation. Acclimation of the hydraulic system has not been observed (Charra-Vaskou et al., 2012b), suggesting that part of these signals might originate from a source(s) other than vessels. Further clarification of embolism formation through UAE analyses requires interpretation of the UAE origins. Using excised branches of walnut (Juglans regia cv. Franquette), in this study, the relationship between the generation of UAEs during various types of freeze–thaw cycles (i.e. changing the number of freeze–thaw cycles and the minimum temperatures) and the PLC after freeze–thawing was clarified and the characteristics of UAEs related to cavitation events in vessels were investigated.

Materials and methods

Many different experiments were performed, and are summarized in Fig. 1.

Plant materials

Samples were harvested from walnut trees (J. regia L. cv. Franquette), grown in an orchard at the INRA UMR-PIAF research station in Clermont-Ferrand, France, during the foliation season (May–October in 2010 and 2011). In the present study, walnut branches harvested during the foliation season were used because sample branches with leaves are easily saturated and dehydrated to desired \( \psi \) values. In addition, these sample branches experienced no freezing event before the experiment subsequent to the previous winter. Branches with a basal diameter up to 2 cm were excised from adult trees and immediately transported to the laboratory. The cut ends of the branches were recut at least three times under water to release
UAE from walnut during freeze–thawing

Detection of acoustic emissions

Ultrasonic emission analyses were performed with a μDiSP flow-meter instrument (Bronkhorst, Montigny-Les-Cormeilles, France). After removal of the plastic film covering the sample segments, 15 cm long samples were cut from the internodal portion of partially dehydrated branches under water. The basal end of samples was connected to the XYL’EM instrument with silicone tubes. The initial conductivity, ψ, which was degassed and filtered through a 0.2 μm filter (Whatman, Maidstone, UK) in advance. The segments were then flushed at a pressure of 150 kPa for 10 min to eliminate gas bubbles from vessels, and the hydraulic conductivity of the segments was determined again. The flushing was repeated until the conductivity reached a threshold (Km). The PLC value was calculated as

\[
PLC = 100 \times \left( K_m - K_0 \right) / K_m
\]

The values of ψ0 (the pressure required for 50% loss of water conductivity without a freeze–thaw cycle) and ψ50 (the pressure required for 50% loss of water conductivity after one cycle of freeze–thawing between 5 °C and −10 °C) were estimated from curve fitting with a sigmoidal function:

\[
PLC = \frac{A - D}{1 + e^{B((C - D)/2) + C}} + D
\]

where A and D represent the asymptotes of the function, B is the sigmoidal gain, and C is the ψ value of the inflection point. In the present study, the maximum and minimum PLC values were fixed at 100% (A parameter) and 0% (D parameter), respectively. The other parameters in Equation 2 were estimated by minimizing the sum of squares using a solver function of Excel 2010 (Microsoft, Redmond, WA, USA).

Evaluation of the level of embolism

The level of embolism was evaluated as the PLC using a XYL’EM flow-meter instrument (Bronkhorst, Montigny-Les-Cormeilles, France). After removal of the plastic film covering the sample segments, 15 cm long samples were cut from the internodal portion of partially dehydrated branches under water. The basal cut end of samples was connected to the XYL’EM instrument with silicone tubes. The initial conductivity, ψ, was measured with a pressure difference of 5 kPa using 10 mM KCl and 1 mM CaCl2 solution, which was degassed and filtered through a 0.2 μm filter (Whatman, Maidstone, UK) in advance. The segments were then flushed at a pressure of 150 kPa for 10 min to eliminate gas bubbles from vessels, and the hydraulic conductivity of the segments was determined again. The flushing was repeated until the conductivity reached a threshold (Km). The PLC value was calculated as

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Statistical analysis

The values of PLC, cumulative number of UAEs, and UAE quality parameters are presented as means ±SEs. The normality of the distribution of the data was tested in a Shapiro–Wilk test. For data that had a normal distribution (PLC values and the cumulative number of UAEs), parametric multiple comparisons were made in a Tukey’s honestly significant difference test. For data not distributed normally (UAE quality parameters), non-parametric multiple comparisons were made in a Steel–Dwass test.

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Results
Freeze–thaw-induced embolism and UAEs induced in sample segments with different water potentials

Typical patterns of UAE generation from saturated sample segments ($\psi = -0.1\,\text{MPa}$) and partially dehydrated sample segments ($\psi = -1.6\,\text{MPa}$) during one freeze–thaw cycle between $5\,\text{°C}$ and $-10\,\text{°C}$ are shown in Fig. 2. In both cases, UAEs were generated during the cooling process. Thereafter, UAEs continued to be registered throughout the freezing process. However, no or few UAEs were observed during the thawing process. A similar pattern of UAE generation was observed in all partially dehydrated samples regardless of water potential, as well as in other experiments described below (Fig. 5; Supplementary Figs S1–S3 available at JXB online). In contrast, no UAEs were detected from oven-dried sample segments (Fig. 2A).

Figure 3 indicates the PLC of partially dehydrated sample segments with or without a freeze–thaw cycle between $5\,\text{°C}$ and $-10\,\text{°C}$. In samples without a freeze–thaw cycle, a distinct increase in PLC was recorded from a $\psi$ of $-1.0\,\text{MPa}$, and $\psi_{50}$ was observed at a $\psi$ of $-2.0\,\text{MPa}$. In the case of sample segments after one freeze–thaw cycle between $5\,\text{°C}$ and $-10\,\text{°C}$, the increase in PLC was initiated at a higher water potential than in the case of sample segments without freeze–thaw treatment (Fig. 3). The increase was observed at slightly negative $\psi$, and $\psi_{50}$ was observed at a $\psi$ of $-0.9\,\text{MPa}$. The shaded area in Fig. 3 shows the difference in PLC with and without freeze–thaw treatment, which suggests conductivity loss only by the effects of the freeze–thaw cycle. The maximum effect of the freeze–thaw cycle on PLC was observed in a range between $-1.4\,\text{MPa}$ and $-1.6\,\text{MPa}$. Thus, in subsequent experiments (repeated freeze–thaw cycles, one cycle of freeze–thawing to $-25\,\text{°C}$ and $-40\,\text{°C}$, and one cycle of stepwise freeze–thawing), sample segments with a $\psi$ in this range ($\psi = -1.6\,\text{MPa}$) were used as partially dehydrated samples.

To evaluate the effect of $\psi$ on the properties of UAEs from sample segments, the counts, amplitude, and absolute energy of signals were compared within three different $\psi$ ranges (from $-0.1$ to $-0.5\,\text{MPa}$, from $-1.3$ to $-1.7\,\text{MPa}$, and from $-2.6$ to $-3.0\,\text{MPa}$) (Table 1). Referring to Fig. 3, it was determined that <20% of hydraulic conductance was lost after one freeze–thaw cycle between $5\,\text{°C}$ and $-10\,\text{°C}$ in the higher $\psi$ range ($-0.1\,\text{MPa}$ to $-0.5\,\text{MPa}$). The effect of the freeze–thaw treatment was most severe in the middle $\psi$ range ($-1.3\,\text{MPa}$ to $-1.7\,\text{MPa}$), and almost all vessels lost their hydraulic functions before the freeze–thaw treatment in the lower $\psi$ range ($-2.6\,\text{MPa}$ to $-3.0\,\text{MPa}$). For all parameters, significant differences were observed among the three $\psi$ ranges, of which the middle $\psi$ range had the highest mean values (Table 1). In particular, the mean absolute energy in the middle $\psi$ range was $>1.6$ times the values in the other $\psi$ ranges.
The relationship between ψ of branches and the cumulative number of total UAEs from sample segments treated with one freeze–thaw cycle between 5 °C and –10 °C is presented in Fig. 4A. In contrast to changes in PLC, no significant differences between each ψ range were observed between 0 and –3.0 MPa.

The cumulative number of total UAEs was separated into four classes (0–0.1, 0.1–1.0, 1.0–10.0, and >10.0 fJ) on the basis of the absolute energy of each hit (Fig. 4B–E). After classification, no significant differences were observed in the cumulative number of UAEs among each ψ range up to 10.0 fJ. In the case of UAEs with absolute energy >10.0 fJ, some significant differences in cumulative number of UAEs among each ψ range were observed, and the mean values of the cumulative number of UAEs had a bell-shaped pattern similar in shape to the upper limit of the shaded area in Fig. 3.

Table 1. Characteristics of freeze-induced ultrasonic acoustic emissions (UAEs) from walnut branch segments at different water potentials, subjected to one freeze–thaw cycle

<table>
<thead>
<tr>
<th>Ψ range (MPa)</th>
<th>Counts</th>
<th>Amplitude (dB)</th>
<th>Absolute energy (fJ)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>–0.1 to –0.5</td>
<td>5.19 ± 0.03 a</td>
<td>51.00 ± 0.02 a</td>
<td>0.93 ± 0.06 a</td>
<td>59 907</td>
</tr>
<tr>
<td>–1.3 to –1.7</td>
<td>6.50 ± 0.02 b</td>
<td>52.24 ± 0.02 b</td>
<td>1.52 ± 0.11 b</td>
<td>116 705</td>
</tr>
<tr>
<td>–2.6 to –3.0</td>
<td>4.94 ± 0.04 c</td>
<td>50.92 ± 0.04 a</td>
<td>0.66 ± 0.04 a</td>
<td>24 138</td>
</tr>
</tbody>
</table>

n, number of signals analysed.
Values are the mean ±SE.
Values within a column followed by the same letter are not significantly different (P<0.05).

Fig. 4. Cumulative ultrasonic acoustic emission (UAE) derived from one cycle of freeze–thawing between 5 °C and –10 °C from walnut branch segments dehydrated to different water potentials (ψ). (A) Cumulative number of total UAEs. (B–E) Cumulative number of UAEs classified on the basis of absolute energy. The range of absolute energy is shown in the upper right corner of each graph. The cumulative number of UAEs was divided by the volume of xylem in the sample segments. Numbers of samples are indicated below the Ψ ranges. Each bar and error bar represent the mean ±SE. For the Ψ range from –2.0 MPa to –2.5 MPa, each point represents an individual measurement. Bars with a different letter are significantly different (P<0.05).
Embolism and UAEs induced by different minimum freezing temperatures

After one freeze-thaw cycle to each of –10, –25, and –40 °C, almost all hydraulic conductivity in partially dehydrated sample segments (ψ = –1.6 MPa) was lost (Table 3). In contrast, the cumulative number of UAEs in each range had a trend similar to the absolute energy of each hit (0.1–1.0 fJ). After classification, UAEs with absolute energy >1.0 fJ were detected mainly in cooling steps from 5 to –10 °C. In the case of UAEs with high energy >10.0 fJ, no significant differences were detected among each minimum temperature for UAEs with absolute energy >10.0 fJ. This result suggests that UAEs with absolute high energy were mainly generated at a temperature above –10 °C.

To confirm the generation of UAEs with high energy at a temperature above –10 °C, sample segments were cooled in a stepwise manner (Supplementary Fig. S3 at JXB online), and the cumulative number of UAEs generated in each step was recorded (Table 4). In the case of UAEs signals were detected mainly in cooling steps from 5 °C to –10 °C. The levels of embolism in partially dehydrated sample segments (ψ = –1.6 MPa) were evaluated by PLC measurement after repeated freeze-thaw cycles. Before the freeze-thaw cycles, the PLC of sample segments dehydrated to a ψ of –1.6 MPa was 38.2 ± 10.1% (data not shown). As shown in previous sections, almost all vessels lost their hydraulic conductivities after one freeze–thaw cycle to –10 °C when the sample segments were partially dehydrated to a ψ of –1.6 MPa (Table 2). Similarly, PLC values of ~100% were observed after two or five freeze–thaw cycles (Table 2).

Embolism and UAEs induced by repeated freeze–thaw cycles

The cumulative number of UAEs was divided by the volume of xylem in the sample segments.

### Table 2. Loss of hydraulic conductivity and cumulative ultrasonic acoustic emission (UAE) derived from 15 cycles of freeze–thawing between 5 °C and –10 °C from walnut branch segments dehydrated to a water potential of –1.6 MPa

<table>
<thead>
<tr>
<th>Freeze-thaw cycles</th>
<th>PLC (%)</th>
<th>CumUAE (hits cm⁻³)</th>
<th>Absolute energy (fJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>95.4 ± 3.8 a</td>
<td>99.1 ± 0.9 a</td>
<td>ND</td>
</tr>
<tr>
<td>Freeze–thaw cycles</td>
<td>0–0.1 fJ</td>
<td>105.8 ± 12.5 a</td>
<td>70.0 ± 10.4 a, b, c</td>
</tr>
<tr>
<td></td>
<td>0.1–1.0 fJ</td>
<td>90.0 ± 6.0 a</td>
<td>46.6 ± 6.3 b</td>
</tr>
<tr>
<td></td>
<td>1.0–10.0 fJ</td>
<td>25.1 ± 16.6 a</td>
<td>9.3 ± 15.6 b</td>
</tr>
<tr>
<td></td>
<td>&gt;100 fJ</td>
<td>4.9 ± 0.1 a</td>
<td>1.4 ± 0.2 b</td>
</tr>
</tbody>
</table>

Values are the mean ±SE for eight replicates. Values within a row followed by a different letter are significantly different (P < 0.05).
CumUAE: cumulative number of UAEs; ND, not determined; PLC, percentage loss of hydraulic conductivity.
counts, 6.64 ± 0.03; amplitude, 52.60 ± 0.02) and from –10 °C to –25 °C (UAE counts, 3.26 ± 0.01; amplitude, 48.92 ± 0.01). From –25°C to –40°C, only a few UAEs were detected (UAE counts, 2.59 ± 0.04; amplitude, 47.86 ± 0.03), and few UAEs were observed during the thawing process (UAE counts, 5.06 ± 0.25; amplitude, 52.38 ± 0.18). Then, the total cumulative numbers of UAEs were classified on the basis of absolute energy of each hit. Although generation patterns of UAEs with energy <1.0 fJ had a trend similar to that of total UAEs, the highest number of UAEs with energy >1.0 fJ was detected from 5 °C to –10 °C among each step. In particular, for UAEs with energy >10.0 fJ, 84% of signals were detected from 5 °C to –10 °C.

Relationship between exothermal phenomena in walnut sample segments and UAEs during freezing

A typical pattern of UAE generation from partially dehydrated sample segments (ψ = −1.6 MPa) during one freeze–thaw cycle to –40 °C is shown in Fig. 5A. In this figure, the difference between xylem temperature and air temperature is also indicated. The curve for this temperature difference has two types of exothermal peaks, even though these peaks overlap to some degree, during the cooling process. The first rapid increase in xylem temperature was observed at about –5 °C (termed the high-temperature exotherm; HTE), and the second increase in xylem temperature occurred at about –20 °C (termed the low-temperature exotherm; LTE). The derivative of the curve of the cumulative number of UAEs and the difference between xylem temperature and air temperature during cooling to –40 °C are shown in Fig. 5B. The generation of UAEs was initiated concurrently with HTE initiation, the maximum number of UAEs was recorded after the HTE peak, and a slight increase in the number of registered UAEs was followed by the LTE peak (Fig. 5B). For UAEs with energy >10.0 fJ, almost all signals were recorded in association with HTE (Fig. 5C).

Discussion

Vessels of walnut have a mean diameter of 60–160 μm (Améglio et al., 2002). Water conduits with a diameter larger than 30 μm are highly vulnerable to freeze–thaw-induced embolism (Davis et al., 1999). In winter, nearly 100% PLC is commonly observed in walnut trees in the field (Améglio et al., 2002). In this study, artificial freeze–thaw treatments...
induced embolism formation even in sample segments with $\psi$ slightly lower than 0 MPa (Fig. 3). The PLC of sample segments partially dehydrated at a $\psi$ of $-1.6$ MPa without a freeze–thaw treatment was $\sim38\%$, which was higher than the PLC estimated from the sigmoidal curve in Fig. 3. A level of native embolism might have remained in some samples even after saturation treatment.

To clarify the relationship between freeze–thaw-induced embolism and UAE, the effects of sample $\psi$, repeated freeze–thaw cycles, and freezing temperatures were investigated. In each experiment, UAEs were first registered concurrent with the initiation of freezing of the sample segments (Figs 2, 5A; Supplementary Figs S1–S3 at JXB online). Across samples with different $\psi$, no correlation between $\psi$ and the cumulative number of total UAEs was observed (Figs 3, 4A). Even in the $\psi$ range from $-0.1$ MPa to $-0.5$ MPa, in which $>80\%$ of vessels retained their hydraulic function after one freeze–thaw cycle to $-10 \, ^\circ\mathrm{C}$, significant numbers of UAEs were generated (Fig. 4A). Repeated freeze–thaw cycles and lower freezing temperatures resulted in increases in the cumulative number of total UAEs (Tables 2–4), although one freeze–thaw cycle from 5 $\, ^\circ\mathrm{C}$ to $-10 \, ^\circ\mathrm{C}$ was sufficient for sample segments to lose most of their hydraulic conductivity (Tables 2, 3). Relaxation of tension in water conduits due to cavitation events in the freezing sap is one of the most likely origins of UAEs during freeze–thawing (Tyree and Sperry, 1989; Mayr et al., 2007; Charrier et al., 2014). However, the discrepancy between the increase in the cumulative number of UAEs and embolism formation indicates that some UAEs originated from sources other than the vessel lumen.

To distinguish UAEs generated by cavitation events in vessels from UAEs of other origins, the parameters of individual freeze–thaw-induced UAEs were investigated for sample segments with three different $\psi$ ranges (Table 1). Significant differences were observed for the parameters of counts, amplitude, and absolute energy among the three $\psi$ ranges. High values of absolute energy were observed particularly in the $\psi$ range from $-1.3$ MPa to $-1.7$ MPa (Table 1), in which the maximum loss of hydraulic conductivity by a freeze–thaw cycle was observed (Fig. 3). Under drought stress, a correlation between the energy of UAEs and xylem lumen dimensions has been observed (Mayr and Rosner, 2011; Ponomarenko et al., 2014), probably because larger water conduits store higher total elastic energy (Ponomarenko et al., 2014). In freeze–thaw conditions, the formation of larger gas bubbles is expected in larger water conduits (Cruiziat et al., 2002). The larger bubbles might induce the release of higher tension, resulting in signals of higher energy. Compared with coniferous trees with narrow tracheids, the absolute energy for walnut branch segments with a $\psi$ range from $-1.3$ MPa to $-1.7$ MPa was $\sim10$ times that detected for P. contorta twigs partially dehydrated at a $\psi$ of $-3$ MPa (Mayr and Sperry, 2010).

When UAEs were split into four groups depending on their absolute energy, the group with the highest absolute energy ($>10.0 \, \mu\text{J}$) was significantly correlated to PLC (Supplementary Fig. S4 at JXB online). It is predicted that these UAEs were probably generated by cavitation events preceding embolism formation during freeze–thaw cycles. Therefore, the progress of freeze–thaw-induced embolism could be estimated using the level of absolute energy of UAEs. However, some technical features of the UAE analysis techniques (e.g. attenuation along the sample, simultaneous arrival times of several independent signals at the sensor, and the lockout time of the acquisition system after a signal has ended) might result in some UAEs being missed. Especially in a heterogeneous material such as wood, the material’s attenuation strongly influences UAE detection (Mayr and Rosner, 2011).
would be better to think that the UAEs reaching the sensor are indicators of events occurring within the wood although not all cavitation events can be detected. Approximately 40% and 15% of UAEs with absolute energy >10.0 fJ were also detected after the first cycle of freeze–thawing from 5°C to –10°C in the repeated freeze–thaw treatment (Table 2) and after the first step at –10°C in the stepwise freeze–thaw treatment (Table 4), respectively. Thresholds separating the different UAE groups (0.1, 1.0, and 10.0 fJ) were assigned by default for descriptive purposes, and should result in the mixing of signals from different sources. More suitable values for the recognition of UAEs that reflect embolism generation might be established by further studies. Nevertheless, correlation between detectable UAEs and hydraulic conductivity is significant (Supplementary Fig. S4).

What are the origins of UAEs other than cavitation events in vessels? In the present study, excised branches were used in experiments without the removal of the bark and cambium. It is thus possible that some UAEs originated from these tissues (Kikuta, 2003). In a preliminary experiment, however, no significant difference in the cumulative number of total UAEs during a freeze–thaw cycle from 5°C to –10°C was observed between sample segments with intact bark and debarked segments (data not shown). Therefore, bark was not a major origin of UAEs. No UAEs were detected from oven-dried walnut sample segments (Fig. 2A), similar to results reported for coniferous trees (Mayr et al., 2007; Mayr and Zublasing, 2010). The lack of UAEs from oven-dried sample segments indicates that cell walls do not induce any acoustic activity. The main components of walnut secondary xylem are wood fibres, vessels, axial parenchyma, and ray parenchyma. In particular, >60% of the walnut secondary xylem is composed of wood fibres (unpublished data). The cavitation events in wood fibres could generate UAEs. Utsumi et al. (1998) reported cavitation and refilling with water not only in vessels but also in wood fibres in some angiosperm trees.

When sample segments partially dehydrated to a ψ of −1.6 MPa were cooled to −40°C, an increase in the number of registered UAEs was observed after the appearance of the LTE for the segments at about −20°C. The UAEs following the LTE did not contain those with energy >10.0 fJ (Fig. 5C). Although some temporal differences were observed between the LTE peak and the peak of the derivative of the curve of the cumulative number of UAEs, the LTE indicates the onset of the freezing process (Fig. 5B). The LTE for xylem tissues containing xylem parenchyma cells that adapt to subfreezing temperatures via a freezing avoidance mechanism (deep supercooling) corresponds to breakdown of the supercooling state of intracellular water in xylem parenchyma cells, while the HTE indicates freezing of water in apoplastic spaces including vessels and wood fibres (Fujikawa and Kuroda, 2000; Neuner et al., 2010). Fukami et al. (2011) detected UAEs from bamboo leaves accompanied by freezing of living cells that adapt to subfreezing temperatures via a freezing avoidance mechanism. Given that no UAEs were generated from winter leaves of angiosperm trees that adapt to subfreezing temperatures by extracellular freezing during the cooling process, Fukami et al. (2011) speculated that UAE generation is related to supercooling breakdown in plant cells. Ristic and Ashworth (1994) and Kuroda et al. (1999) observed protoplasm contraction and cavitation of the intracellular spaces of xylem ray parenchyma cells of angiosperms after cooling of the xylem tissues to a low subfreezing temperature employing transmission electron microscopy and cryo-scanning electron microscopy, respectively. The cavitation events in xylem parenchyma cells might generate UAEs. Weiser and Wallner (1988) reported that there are more acoustic emissions from tree species with xylem ray parenchyma cells that are supercooled at subfreezing temperatures than from those with xylem parenchyma cells that were not supercooled. A seasonal variation in the relationship between freeze–thaw–induced UAEs and temperature, which may be attributed to the winter acclimation of parenchyma cells, was also observed for walnut trees (Charrier et al., 2014).

In this study, UAEs were generated only in the freezing process during freeze–thaw cycles regardless of the temperature regime (Figs 2, 5A; Supplementary Figs S1–S3 at JXB online). This trend also applies to UAEs with energy >10.0 fJ, which probably originated from cavitation events in vessels (see Table 4). Similar results in which UAE generation was observed only during freezing have been reported for coniferous trees (Mayr et al., 2007; Mayr and Zublasing, 2010) and other angiosperm trees (Raschi et al., 1989; Kikuta and Richter, 2003; Charrier et al., 2014). These results seem to contradict the current thaw–expansion hypothesis for freeze–thaw–induced embolism formation (Davis et al., 1999; Lemoine et al., 1999; Utsumi et al., 1999; Hacke and Sperry, 2001; Cruziat et al., 2002; Pittermann and Sperry, 2003). The hypothesis states that small gas bubbles form in water conduits because of the lower solubility of gas in ice when the sap freezes (Sevanto et al., 2012). These bubbles would expand when the pressure of the surrounding sap becomes sufficiently negative to counter the bubble-collapsing force of surface tension during thawing (Cruziat et al., 2002). The generation of UAEs should therefore be expected in both freezing and thawing processes as the formation of gas bubbles and expansion of these bubbles releases tension in the water column. However, given the manner of UAE generation, cavitation events (formation of gas bubbles) might occur during freezing with the generation of UAEs, whereas embolism (i.e. the complete filling of conduits with air) takes place on thawing without the generation of UAEs (Kikuta and Richter, 2003). Furthermore, the difference in ψ at the interface between supercooled water and frozen ice might induce air-seeding from air-filled spaces, releasing vessel tension (Mayr et al., 2007). For pure water, the difference in ψ between supercooled water and ice corresponds to approximately –5 MPa at –5°C (Hansen and Beck, 1988). This tension, much lower than the vulnerability threshold (ψ90) of walnut vessels (~2.0 MPa; Fig. 3), might displace the air–water menisci in xylem pits (Charrier et al., 2014). The different manners of UAE generation from saturated sample segments of walnut versus coniferous trees may reflect the occurrence of air-seeding in angiosperm species. Walnut branch segments emitted a considerable amount of UAEs even without significant tension (Fig. 4A), as was also observed in rachides...
of walnut (Kikuta and Richter, 2003). In contrast, few or no UAEs were observed from saturated twigs of coniferous trees during freeze–thawing (Mayr et al., 2007; Mayr and Sperry, 2010; Mayr and Zublasing, 2010). Even when xylem conduits are full, there are more air-filled spaces such as the pith and empty fibres in the xylem for angiosperm species than for conifers. Anatomical properties of angiosperm xylem can facilitate air-seeding. Finally, the results presented here are in agreement with the recently published hypothetical mechanism of freeze–thaw-induced embolism formation (Charrig et al., 2014). Combinatorial studies of UAE analyses and other assessments, such as centrifuge experiments (Mayr and Sperry, 2010), X-ray tomography (Charrig-Vaskou et al., 2012a; Brodersen et al., 2013; Suuronen et al., 2013), and magnetic resonance imaging (Holbrook et al., 2001), would further clarify freeze–thaw-induced embolism formation.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** Ultrasonic acoustic emission (UAE) generation from a walnut branch segment dehydrated to a water potential ($\psi$) of ~1.6 MPa during 15 cycles of freeze–thawing between 5 °C and –10 °C.

**Figure S2.** Ultrasonic acoustic emission (UAE) generation from a walnut branch segment dehydrated to a water potential ($\psi$) of ~1.6 MPa during one cycle of freeze–thawing between 5 °C and –25 °C.

**Figure S3.** Ultrasonic acoustic emission (UAE) generation from a walnut branch segment dehydrated to a water potential ($\psi$) of ~1.6 MPa during stepwise freeze–thawing.

**Figure S4.** Relationship between cumulative number of ultrasonic acoustic emissions (UAEs) and percentage loss of hydraulic conductivity (PLC) induced by one freeze–thaw cycle.

**Acknowledgements**

This work was partly supported by the Institut National de la Recherche Agronomique (INRA)—Department of Agronomy and Environment, a post-doctoral grant from Blaise Pascal University (Clermont II), and a Grant-in-Aid from the Japan Society for the Promotion of Science (22–5901 to JK). This project was also funded by the French and Austrian research agencies in Aid from the Japan Society for the Promotion of Science (22–5901 to JK). This work was partly supported by the Institut National de la Recherche Agronomique (INRA)—Department of Agronomy and Environment, a post-doctoral grant from Blaise Pascal University (Clermont II), and a Grant-Agronomique (INRA)—Department of Agronomy and Environment, a

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