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Method Article

High-throughput imaging of fresh-frozen plant reproductive samples in a variable pressure SEM

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A B S T R A C T

Conventional light and electron microscopy are the most widely used techniques for examining plant reproductive tissues; however, they are time-consuming or expensive. The anther is the male part of the plant reproductive system. Structural changes drive development, and any structural defect may lead to an increase in fertility or cause sterility; thus, quick detection of structural changes is crucial in reproductive biology. We optimized an existing low-temperature SEM alternative to examine the internal structure of hydrated, fresh-frozen anthers. In contrast with the original technique, our method does not require precooling adhesion (ethanol to fix the specimen), and the cryo-sectioning can be conducted at atmospheric pressure. In addition to enabling the differentiation between aerial and liquid-filled intercellular spaces, this method is expected to facilitate the detection of quick (during a day) developmental changes in plant reproductive tissues, which is a current challenge using conventional approaches.

- This method allows the high-throughput imaging of fresh-frozen plant reproductive samples collected every 10 min, which is important for developmental studies.
- The cryo-images of samples with thickness ranging from 0.2 to 3 mm can be well-preserved at 800X magnification.
- This method does not require chemical processing, critical point drying, customized cryo-accessories, controlled temperature cold stages, or metal coating. This simplified method does not require highly skilled personnel, and it is suitable in most microscopy laboratories.

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Resource availability:	

Method details

The rapid examination of plant reproductive tissues in their “native-hydrated” stage is important in developmental biology studies. The cryogenic scanning electron microscopy (cryo-SEM) technique allows imaging of hydrated frozen tissues at very high resolution (1 nm); however, it is specialized and expensive. Therefore, the development of more feasible and cost-effective techniques than the cryo-SEM has been a continued interest in the research community. In this context, several papers have focused on customized improvements of the SEM technique to facilitate imaging of hydrated frozen tissues. For example, the use of a modified clamping stub [6]; a controlled cold stage for specimen examination [1,2]; a customized cryo-holder with precooling adhesion [4]; and with passive cooling [3]. Here we described an optimized method for imaging the internal structure of hydrated, fresh-frozen anthers in the variable pressure microscope (VP)-SEM. The advantage of our optimized method compared to the low-temperature SEM alternatives includes that it does not require modified clamping stubs, custom-made cryo-holders, attached cold stages, or metal coating. The anther cross-sections were generated by sectioning the cryo-immobilized specimens at atmospheric pressure, minimizing the specimen loss. We observed that the structures of cryo-immobilized fresh-hydrated uncoated anthers of sorghum [*Sorghum bicolor* (L.) Moench] was well-preserved irrespective of the developmental stage. Despite the advantages of examining fresh versus chemically fixed samples, it was not till recently that the first anther study using cryo-SEM and a high-pressure freezing procedure was published [5]. The authors revealed for the first time the transition from a water-filled locule to an air-filled one, adding new insights into this field of study. Our technique is fast (10 min) and cost-effective compared to conventional microscopy techniques (paraffin/resin embedding and cryo-SEM) used for imaging reproductive tissues, including anther. In addition to being suitable in most laboratories, this cryo-VP-SEM alternative applies to a wide range of plant species. We have provided a detailed step-by-step description of this method with all the instrument specifications.

Materials, reagents, and workflow

- Sorghum anther and cotton flower (reproductive tissues from other plant species can be used)
- Tweezers
- Eppendorf tubes
- Water
- Liquid Nitrogen
- Standard SEM stub
- Double-sided carbon tape
- Double edge blade
- Scanning Electron Microscope



Fig. 1. Harvesting and processing of sorghum anthers prior imaging under SEM. The spikelets were collected from sorghum plants at different developmental stages (a). The collected samples were kept hydrated in Eppendorf tubes filled with water (b). The fresh anthers were mounted on the specimen stub with double-sided sticky carbon tape before the cryo-immobilization (c).

Table 1
Recommended SEM specifications for imaging of fresh, frozen specimens.

Parameters	Specifications
Aperture	0
Bias	300
Acceleration voltage	20 kV
Gun Brightness	3
Sample size	50 mm
Vacuum mode	Vp-low
Pressure mode	0=Close
Beam intensity	0 pA

Specimen collection and preparation

Anthers were collected from fresh-hydrated spikelets of floral sorghum panicles. The spikelets were placed in Eppendorf tubes filled with water to keep the samples hydrated (collected specimen should not be wet) (Fig. 1a). Following the collection, the anther samples were vertically placed on the specimen stub using double-sided carbon tape (Fig. 1b) to avoid specimen loss during either the cryo-immobilization, cryo-sectioning, or transference to the SEM vacuum chamber.

Cryo-immobilization, cryo-sectioning, SEM specifications, and specimen imaging

The specimen on the stub was cryo-immobilized (Fig. 1c) using the shock freezing technique (quick cooling) by immersion in liquid Nitrogen ($-196\text{ }^{\circ}\text{C}$) for one minute. Immediately following the cooling, the samples were sectioned using a sharp double edge blade under atmospheric conditions. Afterward, the stub with the resulted cryo-cross sections was transferred to the sample chamber for imaging using the SEM (S-4300 E/N field emission variable pressure scanning electron microscope (FEVPSEM)) with the airlock loading. Before specimen observation, we selected the environmental secondary electron detector (ESDE) mode and set the specifications for imaging (Table 1).

Workflow summary

- Dissect fresh flowers (or rescue anthers) and place them vertically on the specimen stub with double-sided tape.
- Proceed quickly and place the stub with the specimens in the Liquid N_2 bath for 1 min.
- Immediately after the cryo-immobilization, remove the stub from the liquid N_2 and proceed with the cryo-sectioning (quick and firm transverse cuts of the samples).
- Transfer the cryogenic stub and sample into the SEM chamber following the SEM manufacturer's instructions.
 - Select the ESDE mode.
 - Set the instrument parameters as suggested (see Table 1, main text).

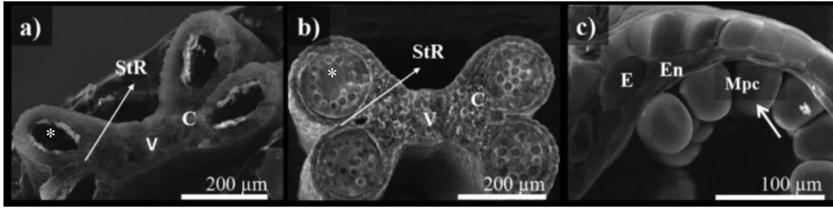


Fig. 2. Cryo-VP-SEM images of fresh-hydrated sorghum anthers at different developmental stages. The white stars indicate the anther locus gas-filled (a, c) or liquid-filled (b). Anther structures: vascular region (V) connective tissue (C); stomium region (StR); epidermis (E); endothecium (En); mother pollen cell (MPC).

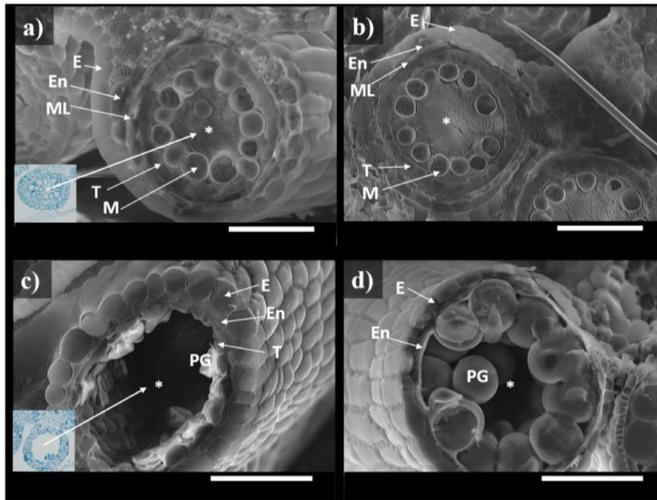


Fig. 3. Cryo-VP-SEM images of fresh-hydrated sorghum anthers at different developmental stages and showing different structures. The white stars indicate the anther locus gas-filled (c, d) or liquid-filled (a, b). Anther structures: vascular region (V) connective tissue (C); stomium region (StR); epidermis (E); endothecium (En); middle layer (ML); tapetum (T); mother pollen cell (MPC). Scale bar represents 200 μm for the whole anther and 100 μm for the single anther lobule. The blue images at the bottom of Fig. 3a and c, represent the light microscopy images, showing a similar anther structure compared to the corresponding SEM image (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

- Proceed to image the samples as soon as the frozen layer melts (sublimation for 1,2 min). The sample examination duration with minimal charging effect is about 30 min. The charging effect and beam damage will increase with time.

Modifications:

This method enables the use of other fast freezing techniques, as it applies to different laboratory conditions and sample types.

Method validation

During the optimization process, the SEM parameters were adjusted to minimize charging and increase image resolution. Fig. 2 shows the cross-sections of the whole anther of sorghum at different developmental stages. We were able to detect the differences between the gas (Fig. 2a) and the liquid-filled (Fig. 2b) intercellular spaces using our method. Fig. 2c shows the image of a well-preserved anther structure, with the two-layer cell wall (epidermis (E), and endodermis (En)), and the mother pollen cell (Mpc). Fig. 3 shows the single anther locule at different developmental stages and the

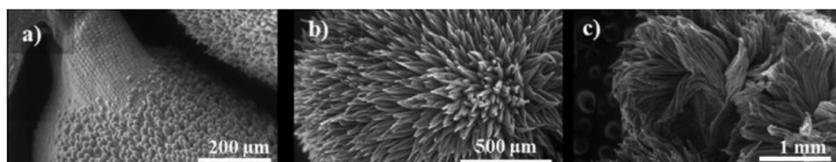


Fig. 4. Cryo-VP-SEM images of cotton fibers. Fiber development at one (a), three (b), and five days (c) after fiber initiation.

distinction between the gas and the liquid-filled sections in the SEM images. However, such distinction was not possible in the light microscopy images. This issue could be associated with the dehydration process required for the plastic embedding sample preparation for light microscopy imaging. Fig. 3a and b show the anther cell wall with the four layers (epidermis (E), endothecium (En), middle layer (ML), and tapetum (T)). Fig. 4 shows the cryogenic images of the cotton fiber at very early developmental stages (first, third, and fifth-day after fiber initiation).

Conclusions

The presented method is an alternative to the cryo-SEM and the low-temperature SEM techniques optimized to image reproductive samples. This chemical-free technique is simpler and faster than the conventional methods (plastic embedding and light microscopy). Besides, it enables high-throughput imaging while avoiding toxic fixatives, staining reagents, critical point drying, cryo-holders, cryo-stages, and metal coating before sample examination. Based on our results, we recommend using the cryo-sectioning-VP-SEM technique for high throughput screening of small (up to 3 mm thick), fresh-frozen plant reproductive samples.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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