Velocity and pattern of ice propagation and deep supercooling in woody stems of *Castanea sativa*, *Morus nigra* and *Quercus robur* measured by IDTA

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Summary  Infrared differential thermal analysis (IDTA) was used to monitor the velocity and pattern of ice propagation and deep supercooling of xylem parenchyma cells (XPCs) during freezing of stems of *Castanea sativa* L., *Morus nigra* L. and *Quercus robur* L. that exhibit a macro- and ring-porous xylem. Measurements were conducted on the surface of cross- and longitudinal stem sections. During high-temperature freezing exotherms (HTEs; −2.8 to −9.4 °C), initial freezing was mainly observed in the youngest year ring of the sapwood (94%), but occasionally elsewhere (older year rings: 4%; bark: 2%). Initially, ice propagated rapidly in the largest xylem conduits. This resulted in a distinct freezing pattern of concentric circles in *C. sativa* and *M. nigra*. During HTEs, supercooling of XPCs became visible in *Q. robur* stems, but not in the other species that have narrower pith rays. Intracellular freezing of supercooled XPCs of *Q. robur* became visible by IDTA during low-temperature freezing exotherms (LTE; −17.4 °C). Infrared differential thermal analysis revealed the progress and the two-dimensional pattern of XPC freezing. XPCs did not freeze at once, but rather small cell groups appeared to freeze at random anywhere in the xylem. By IDTA, ice propagation and deep supercooling in stems can be monitored at meaningful spatial and temporal resolutions.

Keywords: freezing exotherm, infrared differential thermal analysis, infrared thermography, intracellular freezing pattern, persistent supercooling, xylem ray parenchyma cells.

Introduction

Xylem parenchyma cells (XPCs) of many trees from temperate regions exhibit deep supercooling, while cortical and cambial tissues survive by extracellular equilibrium freezing (Sakai and Larcher 1987). During freezing of stem segments of such trees, two separate freezing events are recorded. As ice formation is an exothermic process, heat is released, causing a sudden temperature increase, i.e., a freezing exotherm, that can be recorded by differential thermal analysis (DTA; Burke et al. 1976). The first freezing event is indicated by the so-called high-temperature freezing exotherm (HTE) that is caused by the extracellular freezing of apoplastic water in the bark tissue or of water in the xylem water conduits and non-living cells. The second freezing event, low-temperature freezing exotherm (LTE), occurs at significantly lower temperatures. Temperature depends on the season and ranges from about −20 to below −45 °C in winter (Sakai and Larcher 1987), which is slightly below the homogeneous ice nucleation point of water. LTE is caused by intracellular freezing of XPCs (Tumanov and Krarasvtev 1959, Quamme et al. 1972, George and Burke 1977, Quamme et al. 1982, Ashworth et al. 1988, Malone and Ashworth 1991). XPCs are killed by this process. Consequently, entire trees may die despite survival of the cortical and cambial tissues that show extracellular equilibrium freezing. Such XPC damage is known as ‘black heart damage’ (George et al. 1982). The low-temperature limit of successful deep supercooling in XPCs can restrict the geographical distribution of certain tree species (George et al. 1974, Kaku and Iwaya 1978, 1979, Sakai and Larcher 1987).

DTA uses thermocouples and is commonly used to determine HTEs and LTEs. In addition, infrared video thermography (IRVT; Wisniewski et al. 1997, 2002, Workmaster et al. 1999, Carter et al. 2001, Pearce and Fuller 2001, Ball et al. 2002, Stier et al. 2003, Sekozawa et al. 2004) makes it possible to determine the location of ice nucleation and the pattern of ice propagation. Infrared differential thermal analysis (IDTA) is an improvement on IRVT because it allows the tracking of ice nucleation and propagation in whole plants (Hacker and Neuner 2008) and at considerably higher resolution even down to the plant tissue level (Hacker and Neuner 2007, Hacker et al. 2008).

The first aim of this study was to apply IDTA to the surface of cross- and longitudinal sections of woody stem segments to check whether it might be possible to visualize the temporal spatial ice propagation patterns in different tissues.
of woody stems. Freezing patterns in woody stems are poorly understood, and due to different findings with respect to initial ice nucleation in the bark or the xylem tissue (Pearce 2001), they are somewhat controversial. The advantage of IDTA as compared with other two-dimensional methods for the detection of the location of ice formation, such as proton nuclear magnetic resonance (\(^{1}\)H NMR) microscopy (Repo et al. 2008), is a significantly higher temporal resolution giving a real-time sequence of images with 50 image recordings per second.

Additionally, the spatial resolution of IDTA (200 µm) appeared to be sufficient to visualize the supercooling behaviour of XPCs. Supercooling of XPCs has been demonstrated by several methods until now (DTA, nuclear magnetic resonance (NMR) spectroscopy (see Burke et al. 1976), differential scanning calorimetry (George and Burke 1977, Quamme et al. 1982, Repo et al. 2008), NMR micro-imaging (Ishikawa et al. 1997, Repo et al. 2008) and freeze-fracture SEM (Ashworth 1996, Kuroda et al. 2003)). The high temporal resolution of IDTA contrasts with already applied methods, and we hypothesized that it might be possible to visualize the temporal and spatial patterns of intracellular freezing of XPCs in the xylem.

Materials and methods

Plant material

Three deciduous angiosperm hardwood tree species, i.e., sweet chestnut (Castanea sativa L.), black mulberry (Morus nigra L.) and pedunculate oak (Quercus robur L.), growing in the Botanical Garden of the Institute of Botany of the University of Innsbruck (47°16′ N 11°23′ E, 600 m a.s.l.) were chosen for the experiments. The species were chosen as they exhibit macro- and ring-porous xylem, which was expected to allow the visualization of freezing in single large vessels. The species were also selected as they were expected to have deep-supercooling XPCs (Quercus: Kaku and Iwaya 1979, George et al. 1982, Malone and Ashworth 1991; Castanea: George et al. 1982, Kasuga et al. 2007a, 2007b; Morus: Kasuga et al. 2007a, 2007b). Q. robur was chosen as the width of xylem parenchyma pith rays appeared sufficiently broad to allow the monitoring of supercooling and intracellular freezing under the experimental conditions with the given resolution of the macro lens of the infrared (IR) camera employed.

Infrared differential thermography

Freezing patterns and deep supercooling were monitored with a digital IR camera (ThermaCAM\textsuperscript{TM} S60, FLIR Systems AB, Danderyd, Sweden) that was equipped with a close-up lens (LW 64/150) to achieve a sufficiently high resolution (200 µm). The system was interfaced with a control computer for data transfer via FireWire. Infrared data were then further analysed with the software package ThermaCAM\textsuperscript{TM} Researcher (FLIR Systems AB, Danderyd, Sweden). For IDTA, the method is described in detail by Hacker and Neuner (2007). By the IDTA method, the infrared image immediately before freezing is subtracted from a sequence of images during freezing. In this way, only the temperature change during the release of latent heat is visualized. In addition, background fluctuations of temperature are totally quenched.

Twigs were collected in the Botanical Garden of the University of Innsbruck immediately before the start of the measurement. In the laboratory, twig segments with a mean length of 3 cm and a mean diameter of 1.41 cm were cut off the branch a reasonable distance from the cut surface and placed in the freezing chamber. The IR camera was then focused on the cross-sectional area of the stem piece. Then the temperature was progressively lowered in a temperature-controlled freezing chamber (see Taschler and Neuner 2004) at a mean cooling rate of \(-24 ^\circ\text{C} \text{h}^{-1}\). In addition, thermocouples connected to a datalogger (CR10X, Campbell Scientific, Logan, UT) were placed on the bark below the cut surface of the stem segment to measure absolute temperatures. Plant samples were measured without application of artificial nucleators to identify...
the action sites of intrinsic nucleators (Pearce 2001). A mean total number of 47 IDTA measurements were done per species throughout the course of a whole year. The rate of ice propagation was calculated by an analysis of the time series of IDTA images. The distance ice had propagated in the time span between each IDTA image, i.e., the rate of ice propagation, was measured by image-analysing software (Optimas, Seattle, WA).

Under experimental conditions, IDTA images could only be recorded down to a temperature of about $-25^\circ$C. This temperature limit originated from the cooling capacity of the employed freezing chamber and temperature restrictions of the IR camera. Infrared differential thermal analysis measurements that intended to monitor patterns of intracellular freezing of XPCs in the xylem were conducted in summer when the supercooling capacity of XPCs is lowest (George and Burke 1977, Kaku and Iwaya 1978, Quamme et al. 1982, Wisniewski and Ashworth 1986, Fujikawa and Kuroda 2000, Kuroda et al. 2003, Kasuga et al. 2007a) but within the operating temperature of our measurement system.

**Results**

DTA measurements revealed seasonal patterns of HTE and LTE. The HTE indicates the non-lethal freezing of apoplastic water (Figure 1). Under the experimental conditions, ice nucleation occurred within a temperature span of about 5 °C in a species-specific temperature range (Q. robur: $-3.3$ to $-7.5$ °C, C. sativa: $-2.8$ to $-7.4$ °C, M. nigra: $-5.1$ to $-9.5$ °C). In all species, the seasonal differences were not significant. Lethal freezing of the supercooled XPCs occurred significantly lower temperatures (Q. robur: below 16.4 °C, C. sativa: below 16.9 °C, M. nigra: below 14.5 °C) and produced a second exotherm, the so-called LTE. LTE decreased markedly during winter to their lowest values in January and February in all three species. The decrease in LTE was strongest in Q. robur, with a total seasonal amplitude of 20 °C as compared with 8.8 °C (M. nigra) and 7.1 °C (C. sativa). The frost resistance of XPCs, determined by LTE values in winter, ranked the species as follows: M. nigra < C. sativa < Q. robur.

In all investigated species, particular freezing patterns on the sectional surfaces of the woody stems could be visualized

Figure 2. Cross-sectional view and sequence of IDTA images recorded on the cross-section of a stem segment of Q. robur in October 2007 during a controlled freezing treatment (ice nucleation temperature $-7.3$ °C). Freezing is visualized by a brightening, while unfrozen areas remain black. The numbers in the top left corner of each IDTA image indicate the time in seconds after initial ice nucleation. While ice spread throughout the whole stem segment within 4 s, the XRP tissue supercooled. Freezing of the bark lagged behind.
by IDTA during the HTE. IDTA measurements, in addition to the HTE and LTE, gave information on the location of ice and the sites and patterns of ice propagation in the stem tissue. In *Q. robur*, deep supercooling of XPCs could also be visualized: on the stem cross-section of *Q. robur* shown in Figure 2, initial ice occurred in the sapwood, and from there ice propagated throughout the entire xylem of the stem segment within 4 s. The clearest images of freezing were obtained between 0.4 and 1.6 s. Later images were blurred due to diffusive heat expansion. Particularly between 0.4 and 1.6 s, but also later on, it can be clearly seen that the pith rays remain black. This means that supercooling occurs in the xylem ray parenchyma (XRP) tissue, while the non-parenchymatic parts of the xylem readily freeze, i.e., turn white. The late wood of each year ring also stays darker, maybe because there are only a few narrow vessels interspersed between wood fibres containing a lesser amount of water than in the early wood. Freezing of the bark in

Figure 3. (A) Cross-sectional view and (B–AG) sequence of IDTA images recorded on the cross-section of a stem segment of *Q. robur* in July 2008 during a controlled freezing treatment (ice nucleation temperature −4.0 °C, length of the bars in A and B is 1 cm). In the IDTA images, freezing became visible as a brightening, while unfrozen areas remained black. Time scale after ice nucleation at −4 °C during HTE: (B) 1 s, (C) 4 s, (D) 5 s, (E) 5.4 s, (F) 6 s, (G) 7 s, (H) 9 s and (I) 20 s. Freezing of the XPCs (J–AG) produced small white spots (selected freezing events with highest exotherms are shown) and occurred in a temperature range between −21.3 and −23.3 °C (178–233 min after initial ice nucleation).
the respective stem segment was delayed, starting after the spread of ice throughout the xylem (see Figure 2, last IDTA image at 6.2 s). The pith tissue also remained black during freezing of the bark and the xylem tissue.

After sufficiently low temperatures have been reached, the supercooled XPCs spontaneously freeze intracellularly, producing the so-called LTEs (Figure 3). In summer, it was also possible to visualize intracellular freezing of XPCs in stem segments of *Q. robur*. On the segment shown, initial ice was detected somewhere in the cambial region (Figure 3B). From there, ice readily spread into the sapwood and into the cortical tissue (Figure 3C–E). The non-freezing behaviour of pith rays during HTE can be clearly seen (Figure 3F–H). During the LTE, which occurred in a temperature range between −21.3 and −23.3 °C, intracellular freezing of XPCs is visualized by IDTA images (Figure 3J–AG).

The detection of the LTE by IDTA was difficult as the maximum temperature difference recorded during an LTE was 0.2 °C and lasted only a few seconds. LTEs below the resolution and sensitivity of the IR camera used appear likely. The pattern of XPC freezing is highly random, with single XPC groups freezing separately anywhere in the living xylem and not as one might expect with whole rays freezing at once (Figure 4).

In the other two species, a somewhat similar pattern of ice propagation to that observed in *Q. robur* could be seen in the IDTA images during the HTE. However, supercooling of XRP could only be visualized for pith rays in stem cross-sections of *Q. robur*. In *C. sativa* (Figure 5), the IDTA images

Figure 4. All detectable XPC freezing events from the cross-section of a stem segment of *Q. robur* in Figure 3 are illustrated at the site of their occurrence by grey circles; the chronological order of each LTE is indicated by the number within each circle. The number of the LTE and its corresponding time is displayed in the scale bar (first XPC exotherm is defined as t = 0; it occurred at −21 °C 178 min after initial ice nucleation; see Figure 3). The single-XPC freezing events did not show a distinct two-dimensional pattern but occurred randomly.

Figure 5. Cross-sectional view and sequence of IDTA images recorded on the cross-section of a stem segment of *C. sativa* in June 2007 during a controlled freezing treatment (ice nucleation temperature −6.2 °C). Freezing is visualized by a brightening, while unfrozen areas remain black. The numbers in the top left corner of each IDTA image indicate the time in seconds after initial ice nucleation. Ice spread preferentially in the vessels of the early wood. The ring-porous wood structure of *C. sativa* is clearly visualized in the IDTA images. This freezing pattern diminished when smaller vessels in the late wood started to freeze. Freezing of the bark lagged behind as it did in *Q. robur*. 
of the surface of the cross-section of the stem segment show ice initially appearing in the sapwood of the youngest year ring and then spreading tangentially via the large vessels of the early wood (2 s). Later, on the opposite side of the cross-section, water in the vessels of the early wood in the year ring of the preceding year freezes and also spreads from vessel to vessel. In this way, the ring-porous wood structure of *C. sativa* becomes visible in the IDTA image as the initial spread of ice seems to be restricted to the large vessels of the early wood. After 8 s, the image becomes blurred. Again, the pith tissue remains black. Freezing of smaller vessels is delayed, and freezing of the cortical tissue did not start until after 8 s when the xylem appeared fully frozen. In *M. nigra*, ice also initially propagated tangentially along the early wood of the year rings of the sapwood, making the ring-porous wood structure evident (Figure 6). Freezing of single vessels can be seen even more distinctly than in the other two investigated species as individual vessels in the early sapwood each

Figure 6. Cross-sectional view and sequence of IDTA images recorded on the cross-section of a stem segment of *M. nigra* in October 2007 during a controlled freezing treatment (ice nucleation temperature \(-9.1\, ^\circ\text{C}\)). Freezing is visualized by a brightening, while unfrozen areas remain black. The numbers in the top left corner of each IDTA image indicate the time in seconds after initial ice nucleation. The freezing of individual vessels within the circles of the early wood produces bright spots and shows the circle structure of the xylem.

Figure 7. Longitudinal cross-sectional view and sequence of IDTA images recorded on the longitudinal cross-section of a stem segment of *M. nigra* in April 2008 during a controlled freezing treatment (ice nucleation temperature \(-6.8\, ^\circ\text{C}\), length of the bar in the second image is 1 cm). Freezing is visualized by a brightening, while unfrozen areas remain black. The numbers in the top left corner of each IDTA image indicate the time in seconds after initial ice nucleation. Longitudinal ice propagation in the vessels was much faster than ice propagation in the radial direction (see the arrows in the IDTA image 2.3; rates of ice propagation: longitudinal 1.9 cm s\(^{-1}\), along the radial vector 0.4 cm s\(^{-1}\)).
produce bright white spots in the IDTA image. The pith remains black, and freezing of the cortical tissue starts earlier than in the other species at around 1.2 s.

Ice propagation in longitudinal stem sections occurred preferentially in the longitudinal direction via large vessels (Figure 7). Ice propagation in the longitudinal direction was much faster than in the lateral direction. The IDTA images cannot differentiate between radial and tangential lateral ice propagation or a combination of both. The rate of ice propagation in cross- and longitudinal stem sections was calculated in a three-dimensional manner: longitudinal, radial and tangential rates were determined. Rates of ice propagation were highest in the longitudinal direction. In this direction, they were also highly temperature dependent (Figure 8). The rates differed significantly between directions; for instance, at −6 °C the rates were highest in the longitudinal direction (2.6 cm s⁻¹) and lowest in the radial direction (0.06 cm s⁻¹).

Sites of initial ice propagation in the stem segments did not differ significantly between the three investigated species. Initial ice usually occurred in the sapwood (94%), but was also detected in older year rings (4%) and rarely in the bark (2%). Ice usually spread to adjacent tissues initially through the xylem and only in the final stages of freezing into the bark tissue. In winter, this pattern was very clear cut. In summer, when ice often initially occurred in the youngest sapwood, the bark tissue froze at the same time as the older year rings. The routes of ice propagation did not change seasonally.

Discussion

Deep supercooling

Deep supercooling during HTE and intracellular freezing during LTE were evident in the sequences of IDTA images obtained from monitoring Q. robur stems during freezing. The freezing pattern of XPCs was mottled as the rays did not freeze at once but rather small clusters of XPCs appeared to freeze at random anywhere in the xylem. This XPC freezing pattern suggests the existence of efficient ice barriers to and between XPCs. These observations are in accordance with the early findings of Hong and Sucoff (1980). They examined twigs of various deciduous angiosperms microscopically after freezing down to the temperature range wherein LTEs occur. They found that deep supercooled water in the xylem froze in numerous independent events over a span of as much as 20 °C. The units of intracellular freezing were single cells or small groups of cells, which is fully corroborated by our findings. The observed freezing pattern contradicted the initial suggestion of George and Burke (1977) that one nucleation event triggered intracellular freezing in the cells in one intact xylem ray. The pattern of freezing as seen in the IDTA images may explain the general difficulties in determining LTE in stems as single cells or groups of cells freeze independently of each other throughout a certain period of time, and this releases only very small amounts of heat.

Repo et al. (2008) used ¹H NMR microscopy to study the distribution of free water in the cross-section of Q. robur stems during freezing down to −13 °C. Unfortunately, at −13 °C, they could not locate any non-frozen water in the xylem except for the pith tissue, which led them to suggest that the non-freezing water molecules are strongly bound in proteins and hence not visible in ¹H NMR microscopy.

The mechanisms of deep supercooling of XPCs are under discussion. Water in the form of small isolated water droplets appears to be a prerequisite for deep supercooling (Ashworth and Abeles 1984). This isolation seemed to be realized by specific cell wall structures that allow neither dehydration of protoplasts nor penetration of extracellular ice into the protoplasts of XPCs (George and Burke 1977, Quamme et al. 1982, Ashworth and Abeles 1984, Wisniewski and Davies 1989). In addition to the isolation of protoplasts in some species, deep supercooling appeared to be accompanied by incomplete desiccation (Kuroda et al. 2003). In this way, supercooling down to −40 °C (homogeneous ice nucleation point of water) or sometimes even lower (due to concentration of solutes in protoplasts further depressing the freezing point) should be possible (Gusta et al. 1983, Kuroda et al. 2003). Our results and those of Hong and Sucoff (1980), which show that single cells or small groups of them freeze intracellularly during breakdown of the deeply supercooled state, give further evidence that ice propagation between cells of the XRP must be suppressed.

Furthermore, the seasonal changes in deep supercooling observed in the investigated species have been attributed to the accumulation of certain intracellular substances, such as soluble sugars (Kasuga et al. 2007a, 2007b), unknown substances with anti-ice nucleation activity (Kasuga et al. 2007a, 2007b) or flavonol glycosides exhibiting high anti-ice nucleation activity (Cercidiphyllum japonicum: Kasuga et al. 2008), and to changed gene expression (Larix kaempferi: Takata et al. 2007).
Freezing pattern

The pattern of ice propagation in the investigated species during HTE could be clearly visualized by IDTA measurements on the sectional surfaces of stem segments in some cases down to single vessels. As IR cameras measure the surface temperature, the detected freezing pattern on the cross-sectional surface is a two-dimensional reduction of the three-dimensional freezing process inside the wood sample. An increase in temperature at the sectional surface of the wood sample is detected when freezing has occurred just beneath or at the surface, an event occurring when ice has propagated towards the surface. Hence, ice nucleation sites cannot be localized by measurement on sectional surfaces.

Rates of ice propagation in wood in all three directions (longitudinal, tangential and radial) are defined by the specific anatomical architecture of the wood. Particularly large vessels appeared to promote fast longitudinal ice propagation in the xylem as they lacked major resistance. Similar to observations on vascular tissues of leaves and stems (Hacker and Neuner 2007, 2008), lateral ice propagation was slower but not restricted. Rates of ice propagation in a longitudinal direction significantly increased with decreasing temperature, which corroborates earlier findings (Hacker and Neuner 2007, 2008).

Until recently, ice propagation patterns and nucleation sites in woody stems were poorly understood, but it was believed that the xylem vessel contents froze at some stage during freezing of a plant (Pearce 2001). Our results show that independent of the initial ice nucleation site, the vessel system will promote rapid ice propagation as soon as it comes into contact with ice. It is evident from IDTA observations on leafy stems and in leaves that ice can nucleate anywhere. Initially, nucleation is more likely in the xylem, but after nucleation of the vessel contents, ice spreads fast via the xylem vessels and from there into other tissues (Ball et al. 2002, Hacker and Neuner 2007, 2008, Hacker et al. 2008).

Under the experimental condition where we used stem segments, initial ice was usually detected on the stem surface in the sapwood, but could also be detected in the older year rings during winter and occasionally in the bark. Hence, we still cannot fully answer the question of where initial ice nucleation sites occur in stems. Our observation of frequent nucleation in the sapwood fits the results obtained from stem cross-sections of _Q. robur_ by $^1$H NMR microscopy (Repo et al. 2008) where the distribution of free water in the cross-section of _Q. robur_ stems was studied. On the outer interface of the xylem sapwood of _Q. robur_, some areas with a high concentration of water were found. The role of these spots remained an enigma to Repo et al. (2008), but they nevertheless assumed that the incidence of ice nucleation might be higher in these areas than in areas containing less water.

Ice nucleation in intact plant tissues under laboratory conditions can occur between −1 and −15 °C depending on the species, organ or tissue, the seasonal timing and development stage investigated (Larcher 1985). Our findings of HTEs between −2.8 and −9.5 °C (−6 to −9.4 °C in _Q. robur_; Repo et al. 2008) corroborate laboratory tests, but are too low for natural situations. In their natural environment, most plants will already have formed ice between −0.6 and −2.6 °C (Pearce 2001, Taschler and Neuner 2004, Taschler et al. 2004), except in some organs and tissues that remain supercooled for a long period (Sakai and Larcher 1987). The higher ice nucleation temperature under natural conditions may promote lower ice propagation rates. The seasonal course of HTE showed some variability in the three investigated species, but there were no marked differences between species and measurement date. This may reflect the stochastic nature of ice nucleation.

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