Observation of cavitation and water-refilling processes in plants with X-ray phase contrast microscopy Postprint

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Abstract

With the spatial coherence of X-rays and high flux and brightness of the 3rd generation synchrotron radiation facility, X-ray phase contrast microscopy (XPCM) at Shanghai Synchrotron Radiation Facility (SSRF) can provide high resolution dynamic imaging of low electron density materials in principle. In this paper, we investigated the cavitation and water-refilling processes in rice and bamboo leaves utilizing XPCM at SSRF. The occurrence of xylem cavitation was recorded in vivo. The study also revealed that under different dehydration conditions, cavitation occurs in different degrees, and therefore, the refilling process is different. The results demonstrate that SSRF can provide high enough fluxes to study dynamic processes in plants in real-time, and XPCM is expected to be a promising method to reveal the mechanisms of cavitation and its repair in plants nondestructively.

Full Text

Preamble

Observation of Cavitation and Water-Refilling Processes in Plants with X-Ray Phase Contrast Microscopy

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Abstract
Leveraging the spatial coherence of X-rays combined with the high flux and brightness of third-generation synchrotron radiation facilities, X-ray phase

contrast microscopy (XPCM) at the Shanghai Synchrotron Radiation Facility (SSRF) can theoretically provide high-resolution dynamic imaging of low electron density materials. In this study, we investigated the cavitation and water-refilling processes in rice and bamboo leaves using XPCM at SSRF. The occurrence of xylem cavitation was recorded in vivo. The study also revealed that under different dehydration conditions, cavitation occurs to varying degrees, and consequently, the refilling process differs. These results demonstrate that SSRF can provide sufficient flux for real-time study of dynamic processes in plants, and XPCM is expected to be a promising method for nondestructively revealing the mechanisms of cavitation and its repair in plants.

Keywords: X-ray phase contrast microscopy, Synchrotron radiation, Cavitation/embolism, Water transportation

Introduction

Long-distance water transport plays a crucial role in plant survival and represents an important issue in plant physiology and ecophysiology. As early as the 18th century, debates emerged regarding the mechanism of water ascent in higher plants, puzzling plant physiologists and physicists for several centuries. Over the past few decades, the Cohesion-Tension (C-T) theory has been widely accepted to explain water transmission in trees. However, Zimmermann pointed out that due to the existence of cavitation and embolism, water columns in xylem vessels are not continuous [1], which challenges the foundation of C-T theory. Consequently, xylem cavitation/embolism has become a hot topic in plant physiology and ecology worldwide. Nevertheless, the mechanism of its occurrence remains unclear due to limitations in microscopic techniques.

Since the last century, researchers have conducted numerous studies in this field through theoretical analysis, modeling [2 4], and experimental investigations [5 9]. Lewis et al. observed emboli development in the tracheids of *Thuja occidentalis* L. using optical microscopy in vitro [7]. However, many controversial viewpoints have arisen regarding methods and results because most experimental techniques require destructive sampling, making the results unconvincing. For example, optical microscopy necessitates cutting a stem section or other plant part as a sample, slicing it into ultra-thin pieces, and observing these slices under microscopes during dehydration or refilling. Thus, the results become distorted and may not reflect the original status in plants.

Nowadays, nondestructive or minimally invasive methods exist, such as the Acoustic Emission (AE) method and Nuclear Magnetic Resonance (NMR) imaging. However, NMR suffers from low spatial and temporal resolution, which is insufficient for observing specific xylem vessels. Furthermore, it imposes stringent requirements on samples, such as good toughness and large vessels, and requires specimens small enough to fit the sample chamber. The AE method can only detect the occurrence of cavitation events, but the acoustic emission

signals are usually weak and frequently disturbed by instrumental noise or the surrounding environment, making it difficult to detect true signals. There is an urgent need for a new suitable method to nondestructively observe the inner structures of vessels with higher resolution in real time.

Compared with the destructive and restrictive methods mentioned above, X-rays with their unique penetrating character offer great advantages for nondestructive detection. Since Röntgen discovered X-rays in 1895, their applications have attracted worldwide attention from researchers [10]. At the end of the last century, propagation-based X-ray phase-contrast imaging was proposed for investigating weakly absorbing materials [11]. Since then, this method has been applied across varied research fields, including biology, medicine, materials science, and other important areas. To meet application requirements, great improvements have been made to propagation-based X-ray phase-contrast imaging methods [12 15] using both laboratory X-ray source systems and synchrotron radiation facilities. Synchrotron radiation facilities offer advantages including higher flux and brightness, as well as superior spatial and temporal resolution, without requiring slicing, reagent injection, or other special sample preparations.

Considering that xylem cavitation/embolism and refilling processes occur over short periods, X-ray phase contrast microscopy (XPCM) based on synchrotron radiation is an ideal technique for investigating these dynamic processes nondestructively in plants, which are mainly composed of low-Z elements. Lee et al. have attempted to employ X-ray micro-imaging to study the water-refilling process in bamboo leaves to understand the mystery of water transport [16,17]. In this paper, we utilized a wiggler source beamline—the X-ray Imaging and Biomedical Application Beamline (BL13W1) at the Shanghai Synchrotron Radiation Facility—to investigate cavitation and water-refilling processes in plants (both rice and bamboo leaves) using X-ray phase contrast microscopy.

2.1 Experimental Setup

BL13W1 is one of the initial beamlines of SSRF, employing an in-line setup to realize X-ray phase contrast microscopy. X-ray in-line phase contrast microscopy has a simple layout and moderate requirements on beam chromatic coherence compared to X-ray interferometry and holography. The factors affecting imaging quality of in-line phase contrast microscopy have been systematically investigated [18,19], and experimental parameters were optimized accordingly. Fig.1 shows the BL13W1 setup, which uses a wiggler source to radiate X-rays across a large energy range from 8 keV to 72.5 keV. The flux density of BL13W1 is 2.2$\times$10^{10}$ phs/s/mm^2@20 keV@200 mA, which is high enough for dynamic imaging at the sample stage 34 m downstream from the source. The low emittance of 3.9 nm$\cdot$rad at SSRF ensures high brilliance for phase contrast imaging. X-ray CCDs with pixel sizes from 0.37 to 24 microns and field of view from 0.74 to 50 mm are equipped to record X-ray images statically or dynamically.

2.2 Experimental Parameters

To meet requirements for high resolution and suitable exposure time, a HAMAMATSU X-ray CCD was used in the experiments, offering two optional modes with resolutions of 4.5 μm and 1 μm. For the higher spatial resolution camera lens, an exposure time of 5 10 s is needed, which is too long to observe dynamic phenomena in living plants. For the 4.5 μm camera lens, exposure time is significantly reduced to only 1 2 s or even hundreds of milliseconds, which is very appropriate for dynamic experiments in this study. Since the structures to be observed in these experiments range from ten microns to several tens of microns, 4.5-μm resolution is suitable. Therefore, a CCD with 4.5 μm resolution was selected, providing high enough resolution to visualize the structures of interest while maintaining acceptable exposure times.

In the cavitation experiments, a 150 Watt High Pressure Sodium Lamp (HPSL) was used to simulate sunlight, with initial lumens of 13500 lm and mean lumens of 12150 lm. During the experiments, a photon energy of 15 keV, sample-detector distance of 9 cm, and exposure time of 600 ms were selected.

2.3 Sample Preparation

Two plant types—bamboo (*CV. Ventricousinternode* was used in this study) and rice leaves—were employed as samples to dynamically investigate cavitation and related repair processes. The imaging targets were dynamic behaviors in xylem vessels, which range from several microns to tens of microns in diameter. Rice is a monocotyledonous annual herb, while bamboo is a perennial plant. Both rice and bamboo belong to Gramineae and have lignified leaves, expecting to show high contrast under X-rays.

Before observing xylem cavitation, the whole rice plant was immersed in water for several hours to ensure full leaf hydration. Then, one leaf piece was cut from the plant at the joint of the leaf blade and sheath, and the leaf was fixed on the sample stage for XPCM experiments. Meanwhile, the HPSL was fixed 30 cm from the sample at approximately 45° to the rice leaf, acting as a simulated sunlight source to accelerate transpiration and cavitation occurrence.

Before observing water refilling, leaves were first cut at the joint of leaf blade and sheath, then dehydrated in air for at least several hours, allowing air to enter the leaves from the cut and cavities to generate in the vessels.

3.1.1 Changes in Inner Vessels

An image sequence was taken to reveal the xylem cavitation process. Fig.2 shows cavitation processes that occurred approximately 11 minutes after HPSL irradiation. Fig.2(A) shows the structure before cavitation, while Fig.2(B) shows the structure after cavitation occurred. The exposure time for both Figs.2(A) and 2(B) was 600 ms. The two images were recorded in temporal sequence with an acquisition interval of 1160 ms. As indicated by the arrows, vessels in

Fig.2(B) are clear, while those in Fig.2(A) are indistinct. This is because vessels in Fig.2(A) are full of water, which blurs the XPCM imaging contrast of vessel walls, whereas those in Fig.2(B) are full of cavities, which enhances the contrast instead. This implies that cavitation occurred in the vessel.

From Fig.2(B), the wall between two adjacent vessel elements could be clearly identified, as indicated by the lower arrow. The instantaneous occurrence of xylem cavitation was recorded in vivo for the first time domestically, which will be very useful for studying cavitation and embolism in intact plants. XPCM is expected to be a helpful method for understanding the mechanism of cavitation occurrence in plants under different stresses.

3.1.2 Changes in Other Leaf Parts

Due to the limited field of view of the detector, we cannot obtain the dynamic cavitation process of the whole sample simultaneously. Therefore, to capture overall cavitation changes across the entire blade, images of several other sample positions were also collected before and after real-time acquisition. During the experiment, the high pressure sodium lamp was also used to simulate sunlight.

Results showed that after tens of minutes or even a few hours of simulated sunlight illumination, most parts of the leaves underwent cavitation. Fig.3 shows tissue changes in different parts of a rice leaf before and after a 1-hour cavitation experiment. In region 1, distinct cavitation occurred in the vessel pointed to by the arrow. In region 2, more cavitations occurred, and we could even distinguish two adjacent vessel elements (pointed to by arrows). The wedge-shaped structure connecting two vessel elements is also very clear. Meanwhile, the sample underwent obvious contraction after the cavitation experiment, allowing us to obtain more microstructural information of the leaf within the same CCD field of view. This is also verified by images of region 3, which show that after the cavitation experiment, the contrast of the vessels is enhanced and the contraction effect is more obvious.

3.2.1 Rice Leaf

During sample preparation for rice leaf water refilling experiments, rice leaves were first cut at the joint of leaf blade and sheath, then dehydrated in air for 3 hours. During this time, we suppose that air entered the leaves from the cut and cavities generated in the leaf vessels. We verified this supposition in the following observations. The leaf was fixed vertically on the sample stage with the cut end immersed in water to rehydrate, while simultaneously being observed in real time using X-ray phase contrast imaging. The inner structures of embolized xylem vessels and the water refilling processes were recorded in temporal sequences.

During rehydration, 60 images of the dynamic water refilling process in a rice leaf were obtained, in which water transport in rice leaf vessels was observed, as shown in Fig.4. Fig.4 shows two frames of the dynamic refilling process: the

first image of the refilling sequence (Fig.4A) shows the structure before refilling, and the last image (Fig.4B) shows the structure after refilling. From Fig.4, it is obvious that some vessels in the last image have worse phase contrast than those in the first image (Fig.4A). This suggests that vessels in the last image are essentially full of water, while those in the first image are full of cavities.

Fig.5 provides a comparison of a magnified vessel area before and after refilling, from which a contrast difference can be easily distinguished. In Fig.5A, the vessel pointed to by the arrow is embolized before refilling; the air in the vessel enhances its relative contrast to surrounding tissues. Fig.5B shows vessels refilled with water, which blurs the vessel contrast relative to surrounding tissues. Besides the differences in phase contrast, the leaf became swollen in the vertical direction of the veins after refilling, because the diameter of xylem vessels in plants varies with the moisture content of the surrounding matrix. The wilted plant will recover after refilling for a period of time. The results reported here may open a new way for investigating drought-resistant trees and crops, and could be valuably used to investigate different lethal thresholds of various plants under different drought stresses.

3.2.2 Bamboo Leaf

Water-refilling experiments on bamboo leaves were also carried out. The preparation and operation were the same as for rice leaves, with the only difference being the dehydration time, which was about 1 day—much longer than the rice leaf experiment. The entire refilling process was recorded in sequence, with a total of 196 frames recorded at an exposure time of 2 seconds per frame. Frame No.60 and No.100 are shown in Fig.6, in which arrows indicate the “wave front” of refilling water. We could see that vessels remained visible in good contrast relative to surrounding tissues after refilling because they were permanently embolized. Therefore, during refilling, most vessels could no longer transport water. Water could only rise along the dehydrated mesophyll located between the upper and lower epidermis, so the refilling water in the leaf rose as a whole rather than through specific vessels for transportation.

Embolization is the leading cause of plant death. If permanent embolism occurs in a certain percentage of vessels, the plant will die. Some studies have already examined the relationship between embolism and permanent wilting [21]. However, previously there has been no suitable method that could accurately determine this relationship. Up to now, the only effective way to determine whether a plant that has undergone drought stress will survive or die is to observe whether it can recover.

4 Conclusion

In conclusion, the experimental results presented here, carried out at SSRF, demonstrate that cavitation and water-refilling processes in plants can be visualized in vivo with X-ray phase contrast microscopy. As we know, embolism

repair is affected by many factors, but the most important element is hydraulic conductivity in plant vessels, which determines the extent of embolism and its repairing mechanism. Among all available techniques, XPCM appears to be the most promising for investigating this phenomenon in vivo due to its high spatial resolution and non-destructive nature. With the high flux density available at third-generation synchrotron X-ray sources, it is also possible to obtain microscopic images with high frame rates. Moreover, the low emittance at SSRF ensures high coherent flux for imaging live plant cells via phase contrast.

To sum up, the X-ray phase contrast microscopy method opens a potential way for observing inner microscopic structures of plants in vivo. This is of great significance not only for investigating xylem cavitation, embolism, and repairing processes in plants, but also for studying drought-resistant mechanisms and lethal thresholds of crops and trees.

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Note: Figure translations are in progress. See original paper for figures.