Inspection of gas bubbles in frozen Betula pendula xylem with micro-CT: Conduit size, water status and bark permeability affect bubble characteristics

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Funding information
Academy of Finland, Grant/Award Numbers: 310375, 323843, 337549; European Synchrotron Radiation Facility

Edited by I. Willick

Abstract
Bubbles of gas trapped in the xylem during freezing are a major cause of damage for trees growing at high altitudes or latitudes, as the bubbles may cause embolism during thawing. Yet the factors controlling bubble formation upon freeze–thaw cycles remain poorly understood. Especially the size of the bubbles formed in the ice is crucial for winter embolism formation. We used high-resolution X-ray microtomography combined with freezing experiments to investigate the size and shape of 68,343 gas bubbles in frozen conduits in branches of Betula pendula. We also studied how conduit size, tree water status (−0.2 MPa vs. −0.6 MPa) and bark permeability to gases (decreased by Vaseline-coating) affect the gas bubbles characteristics. High-resolution X-ray images allowed us to detect gas bubbles down to 1.0 μm in diameter and revealed that not only small spherical gas bubbles but also gaseous volumes of various shapes and sizes were found from the frozen xylem indicating that gas bubbles may have started to grow already during the freezing propagation. Most of the gas bubbles were found in fibers, but the rare gas bubbles found in the vessels were larger than those in the fibers. Bubble volume increased with conduit volume in both fibers and vessels, but conduit size alone could not explain gas bubble volume. Low water potential and restriction of gas escape from the branch seem to cause more, larger, and less spherical bubbles and thus increase the risk of embolism formation. These findings open new research avenues for further studies of winter embolism formation.

1 | INTRODUCTION

In high latitudes and altitudes, cold and frost are key factors for plant survival and distribution. Frozen xylem sap can damage living cells by plasmolysis or intracellular ice formation (see review by Arora, 2018; Ristic & Ashworth, 1993). Freezing and thawing can also cause embolism in xylem and, therefore, loss of xylem hydraulic conductivity (Mayr et al., 2007, 2020). Winter embolism forms because gases dissolved in liquid sap are hardly soluble in ice and thus form bubbles when the sap freezes, and the bubbles may expand and fill the conduits with gas during thawing (Sperry & Sullivan, 1992; Sucoff, 1969). The fate of gas bubbles during thawing, that is whether they collapse or expand to embolize xylem conduits, depends on the water potential of the surrounding xylem sap and the bubble size (Pittermann & Sperry, 2006). Although the gas bubble size is crucial in winter embolism formation, the critical factors affecting bubble formation and
subsequent bubble size during freezing are unclear (Charrier et al., 2015; Lintunen et al., 2020).

What determines the gas bubble characteristics in frozen xylem? Vulnerability to winter embolism is known to increase with increasing xylem conduit size (Davis et al., 1999; Pittermann & Sperry, 2003), and this linkage has been explained by larger gas bubbles forming in larger conduits assuming proportionality between gas bubble volume and water volume (Pittermann & Sperry, 2003, 2006; Sperry & Sullivan, 1992). However, this explanation is theoretical and has not been directly measured to our knowledge.

A second factor potentially affecting the gas bubble size is the escape of gases from the xylem during the freezing process. It has been generally assumed that all the gas dissolved in the xylem sap is trapped within the conduits and forms bubbles during freezing, but we have previously shown that this is not the case as substantial freezing-related bursts of CO2 are released from the stem during ice propagation (Lintunen et al., 2014, 2020). The size and/or number of gas bubbles in the ice are likely to change with the amount of gas diffusing or being pushed out by the high pressures that develop during the freezing process (Robson & Petty, 1987).

In addition to conduit size and gas extraction, xylem sap water potential during the freezing process can affect the size of gas bubbles. It has been frequently shown that hardly any winter embolism formation occurs in laboratory conditions if the wood samples are saturated with water whereas decreasing the sample water potential prior to freezing allows detection of winter embolism formation (Mayr et al., 2007; Mayr & Zublasing, 2010). This has been suggested to be linked to bubble formation (Charrier et al., 2014), because decreasing the sap water potential prior to freezing causes more acoustic emissions from wood detected during the freezing process (Mayr et al., 2007; Mayr & Zublasing, 2010). However, Mayr and Sperry (2010) observed in Pinus contorta that decreasing the water potential during freezing only affected acoustic emissions, not branch hydraulic conductivity, whereas decreasing the water potential during thawing did decrease hydraulic conductivity. The origin of acoustic emissions during drought is the rapid tension release in the conduit walls when liquid water is very rapidly replaced by water vapor (Charrier et al., 2015; Mayr & Rosner, 2011; Ponomarenko et al., 2014; Saleo & Lo Gullo, 1986; Tyree & Dixon, 1983), but during freezing, the origin of acoustic emissions is less clear. However, increasing evidence indicates that acoustic emissions during freezing are caused by cavitation in the xylem sap due to low water potential at the ice–liquid interface (Charrier et al., 2014, 2015, 2017; Mayr et al., 2007).

Measuring gas bubbles within ice inside wood is challenging because of the high resolution required for the imaging, as the diameter of the bubbles is ~1 μm (Robson et al., 1988). Gas bubbles in frozen xylem have been visualized only a few times before using an optical microscope (Sucoff, 1969) or a (cryo-) scanning electron microscope (Robson et al., 1988; Utsumi et al., 1999), and even in those cases, only a few bubbles were captured in the sampled frozen microscopic wood sections. Recent developments in X-ray microtomography (micro-CT) techniques allow high-resolution imaging of the xylem. Micro-CT can visualize the density differences inside samples in 3D with resolutions reaching much below 1 μm. While the method has increasingly been used in studying plant embolism by quantifying embolized xylem conduits (Cochard et al., 2014; Mayr et al., 2020), it has not yet been used in studying gas bubble characteristics in the ice leading to winter embolism formation.

We used micro-CT with the aim of inspecting gas bubbles in frozen xylem of diffuse-porous Betula pendula (Roth). To utilize micro-CT to study gas bubble characteristics and their drivers in frozen xylem, branches with three different treatments were frozen in controlled conditions prior to the micro-CT: (1) saturated control samples, (2) saturated samples with decreased bark permeability by Vaseline-coating, and (3) dehydrated samples. We hypothesized that (1) gas bubbles can start to grow already during the ice propagation in the xylem; (2) gas bubble size and number increase with increasing conduit diameter, thus being larger in vessels than in fibers; (3) gas bubble size and number increase with decreasing bark permeability (due to decreased extraction of gases from the xylem during the freezing process); and (4) with decreasing sample water potential. This study aims to bring new understanding of the mechanism of winter embolism formation in trees.

2 | MATERIAL AND METHODS

2.1 | Sample collection and preparation

Nine 1-m-long branches of B. pendula were sampled from mature trees on the 7th of May in 2018 in Turku, southern Finland (N 60° 26’4, E 22° 14’0”). In early May, trees in Southern Finland are already dehardened after winter and the branches had small leaves when sampled. This should not, however, interfere with our analyses as we did not measure/analyze living cells or any processes during thawing (including embolism formation), only the physical freezing process. The branches were immediately recut under water and saturated by keeping them in water for 48 h covered with a black plastic bag.

The branches were randomly divided into three subsets, saturated control branches, saturated branches coated with Vaseline (to test if a barrier to gas diffusion out of the branch during the freezing process would impact bubble formation), and branches dehydrated with bench-drying (to test the effect of branch water potential on bubble formation, Table 1). Vaseline coating was applied to saturated branches just prior to the start of the freezing experiment. The water potential of the branches was measured with a pressure chamber (PMS Instrument Company Model 1000). Based on previous studies, it is also known that xylem water potential and ice nucleation temperature are positively linked (Arias et al., 2017; Lintunen et al., 2018; Medeiros & Pockman, 2011; Sperling et al., 2017), so branched dehydrated with bench-drying also should have lower apoplastic ice nucleation temperature. The difference in water potential and apoplastic ice nucleation temperature between the saturated and dehydrated samples was 0.34 MPa and 1.3°C, respectively, whereas there was no difference in the water potential or apoplastic ice nucleation...
temperature between the saturated samples and saturated Vaseline-coated samples (Table 1).

2.2 | Freezing of the samples

The freezing experiments were conducted in a test chamber (Weiss Umwelttechnik WK11 2340/40) at the University of Helsinki. The cut end of each branch was sealed with self-amalgamating tape and placed in the test chamber vertically in a dish. A T-type thermocouple was attached to the surface of each branch and temperatures were recorded every 10 s on a data logger (CR1000, Campbell Scientific) to detect the freezing exotherms (i.e. heat released during phase transition). The temperature was first dropped from room temperature to +5°C, kept there for 15 min, and decreased to −15°C in 1 h 45 min. After freezing the branches, they were moved to a −20°C freezer and cut frozen to 80-mm-long samples with a diameter of ~5 mm. Each sample was sealed in a plastic tube and stored in the freezer for a week. After this, the samples were shipped in dry ice to the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. At the ESRF, the samples were stored at −80°C for 1 week before the synchrotron measurements.

2.3 | X-ray phase microtomography inspection

The sample holder (Figure 1) consisted of a custom-made cup of expanded polystyrene with a lid to isolate the sample from room temperature. The cup was glued to a metal plate that could be attached with four screws to the sample stage of the X-ray facility. A layer of dry ice was inserted on the bottom and the upper part of the cup to keep the sample frozen during the measurement. The upper layer of dry ice was separated with a mounted cardboard plate so that there was a part in the middle where the X-rays could transmit through without encountering dry ice. Inside the cup, there was a hole with a depth of approximately 10 mm on the bottom and in the lid, where a tube with a sample could be firmly mounted. The tube was made of cryo-durable plastic (a piece cut from a 15 ml falcon tube [VWR]). On both ends of the tube, custom-made plastic plugs were attached with the size and shape exactly corresponding to the holes made to the bottom and the lid of the cup and the inner diameter of the tube.

A frozen sample, recut to about 40 mm in length, was placed inside the tube before the tube was mounted to the cup. Since it was important to have the branch situated in the middle of the sample holder and to keep it perfectly still during the scans, the branch sample was attached from the base and tip with playdough to the plastic plugs. The playdough was soft when the sample was pressed in and rigid when frozen, thus keeping the branch firmly and preventing movements of the sample during the measurements. All preparations were made in a cool box above the dry ice, ensuring that the sample stayed frozen during the preparations.

We used the beamline ID19 with a pink beam of 35 keV for the micro-CT measurements. Such high energy is required for sufficient transmission through the sample and the sample holder cup while minimizing the scattering background in the images. The resolution of ID19 going to sub-micron enables the detection and quantification of the small gas bubbles. The high flux enables high throughput allowing

### Table 1

| No | Treatment               | Ice nucleation T (°C) | No of high resolution scans |
|----|-------------------------|-----------------------|----------------------------|
| 1  | Saturated (S)           | −0.7                  | 3                          |
| 2  | Saturated (S)           | −1.0                  | 5                          |
| 3  | Saturated (S)           | −1.8                  | 5                          |
| 4  | Saturated and coated (C)| −1.6                  | 5                          |
| 5  | Saturated and coated (C)| −0.8                 | 5                          |
| 6  | Saturated and coated (C)| −1.1                  | 5                          |
| 7  | Dehydrated (D)          | −2.4                  | 6                          |
| 8  | Dehydrated (D)          | −2.5                  | 5                          |
| 9  | Dehydrated (D)          | −2.4                  | 4                          |
to image sufficiently large volumes of interest in the sample. First, a low-resolution scan with a complete cross-sectional view was taken from each sample with a pixel size of 3.61 μm. Based on this scan, locations for high-resolution scans with 0.36 μm pixel size and 0.7 mm field of view were selected for each sample along a radial gradient (resulting in 13–15 scans per treatment, Table 1).

2.4 Analyses of gas bubble characteristics from the scans

For the data analysis, the conduits and the gas-filled volumes were first segmented from the 3D images. The segmentation procedure was performed in the ImageJ (National Institutes of Health) utilizing the MorpholibJ plugin. The conduits were segmented based on a grayscale threshold (manually chosen, same for all samples) that separated the conduit lumens from the cell walls. Binary morphology operations were then used to clean out any remaining structures that were not sufficiently large and long enough in the vertical direction (this removed especially structures associated with the rays). This was followed by a 3D morphological erosion to create seed areas inside each conduit that were then used as starting points for distance transform watershed that established the final segmentation of the conduits. The gas-filled volumes were segmented using a threshold (manually chosen, same for all samples) and then labeled using a flood-fill algorithm. Finally, gas-filled volumes and conduits that touched image borders were excluded from further consideration.

An additional classification step using a convolutional neural network (CNN) was performed to avoid false-positive gas voids. For each segmented gas void, three orthogonal slices (3 × 128 × 128 pixels) centered at the void were used, and the void was classified based on its visual appearance on those three slices. To generate training and validation data sets, visual inspection was used to classify positive (n = 3300) and negative (n = 3300) examples (training/validation split 70/30), taken from 15 different scans from 7 different samples. The architecture of the classifier was based on a pretrained VGG-16 network (with fixed weights) as included in the Keras framework for python (version 2.1.6-tf) and replacing the top (fully connected) layers with a small fully connected network for training (4 layers with 512, 64, 32, and 2 nodes, with dropout layers between each layer with 15% dropout rate). The training data was augmented offline 30-fold by sampling from the original 3D data sets with random rotations around the branch axis (±1 pixel displacements in each direction). These augmented data were then passed through the VGG-16 network (minus the top layer) to create bottleneck features that were saved to disk to speed up the training. The achieved classification performance in the validation set was 93% accuracy with a loss of 0.21 (for the training set the values were 94% accuracy, 0.15 loss).

This model was then used to classify all the remaining gas voids to give them a CNN score that was used as a criterion in further analyses. However, due to the rare occurrence of larger voids in the training data and the chosen limit of 128 × 128 pixel ROI for the CNN classification (46 × 46 μm), larger voids were not reliably classified by this method. Therefore, the CNN score was only used for voids smaller than the mean volume in this prefinal dataset (429 μm³). The CNN check for these voids was passed if the CNN value was 0.8 or higher. Regarding the larger voids, all gas volumes filling 80% or more from the surrounding conduit were excluded from the data to avoid including fully embolized (i.e. gas-filled) conduits in the analysis. Also, all gas voids in conduits shorter than 100 μm were excluded from the data to ensure no gas voids in living tissue remained in the analyzed data.

After these steps of data cleaning, the number of remaining gas voids (hereafter called “gas bubbles”) was 68,343.

The gas bubbles were classified as being inside a vessel or inside a fiber by using a visual inspection of all valid bubbles that were in a conduit whose luminal diameter was more than 12 μm, which can be assumed to be a minimum diameter for vessels in *Betula pendula* branches (Lintunen & Kalliokoski, 2010). For all true bubbles inside vessels, the vessels were manually segmented by tracing their boundaries on a few slices (10–20 for each vessel) and interpolating the

![Figure 2](image)

**FIGURE 2** Gas bubble volume vs. bubble sphericity in (A) fibers and (B) vessels. Observation frequency is shown with a color scale. Some sphericity values are over 1, and these are artifacts that can appear for small objects due to underestimation of the object surface area from the voxel data.
shape for the rest of the slices. In the analysis where conduit volumes were compared against gas bubble volumes (in Figure 6), we deleted such fibers with a cross-sectional area larger than a mean plus one standard deviation (equal to 125 $\mu$m$^2$ and diameter of 12.6 $\mu$m). This was done because sometimes the segmentation failed to separate every fiber from each other, resulting in unrealistically large fibers.

**Figure 3** Examples of high-resolution images with (A) cross-sectional whole field of view in a saturated sample and (B) a dehydrated sample. (C) Longitudinal views on saturated samples and (D) dehydrated samples to demonstrate different sized and shaped bubbles. The denser the material is, the brighter is the color in the picture (i.e. white refers to cell walls, light gray to ice and dark gray to gas).

**Figure 4** Histograms of (A) gas bubble volume in fibers and (B) vessels. Different treatments are shown separately ($S =$ saturated, $C =$ saturated and coated, and $D =$ dehydrated). x-axis is cut for readability. Note that the y-axis scale is different in (A) and (B). Note also that the saturated treatment has two scans less than the other two treatments for technical reasons (13 vs. 15 scans, Table 1); see the exact differences between the treatments in the number of bubbles per scan volume in Figure 5E,F.
Finally, the number of gas bubbles, the volumetric size and shape of each gas bubble and the volumetric and cross-sectional size of each associated conduit was measured from the cleaned data. The shape of all gas bubbles was characterized by a sphericity index (Legland et al., 2016) normalized such that the value for a perfect sphere equals one.

We made an error analysis for the number of false-positive gas bubbles in different size categories of conduits by dividing the conduits into four size categories based on their volume and evaluating up to 50 bubbles in each size category for each sample (total number of evaluated bubbles in the four conduit size categories were 118, 244, 308, and 289, spread across all 9 samples): the fraction of
false positives was 25% in conduits smaller than 5000 μm³, 8% in conduits with a volume between 5000 μm³ and 10,000 μm³, 3% in conduits between 10,000 μm³ and 20,000 μm³, and 6% in conduits larger than 20,000 μm³.

The linkage between gas bubble volume and sphericity, as well as gas bubble volume and fiber/vessel volume, were visualized with frequency maps (sghplot procedure) produced in Statistical Analysis System (SAS, version 9.4, SAS Institute Inc.). Gas bubble volume histograms were produced with the univariate procedure in SAS. Results for the different treatments are shown with box plot figures (sghplot procedure, SAS). The correlation between mean gas bubble volume in a sample and sample ice nucleation temperature was tested using a general linear model (glm procedure, SAS).

3 RESULTS

Most gas bubbles found in frozen conduits of B. pendula were in fibers and only 0.2% of the total number of gas bubbles and 4.4% of the total gas bubble volume in vessels. Gas bubbles of various shapes and sizes were found (Figures 2 and 3). Within a conduit, gas bubbles occurred either as individual bubbles or series of bubbles, with typically various shapes and sizes in the latter case (Figure 3). The smallest gas bubbles were spheres, and the sphericity decreased with increasing bubble size in both fibers and vessels (Figure 2). Accordingly, the largest gas bubbles were more like gas pockets that filled a significant share of a conduit with gas, while the remaining conduit sections were filled with ice (Figure 3B,D).

More gas bubbles were found in fibers than in vessels (Figures 4 and 5E,F). Fewer gas bubbles were detected in the saturated samples than in the dehydrated or coated samples in fibers and vessels (Figures 4 and 5E,F). From the saturated samples, only one single gas bubble was found in the case of vessels (Figure 4). Also, more gas bubbles were found in the dehydrated than coated samples in fibers and vessels (Figures 4 and 5E,F). Variation in the number of gas bubbles between scans was especially high in the case of the coated samples (Figure 5E,F).

The gas bubbles were larger in vessels than in fibers (Figures 4 and 5C,D). The mean and median gas bubble volumes in the whole data set were 1735 μm³ and 57 μm³ in fibers and 31,940 μm³ and 4901 μm³ in vessels, whereas the mean and median gas bubble diameters (calculated from cross-sectional areas assuming circular cross-sections) were 3.7 μm and 3.5 μm in fibers and 15.3 μm and 14.6 μm in vessels. Mean gas bubble volume was the largest in the dehydrated samples in fibers and vessels (Figures 4 and 5). Gas bubbles in the coated samples were, on average larger than those in the saturated samples in fibers (in vessels, only one gas bubble was detected in the saturated samples, Figures 4 and 5C,D).

Gas bubble sphericity was larger in fibers than in vessels, the overall mean being 0.64 for fibers and 0.47 for vessels (Figure 5). Gas bubbles were less spherical in the dehydrated samples than in the saturated or coated samples (Figure 5A,B). Also, gas bubbles in the saturated samples were more spherical than those in the coated samples in the case of fibers (in vessels, only one gas bubble was detected in the saturated samples, Figure 5A,B).

Gas bubble volume detected in the conduits (i.e. sum of all bubbles in a conduit) increased with increasing conduit volume (Figure 6). In vessels, this relationship was rather linear (Figure 6B), whereas, in fibers, there was a large variation in the gas bubble volume that could not be explained by the conduit size (Figure 6B). There seem to be two separate trends in the relationship between bubble volume and conduit volume, especially in the fibers: (1) bubble volumes that increase in size when the conduits get larger as conduit volume seems to set a maximum for the bubble volume, and (2) small gas bubbles that are present in any size of conduits (Figure 6).

Because the bench-dehydration prefreezing treatment affected not only branch water potential but also its apoplastic ice nucleation temperature, we analyzed the relationship between mean gas bubble volume of a sample and sample ice nucleation temperature. A negative correlation was found between sample gas bubble volume and ice
Mean volume of gas bubbles in (A) fibers and (B) vessels in a sample vs. sample ice nucleation temperature. Samples with different treatments are shown with letters (S = saturated samples, C = saturated and coated samples, D = dehydrated samples). Linear regression lines are shown.

**FIGURE 7** Mean volume of gas bubbles in (A) fibers and (B) vessels in a sample vs. sample ice nucleation temperature. Samples with different treatments are shown with letters (S = saturated samples, C = saturated and coated samples, D = dehydrated samples). Linear regression lines are shown.

nucleation temperature for fibers, but the correlation was not significant for vessels (Figure 7).

**4 | DISCUSSION**

We show with high-resolution micro-CT that small spherical gas bubbles and larger gas voids are observed in frozen fibers and vessels of *B. pendula* (Figure 3). Similarly, Sucoff (1969) found that in the branches of two pine species, ice in tracheids contained small bubbles with ~1 μm in diameter, but in some tracheids, large gas pockets filling 10%–90% of the tracheids were found. Other studies that have reported gas bubble shapes and diameters in frozen xylem conduits are the studies by Robson et al. (1988), who reported spherical or ellipsoidal gas bubbles, ~2 μm in diameter in the frozen tracheids of *Pseudotsuga menziesii*, and by Utsumi et al. (1999), who found bubbles of ~10 μm in diameter in frozen vessels of *Fraxinus mandshurica var japonica*. In agreement with these previous studies, the median bubble diameter was 4 μm in frozen fibers and 15 μm in frozen vessels of the *B. pendula* branches studied here.

Our first hypothesis was that small gas bubbles can start to grow already during the ice propagation in the xylem, not only at thawing. This hypothesis gets support from the results of this study (Figures 2 and 3). We hypothesize that small spherical gas bubbles are born at the advancing ice front as the gas concentration increases and the sap becomes supersaturated, and the difference in the pressure of the gas phase and liquid phase increases to be large enough that the bubbles nucleate (Sevanto et al., 2012). Supersaturation can also occur when xylem sap water potential decreases and/or temperature increases (Schenk et al., 2016), which could be possible also during the xylem ice propagation. The nucleated, small bubbles will lead to embolism formation at thawing if the tension of the xylem sap is sufficiently high (Pittermann & Sperry, 2003, 2006; Sperry & Sullivan, 1992). In addition to small spherical gas bubbles, we observed larger, nonspherical gas bubbles that were more likely to occur in coated or dehydrated wood (Figure 5). In this case, the cavitated gas bubbles may have started to grow already during the freezing process, which would increase the risk of embolism formation during thawing. However, it should be noted that we did not have sample controls instantaneously frozen with liquid nitrogen, which would have allowed a direct comparison of the situation before and after the ice propagation. Instead, our conclusions rely on the assumption that in the absence of positive pressure in conduits, a partly embolized (or partly filled) conduit should not be possible prior to freezing. *B. pendula* is a species that can create positive pressures in the stem in springtime before the budburst to refill embolized conduits (Hölttä et al., 2018). However, our experiment was conducted after budburst, and we did not observe the presence of positive pressures during sample collection or in the water potential measurements after the rehydration. Also, to fully refill a branch should not take more than 5 h when in contact with water (Knipfer et al., 2016; Salmon et al., 2018), and we let the branches saturate for 48 h. This should guarantee that any possible refilling process was completed before the freezing experiment. In the freezing experiment, the branches were placed vertically in a dish with no water contact (cut ends sealed). A similar observation of partly embolized frozen conduits was reported by Sucoff (1969) on two pine species not known to create positive stem pressures.

This is, to our knowledge, the first study where the relationship between the size of a gas bubble and the size of the frozen conduit bearing the gas bubble has been empirically studied. We hypothesized that gas bubble diameter is positively correlated with conduit diameter. This hypothesis was shown to be partly true in *Betula pendula* as the maximum detected gas volume increased with conduit size (Figure 6). This finding is in accordance with the theory presented by Pittermann and Sperry (2006). However, the conduit size alone could not explain gas bubble size as a large variety of different sized gas volumes were found in conduits of a given size. Also, not all gas bubbles had a spherical shape, and they were not evenly distributed inside conduits as assumed in theory, implying that the reality is more versatile. We found larger gas bubble sizes in large vessels compared to small fibers (Figure 5), but often vessels without any gas bubbles were surrounded by fibers with gas bubbles (Figure 3), although the size difference between the two conduit types suggests otherwise (Pittermann & Sperry, 2003, 2006; Sperry & Sullivan, 1992).
Our results suggest that in addition to conduit size (within conduit type), the other factors affecting gas bubble number and size in frozen conduits are xylem water potential and bark permeability to gases. In line with our hypothesis, we found that decreasing the branch water potential by 0.34 MPa prior to freezing by bench-dehydration resulted in more gas bubbles than in the saturated samples (Figures 4 and 5). The gas bubbles were also larger and less spherical in the dehydrated samples compared to the saturated samples in fibers, whereas in vessels, such comparison is impossible since only one gas bubble was found in the vessels of the saturated samples. Based on these results and previous literature, we hypothesize that more, larger and less spherical bubbles are grown due to the low water potential in the remaining sap at the ice front, which locally increases tension in the sap, inducing the formation of large gas voids and acoustic emissions (Charrier et al., 2014, 2015). Previous studies have shown that the amount of acoustic emissions detected from freezing xylem is typically small for saturated samples and increase with increasing dehydration until the majority of the xylem gets embolized due to the prefreezing dehydration (Charrier et al., 2015; Lintunen et al., 2020; Mayr et al., 2007).

Dehydrated branches also had 1.3°C lower apoplastic ice nucleation temperature (i.e. higher degree of supercooling) than the saturated samples (Figure 7). Ice nucleation temperature can affect the size of gas bubbles since ice propagation velocity in trees is negatively correlated with xylem ice nucleation temperature (Charrier et al., 2015; Hacker & Neuner, 2007; Robson & Petty, 1987), and the faster the ice propagates, the more and the smaller should be the gas bubbles formed in ice (Carte, 1961; Robson & Petty, 1987). This means that low ice nucleation temperature causes fast ice propagation, and small gas bubbles get trapped in ice. However, we found a negative correlation between ice nucleation temperature and gas bubble size (Figure 7), which suggests that the difference in water potential rather than ice nucleation temperature explains the difference in the gas bubble size between the saturated and dehydrated samples.

We also found that coating the branches with Vaseline to decrease their bark permeability to gases tended to result in increased number of gas bubbles in both fibers and vessels. The bubbles also tended to be larger and less spherical, although this is only evident in fibers due to the lack of bubbles in the vessels of the saturated samples (Figure 5). These observations are consistent with previous studies that show significant gas bursts coming out from xylem during ice propagation (Lintunen et al., 2014, 2020). Together these results suggest that bark that is permeable to gases can decrease the risk of embolism formation in trees. Lintunen et al. (2020) measured simultaneously gas bursts and acoustic emissions during a freezing process from branches of Picea abies and Salix caprea and concluded that the expanding ice front in the freezing xylem was responsible for both gas bursts and acoustic emissions and both processes were also affected by branch water potential. However, they could not prove a direct link between the processes as decreasing the sample water potential from saturated to ~3.3 MPa increased acoustic emissions significantly but decreased gas bursts nonsignificantly (Lintunen et al., 2020). The species studied here and in the previous work by Lintunen et al. (2014, 2020) have been boreal and Alpine species adapted to cold environments. In contrast, Sperling et al. (2015) studied woody species from a temperate environment and found no evidence of freezing-related gas bursts, only an increase in CO₂ efflux from the stem just before the freezing of the stem, hypothesized to be connected to accelerated nonstructural carbohydrate consumption. In the future, it will be important to study gas dynamics during the freezing process in more tree species and from various environments.

The micro-CT method was successfully used to study the bubble characteristics in frozen xylem samples. Analyzing the micro-CT data included many steps, one being a quality check for the gas bubbles identified from the images. In Figure 4, it can be seen that there is a step-change in the number of gas bubbles at a bubble volume around 435 μm³, that is corresponding to the definition of large bubbles that did not need to pass the “CNN check” (Section 2.4). This is an artifact and not a real property in the data. In bubble volumes smaller than 435 μm³, the CNN check was applied, thus rejecting some of the voids. A reliable CNN check was unavailable in bubble volumes larger than 435 μm³, and the data thus includes more false positives. However, all samples were treated the same, and thus this does not affect the main conclusions of the study.

One of the largest questions that is left unsolved is why fibers seemed to be more likely to embolize based on the number of bubbles than larger vessels in B. pendula, although larger conduit sizes are generally connected with larger vulnerability to winter embolism formation (Davis et al., 1999; Pittemann & Sperry, 2003). Especially in the saturated samples, gas bubbles were absent from the vessels. Fibers are structurally very different from vessels, not only in their size. B. pendula has vascentric fiber tracheids with bordered pits along libriform fibers with simple, slit-like pitting (Terävä & Kanervo, 2008). Fibers are less conductive than vessels and better serve the function of mechanical support. It is possible that the gas bubbles in the vessels are smaller than our detection limit (~1.0 μm in diameter). The size of bubbles nucleated in bubble formation events can be calculated using LaPlace’s law using the surface tension of water and the difference in pressure between the gas and liquid phases. If the difference in pressure between the gas and liquid phase is larger than 0.3 MPa, nucleation bubbles will be smaller than the detection limit, that is smaller than 1.0 μm in diameter. As far as we know, there are no measurements of this pressure difference, but according to theoretical calculations by Sevanto et al. (2012), pressure differences larger than this are likely to exist, meaning the bubbles are smaller than our detection limit at least before they have had a chance to grow sufficiently. However, very small bubbles should also not form a big risk for winter embolism formation as they are likely to collapse back to xylem sap when it melts (Pittemann & Sperry, 2003, 2006). It is also possible that gases could escape more easily from vessels than from fibers. This aspect needs further studies and needs to be combined with studies of winter embolism formation. In an ideal experiment, the micro-CT imaging would be done prior to freezing, during a frozen state and after thawing to observe the native embolism, avoid any possible changes during storing or transport of frozen samples, and be able to link the bubble characteristics in apoplastic ice directly to embolism formation.
5 | CONCLUSIONS

To summarize, high-resolution X-ray microtomography was successfully used to study gas bubble characteristics in frozen xylem. Our results suggest that gas bubbles can start to grow already during the ice propagation in the xylem, not only at thawing. If this observation is confirmed with other tree species, it gives a new perspective to the prevailing understanding of freeze–thaw embolism formation. We also found that larger conduits enable larger bubbles to form in line with the prevailing theory of freeze–thaw embolism formation, but there is also a large variation in the gas bubble volumes that cannot be explained by the conduit size alone. Our results also suggest that it is more likely that a gas bubble is born in a fiber than in a vessel during the ice propagation, and that in addition to conduit size, gas bubble number and size increase with decreasing sample water potential and decreasing bark permeability.

Improved understanding of the vulnerability of trees to winter embolism is needed since this has clear implications for the performance and survival of trees in high latitudes and altitudes. This study suggests that wood dehydrated prior to freezing is more vulnerable to winter embolism than saturated wood, because large gas bubbles are formed inside frozen conduits during ice propagation. Our results together with earlier studies showing that winter embolism does not occur in branch samples in laboratory conditions unless they have been dehydrated during thawing (Mayr & Sperry, 2010) suggest that as the likelihood of drought will increase in future in many areas globally, also in areas where frost is co-occurring, exposure to drought will increase trees’ vulnerability to winter embolism. However, bark permeability also plays a role in tree performance in these conditions as it increases tree’s vulnerability to drought (Lintunen et al., 2021) but seems to decrease tree’s vulnerability to winter embolism formation. It remains to be studied how different tree species can acclimate/adapt to the changing environmental conditions.

AUTHOR CONTRIBUTIONS
All authors contributed to planning of the study. Anna Lintunen and Yann Salmon made the freezing experiments and Anna Lintunen, Heikki Suhonen, and Yann Salmon the synchrotron measurements. Heikki Suhonen had the main responsibility in analyzing the micro-CT images and Anna Lintunen in writing the manuscript. All authors commented the manuscript.

ACKNOWLEDGMENTS
We acknowledge the European Synchrotron Radiation Facility for provision of synchrotron radiation facilities and we would like to thank Elodie Boller and Lukas Helfen for assistance in using beamline ID19. Funding for this project was provided by the Academy of Finland (grants 310375, 323843, 337549).

DATA AVAILABILITY STATEMENT
The cross-sectional slices are available via IDA data service: https://doi.org/10.23729/5f296588-f17a-49e1-be4c-fddc42d7f153.

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