Visualization of embolism formation in the xylem of liana stems using neutron radiography

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INTRODUCTION

Plants employ a fascinating mechanism that facilitates water transport against the gravitational potential. According to the cohesion–tension theory, evaporation at the nanoporous walls of mesophyll cells and subsequent transpiration induces capillary forces driving the long-distance water transport without additional metabolic energy input. Water is conducted by the xylem tissue, a transport structure that basically consists of dead capillary-like cells (Tyree and Zimmermann, 2002). While transpiration at the leaves generates suction that pulls the water through the plant, pressure inside the xylem tissue can fall below vapour saturation pressure or may even become negative, i.e. the water is under tension. This thermodynamically metastable state favours the (unlimited) growth of microscopic gas bubbles, leading to embolisms (Tyree and Sperry, 1989). As a consequence, the affected xylem vessels are blocked and lose their capacity for water transport.

Plants have developed various safety strategies to ensure the reliability of their water management even under most unfavourable conditions. A central feature is the active control of the transpiration rate by stomata. Specialized guard cells regulate the stomata opening to avoid unnecessary or excessive leaf transpiration that would otherwise lead to a critical drop of xylem pressure (Brodribb et al., 2003). Nonetheless, there are situations, e.g. during periods of persistent drought, where xylem pressure can fall under a critical threshold below which embolism events are promoted. For this reason, the xylem has a dedicated hydraulic architecture to cope with these situations. Individual xylem vessels are interconnected to form a complex transport network with a high degree of flow path redundancy. Hydraulic junctions between the xylem members, so-called pits, act like biologic safety valves. They allow the passage of liquid water but form barriers for the propagation of gas–liquid interfaces, thus preventing the uncontrolled spread of gas emboli (Crombie et al., 1985; Sperry, 2003; Wheeler et al., 2005).

Due to the great redundancy of flow paths represented by the high number of single interconnected conduits, plants can easily compensate the dysfunction of individual vessels. However, with increasing numbers of embolisms the overall conductance of the xylem decreases. Conduits can remain functional for just a few days or for >100 years (Tyree and Zimmermann, 2002), but the first step towards a state of permanent dysfunction probably involves a state of embolism. Repair mechanisms for embolized vessels could, therefore, have an important stabilizing effect on the plant water supply.

There is already theoretical and experimental evidence that embolized xylem vessels can be repaired. Even the refill of empty conduits under negative pressure is possible, as has
been shown in various studies (Tyree et al., 1999; Hacke and Sperry, 2003; Salleo et al., 2004). Living tissues in the xylem, rays and xylem parenchyma can play an important role in the refilling process, particularly during the much-debated embolism repair under negative pressure (Vesala et al., 2003; Salleo et al., 2006; Nardini et al., 2011). Most studies on embolism repair apply conventional experimental methods, e.g. the measurement of hydraulic conductivity whose temporal changes demonstrate vessel embolism or refill indirectly (Sperry et al., 1987; Zwieniecki and Holbrook, 1998; Cochard et al., 2001; Vogt, 2001). These methods require destructive sampling and therefore make in vivo measurements impossible.

To understand refilling mechanisms thoroughly, direct observations of embolism formation and/or refill are desirable, i.e. in vivo visualization of formation, change and disappearance of gas spaces inside conduits. Recently, the rapid technological progress of imaging techniques has offered novel experimental approaches for studies of embolism formation and refilling. One method to realize in vivo observations of embolism formation is nuclear magnetic resonance imaging (MRI) (Holbrook et al., 2001; Clearwater and Clark, 2003; Windt et al., 2006; Scheenen et al., 2007). The resolution (<100 μm) is sufficiently high to distinguish individual xylem vessels in plants with very wide vessels (D > 100 μm), e.g. some liana species (Ewers et al., 1990). However, the low temporal resolution (17 min per acquisition) hampers the observation of dynamic changes of embolism or refill. Further limitations to MRI studies of xylem transport are given by the physical restrictions on plant samples which must be fitted into the sample with a thickness d = 30 μm and diameter ≤ 2 cm. Although the spatial resolution is about 20–50 μm (Boillat et al., 2008, 2010; Hickner et al., 2008; Williams et al., 2012) which is appropriate to study the embolism formation in wider vessels.

**MATERIALS AND METHODS**

**Neutron-imaging technique**

The experiments were performed at the CONRAD measuring station located at the end of a curved neutron guide, which faces the cold-neutron source of the BER-II research reactor at the Helmholtz-Zentrum Berlin für Materialien und Energie (HZB). The beam is passed through a collimation system with a circular aperture of 2 cm providing neutrons with wavelengths between about 2 and 12 Å (peaking at 3–1 Å) and a neutron flux of approx. 1.6 × 10^7 cm^{-2} s^{-1} (Kardjilov et al., 2009, 2011b). A 10 × 10 cm² lithium fluoride scintillator screen with a thickness of 50 μm was used to convert impinging neutrons into visible light. The absorption image formed on the scintillator screen is projected onto the 16-bit 2048 × 2048 CCD chip of the camera (Andor DW436N-BV) via a mirror and a 105 mm-focus Nikon camera lens (Kardjilov et al., 2011a). Total image acquisition time was 24 s; 20-s exposure plus 4-s readout.

**Image processing**

The visualization of embolism events by neutron imaging requires appropriate image processing. This includes dark-field and flat-field correction of the neutron absorption images to eliminate the CCD dark current signal and the inhomogeneities of the beam profile, respectively. Moreover, a median filter was applied over five consecutive images to reduce the image noise. The initial image of each sequence served as a reference image for the calculation of quotient images. This approach enhances the sensitivity for the detection of any sample changes occurring during the experiment and allows estimating variations in the sample water content quantitatively.

The normalization procedure is based on the Lambert–Beer law which describes the beam attenuation as a function of sample thickness and composition (Banhart, 2008). At time \( t = n \), the intensity \( I_n(x, y) \) of a neutron beam passing the sample with a thickness \( \delta_n \) in z-direction is given by

\[
I_n(x, y) = I_{0,n}(x, y) e^{-\mu(x, y, z)\delta_n}
\]

where 

\[
\mu(x, y, z) = \int_0^z \rho(x, y, t) \, dt
\]

\( \rho(x, y, t) \) is the number density of oxygen atoms in the sample that can be approximated by a Gaussian function.

\( \delta_n \) is the sample thickness in z-direction, and \( \mu(x, y, z) \) is the linear attenuation coefficient of the sample.

\[ I_{0,n}(x, y) \] is the intensity of the incident beam at time \( t = n \).

\[ \int_0^z \rho(x, y, t) \, dt \] is the integral of the number density of oxygen atoms in the sample over the thickness of the sample.

\[ \mu(x, y, z) \] is the linear attenuation coefficient of the sample at time \( t = n \).

\[ \delta_n \] is the sample thickness in z-direction.

\[ I_n(x, y) \] is the intensity of the incident beam at time \( t = n \).

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\[ \delta_n \] is the sample thickness in z-direction.

\[ I_{0,n}(x, y) \] is the intensity of the incident beam at time \( t = n \).
Where \( \mu (x, y, z) \) denotes the distribution of the local attenuation coefficient in the sample and \( I_{0,n} (x, y) \) the initial beam intensity distribution.

To evaluate eqn (1) further, we assume (a) that the plant sample can be partitioned into a water fraction and a ‘dry biomass’ fraction and (b) that the variation of the respective attenuation coefficients \( \mu_{\text{water}} \) and \( \mu_{\text{dry}} \) with respect to the \( x-, y-, z- \) co-ordinates is negligible. Thus, eqn (1) can be approximated as

\[
I_n(x, y) = I_{0,n}(x, y) e^{\left[\mu_{\text{dry}} \delta_{\text{dry},n}(x, y) + \mu_{\text{water}} \delta_{\text{water},n}(x, y)\right]} \tag{2}
\]

\( \delta_{\text{water}} (x, y) \) and \( \delta_{\text{dry}} (x, y) \) refer to the hypothetical thickness of water and biomass fraction. Taking the initial image (\( t = \text{start} \)) as a reference, image normalization of the \( n \)th image amounts to

\[
\frac{I_n(x, y)}{I_{\text{start}}(x, y)} = \frac{I_{0,n}(x, y) e^{\left[\mu_{\text{dry}} \delta_{\text{dry},n}(x, y) + \mu_{\text{water}} \delta_{\text{water},n}(x, y)\right]}}{I_{0,\text{start}}(x, y) e^{\left[\mu_{\text{dry}} \delta_{\text{dry},\text{start}}(x, y) + \mu_{\text{water}} \delta_{\text{water},\text{start}}(x, y)\right]}} \tag{3}
\]

As we further assume \( \delta_{\text{dry}} \) and the beam intensity \( I_0 \) to remain constant during the experiment, eqn (3) simplifies to

\[
\frac{I_n(x, y)}{I_{\text{start}}(x, y)} = e^{\left[\mu_{\text{water}} \delta_{\text{water},n}(x, y) - \mu_{\text{water}} \delta_{\text{water},\text{start}}(x, y)\right]} \tag{4}
\]

Defining \( \Delta \delta_{\text{water},n}(x, y) = \delta_{\text{water},n}(x, y) - \delta_{\text{water},\text{start}}(x, y) \) and re-arranging eqn (4) one obtains

\[
\Delta \delta_{\text{water},n}(x, y) = \frac{1}{\mu_{\text{water}}} \ln \left[\frac{I_n(x, y)}{I_{\text{start}}(x, y)}\right] \tag{5}
\]

This expression connects the difference of water thickness in the \( z- \) direction between times \( t = \text{start} \) and \( t = n \) at any position \( (x, y) \) in the detector plane with the corresponding measured beam intensities. Thus, the normalization procedure transforms the grey value of a pixel at position \( (x, y) \) into the thickness variation of water at this position.

**Experimental set-up**

The aim of these experiments was the detection and visualization of embolism events and potential repair mechanisms in the xylem of liana plants. Some general advantages of this plant type for radiographic experiments are the flexible stems which can be fixed to a supporting structure and positioned into the field of view. Moreover, the xylem tissue contains extraordinarily wide vessels with diameters up to \( > 100 \mu \text{m} \) which meets the resolution capability of the imaging method. The focus of the experiments was on three different liana species: *Adenia lobata*, *Aristolochia macrophylla* and *Parthenocissus tricuspidata*. The plants were cultivated in flower pots in the Botanical Garden of the Universität Tübingen. At the time of the experiment, the plants were between 6 months and 12 months old. Additionally, stems of outdoor plants of *P. tricuspidata* were used that climbed the façade of one of the HZB buildings.

To provide basic physiological conditions appropriate to observe embolism formation and subsequent repair we organized the radiographic experiments in the following manner.

(a) During the first period the liana plants were subjected to water stress to induce embolism events. This was achieved by stopping watering for 2–4 d prior to the experiments. (b) Additional invasive measures such as partial cutting of xylem tissue were applied to enhance water stress and, thus, the probability of embolism events. (c) Then, plant samples were watered and left in the dark for several hours, thus providing favourable physiological conditions for embolism repair. Neutron radiographic images were taken throughout the whole sequence of steps, except for a short interruption needed for watering.

The general experimental set-up is illustrated in Fig. 1. The liana plants were mounted on an aluminium frame and placed in the neutron beam. The stem sections under investigation were arranged within the field of view and firmly fixed to a perforated aluminium sheet which was positioned 1 cm in front of the scintillator screen. The stems were clamped to the perforated plate to prevent sample movement that would decrease image quality and accuracy of water quantification.

A high-pressure sodium vapour lamp with an electrical power of 600 W was used to illuminate the plant samples in the measurement room. The average light intensity (PAR) at the leaf surface was about 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and the relative humidity was 30 %. The stomatal conductivity of leaves was measured with a Delta-T porometer of the type AP4. *Parthenocissus tricuspidata* showed mean values of 22.3 mmol m\(^{-2}\)s\(^{-1}\), *Adenia lobata* 19.8 mmol m\(^{-2}\)s\(^{-1}\) and *Aristolochia macrophylla* 23.7 mmol m\(^{-2}\)s\(^{-1}\).

**RESULTS AND DISCUSSION**

**Testing the imaging method**

In this study neutron radiography was applied as a novel method to visualize embolism formation and potential
refilling. The suitability of the experimental set-up was tested in a preliminary experiment. The water ascent in cut liana stems was visualized using heavy water (D$_2$O) as a contrast agent. Plant samples were arranged to fit into the field of view and fixed to a perforated aluminium sheet as described above. The stems were cut and submerged into a D$_2$O reservoir. Upon illumination, the plant began to transpire water from its leaves, replacing increasing amounts of xylem water with heavy water. As D$_2$O attenuates neutrons much less than H$_2$O, the ascending D$_2$O front in the xylem tissue could be tracked as a contrast change in the neutron images. The image sequence of Fig. 2 visualizes the D$_2$O transport in cut stems of Aristolochia macrophylla and Parthenocissus tricuspidata. In fact, water and D$_2$O are transported simultaneously. The water column inside the stem xylem is moving upwards and pulls the D$_2$O column into the xylem vessels. The consecutive images are normalized with respect to the initial image of the sequence. The quotient pictures reveal the exchange of water for D$_2$O over a 3-h period, illustrating the ascending water. Note the stems of both samples are cut in half to study the effect of severed vessels on the water balance of the tissue located above. For both samples, the image sequences prove that the D$_2$O uptake takes place in both the intact and the injured parts of the stem. Apparently, lateral interchange, e.g. due to interconnectivity and tortuosity of vessels and diffusive transport, can compensate the loss of transport capacity in the transected vessels.

Transpiration rates inside the facility were always quite low, and amounted to about 20–40 mmol m$^{-2}$ s$^{-1}$. This was very probably due to the environmental conditions. Low transpiration rates were also observed elsewhere on study plants under similar conditions (Clearwater and Clark, 2003).

Observation of embolism formation

Preliminary neutron radiographic experiments showed that water stress caused by substrate desiccation, i.e. no watering of the pots over several days, failed to trigger embolism events in the considered plant species. Therefore, various methods were tested to intensify the water stress and to promote embolism, for instance, partial and complete stem cutting as well as exposure to ultrasonic waves. Stem cutting, however, turned out to be the most effective method. We will focus on selected results obtained by partial and complete stem transection.

A potted Adenia lobata plant was subjected to water stress by interrupting the daily watering procedure for 2 d. Before starting the radiographic measurement approximately half of the xylem tissue was cut in such a way that a complete wedge was removed from the stem exposing the cut area to the air.

Figure 3A displays a (flatfield- and darkfield-corrected) radiograph of the sample showing the general shape and structure of the stem. In Fig. 3B a detail of the stem section around the cut is displayed as a normalized image revealing changes of the stem water content occurring within 10 min after notching the stem. The bright rhomboid area surrounding the lesion turns up after cutting, intensifies and expands for 10 min before its size and intensity stabilizes. Obviously, the contrast change is the result of evaporative water loss of the plant tissue adjacent to the lesion that was exposed to air. The evolution of the bright shape is probably linked to the process of wound closure which counteracts evaporative loss of injured tissue. Wound closure seems to be completed after 10 min since no further significant contrast change is observed. However, no other changes were observed that could be attributed to embolism events. This is remarkable since the disconnection of a significant portion of the conducting tissue was expected to affect the water system substantially, thereby, leading to embolism. However, no embolism could be detected during several hours after the manipulation. Figure 3 demonstrates the robustness of the water transport despite stem injury. It proves that only a small stem region around the cut is affected by water loss, suggesting that wound closure quickly counteracts evaporative water loss of stem tissue.
The overall difficulty to provoke embolism may be attributed to the conditions in the experimental chamber, particularly the low-light conditions affecting stomatal conductance. Embolism tends to be markedly reduced under these conditions. However, there are also other reports that show that it can be difficult to promote embolism during non-invasive imaging. Kim and Lee (2010) dehydrated plants of *Oryza sativa* until severe wilting occurred to observe embolism via X-ray imaging methods. However, no embolism event could be detected unless the leaves were cut to generate air/water interfaces inside the conduits. Also no embolism could be provoked in intact *Ripogonum scandens* plants during MRI imaging and it was necessary to place the cut end of a severed stem into a PEG solution with high osmotic potential (Clearwater and Clark, 2003). The conditions under which embolism events set in is strongly species dependent and various taxa appear to avoid embolism (Vogt, 2001). Remarkable in our studies is the circumstance that none of the liana species considered showed embolism with intact stems, even if the stem was severely damaged by a wedge cut with a large area of stem tissue being exposed to air.

Since the partial transection of the xylem was not sufficient to trigger embolisms in either *A. lobata* or in other species (data not shown), the procedure was changed and in another experiment a liana stem (*Parthenocissus tricuspidata*) was completely cut through and subsequently radiographed for 1.5 h. While leaves were illuminated to initiate transpiration and the build-up of water stress, no water was supplied to the cut end of the stem. The sequence of normalized images in Fig. 4 documents the development of the stem water status. Within the first 36 min the intensity was quite stable for the whole stem section except for the stem edges which brighten (Fig. 4A). This effect continues throughout the whole experiment and can be explained by the contraction of elastic portions of the xylem tissue. This contraction is caused by the decreasing water potential and leads to a slight shrinkage of the liana stem. A coupling between stem water content and stem diameter was also recently demonstrated by using MRI (De Schepper et al., 2012). However, after 38 min a fibre-like bright structure appears along the stem axis (indicated by the arrow in Fig. 4B). In the subsequent images (Fig. 4C and D, *t* = 40 and 42 min) the shape and intensity of the bright structure further increases and stays almost constant for the rest of the experiment. The shape of the affected region as well as its rapid development suggests that xylem embolism has occurred.

As described in the Methods, the normalization procedure allows a quantitative estimation of the change of water thickness which is transmitted by the neutron beam. The grey value of each pixel denotes the reduction in water thickness in the z-direction at the detector position (in μm). In Fig. 5 the variation of the mean water thickness during the experiment was evaluated for a stem section that was obviously affected by an embolism (area marked red in Fig. 5B) and, for comparison, a section located next to the embolized vessel (area marked white in Fig. 5B).

According to Fig. 5A, the evolution of the water thickness in the stem section marked red (see Fig. 5B) is characterized by two phases (0 min...38 min and *t* = 42 min...85 min) of continuous and approximately linear thickness reduction interrupted by a short intermediate phase of rapid water loss between *t* = 38 min and *t* = 42 min (see also data points 1, 2, 3 in Fig. 5). The early and late phases of moderate thickness reduction can be explained by the contraction of elastic tissue caused by the falling plant water potential (equivalent to increasing water stress). This amount to shrinkage of the stem...
diameter at a rate of about 1.7 μm min⁻¹, which corresponds to the increasingly bright stem edges observed in Fig. 4. Between data points 1 and 3 the water thickness reduction rate (about 25 μm min⁻¹) is one order of magnitude higher than before and afterwards. During these 4 min, the water thickness is reduced by about 100 μm. Since typical vessel diameter of this species range between 50 and 100 μm, it is most likely that an embolism was formed in a xylem vessel. This interpretation is corroborated by the sudden emergence of the fibre-like structure observed in the normalized images of Fig. 4. For comparison, the evolution of the water thickness is also plotted for a stem area next to the embolized vessel (see area marked white in Fig. 5B). While in the first 36 min the effective water thickness decreases in both regions simultaneously at the same rate, the rapid water loss between \( t = 38 \) and \( t = 42 \) min is only detected in the stem section affected by the embolism. In contrast, in the stem section next to it the approximately linear trend of water thickness reduction occurring continuously throughout the measured period is caused by the contraction of elastic tissue due to increasing water stress.

**Behaviour after watering**

In the second part of the experiment it was attempted to observe rehydration and, ideally, refilling processes in the stem of *P. tricuspidata*. To provide appropriate rehydration conditions the plant was watered by submerging the end of the stem into a water reservoir after cutting. Furthermore, the plant illumination was switched off to minimize leaf transpiration. Radiographs were taken for another 2.5 h; the initial image of this series served as a reference image for the normalization procedure.

It is to be expected that rehydration leads to an increase in the thickness of the xylem tissue and, therefore, the water column within it. The evolution of the liana stem is documented in Fig. 6 by a sequence of normalized images including the evaluation of the mean change in water thickness for two selected stem regions. The sequence of images shows that with elapsing time the whole stem area brightens, i.e. the stem tissue does not rehydrate but continues to lose water. Moreover, several additional embolisms form in the xylem which is reflected by the emergence of bright fibre-like structures, e.g. at \( t = 12 \) min and \( t = 122 \) min after watering (Fig. 6B, D). The embolism events correlate with a sudden reduction in the water thickness in the affected stem areas (cf. Fig. 6).

The appearance of additional embolism events indicates that no rehydration via the cut surface occurred. Possibly the stem region above the cut was already too dehydrated for water uptake. Consequently, the drought stress in the plant increased further, entailing additional contraction of elastic tissue. The shrinking rate of about 0.9 μm min⁻¹ is much lower compared with that observed before watering (1.7 μm min⁻¹). Probably, the turning-off of the illumination leads to stomatal closure, which reduced leaf transpiration significantly. Unaffected by the lower shrinking rate due to contraction of living stem tissue, embolism events show the same dynamic behaviour, i.e. reducing the water thickness by 50–100 μm within a few minutes.

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**Fig. 5.** (A) Plots of the mean water thickness change for the stem regions marked red and white in (B). Data points 1, 2, 3 and 4 correspond to the labels in Fig. 4A, B, C and D, respectively. (C) Schematic representation of \( \delta_{\text{water}} \) at \( t = 0 \), \( t = 36 \) min and \( t = 42 \) min, due to stem shrinkage and embolism. Note that the diagram is not drawn to scale and that the position of the embolized vessel is speculative with respect to the \( z \)-direction.
formation and repair occur. It is suggested that this method represents a very promising tool for gaining more information on the in vivo processes occurring during embolism and vessel refill.

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LITERATURE CITED


Conclusions

The observations made within this study show that neutron radiography is a suitable tool for direct monitoring of gas spaces within the xylem by detecting changes in transmitted water thickness in whole plant stems. The temporal resolution of the observed embolism events allowed for quite exact determination of the discharge time of a single vessel; this was completed in our study in about 4 min. To our knowledge, this was the first attempt to use neutron imaging for the visualization of embolism formation in plants. One of the advantages of neutron radiography is that a considerable portion of a stem can be observed with a quite acceptable temporal resolution. The results of this study demonstrate the great potential of neutron imaging for biological studies of water transport phenomena in plants. The approach should principally allow observation of gas spaces in the xylem of intact plants under suitable conditions. Further work is necessary to address the conditions and circumstances under which embolism