An alternative simple method for preparing and preserving cross-section of leaves and roots in herbaceous plants: Case study in Orchidaceae

Cite as: AIP Conference Proceedings **1862**, 030113 (2017); https://doi.org/10.1063/1.4991217
Published Online: 10 July 2017

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An Alternative Simple Method for Preparing and Preserving Cross-section of Leaves and Roots in Herbaceous Plants: Case Study in Orchidaceae

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Abstract. This alternative method provides a simple and faster procedure for preparing cross-sections of leaves and roots in herbaceous plants, especially for living specimens of orchids (Orchidaceae). This method used a clamp-on hand sliding microtome to make cross-sections of leaves and roots, with sections preserved inside the microtubes containing preservation liquid. This preservation technique allowed the sections to be restained and to be used for further usage in future. This method was more practical than the paraffin embedding method because it does not need the additional steps of paraffin embedding and deparaffinization. It may also provide better cross-section results than free-hand sectioning method. The procedure is very feasible and is recommended for use in plant anatomy observation.

Keywords: botanical research, plant science, plant anatomy, cross-sectioning method, cross-section, herbaceous, orchid

INTRODUCTION

Anatomical studies on herbaceous plants, especially in Orchidaceae, are still an important approach for understanding their taxonomy, physiology, ecological adaptation, and evolutionary biology [1-5]. Therefore, improving the current methods used to prepare and preserve plant material in anatomical studies are an important focus for research.

A free hand section is widely recognised as the simplest method to prepare plant cross sections for microscopic observation. It is appropriate for a broad variety of plant materials [6], although the resulting sections are usually not thin enough, and their thickness is sometimes not uniform. It also faces great difficulties when the plant material is too thin (for example thin leaf lamina) or too soft, such as herbaceous roots with a very small diameter. In addition, to get good cross sections, using this simple technique requires patience, long intensive practice, and outstanding skill [7].

The paraffin embedding method has been used for many years to provide thin sections for anatomical observation in botany. In this procedure, plant materials are hardened by replacing the intracellular water with paraffin so that they can be cut as thin as possible [8]. However, the steps required in order to obtain a good permanent slide using the paraffin embedding method are time-consuming and require much effort [8-10]. The process consist of many steps, including fixation, dehydration, infiltration, evaporation, mounting, and staining [8, 11].

Therefore, the purpose of this paper is to demonstrate a simple method of preparing and preserving cross sections of leaves and roots, as an alternative to the free hand section and paraffin embedding methods. This simple method has been used many times in our laboratory for over three years, predominantly with specimens from the Orchidaceae family.

International Symposium on Current Progress in Mathematics and Sciences 2016 (ISCPMS 2016)
AIP Conf. Proc. 1862, 030113-1–030113-6; doi: 10.1063/1.4991217
Published by AIP Publishing. 978-0-7354-1536-2/$30.00

MATERIALS AND METHODS

Samples were mostly from orchid species, both thick and thin-leaved. Samples were also taken from both wet herbarium specimens or liquid conserved collections, and fresh specimens. We have used this method to carry out cross-sectioning of leaves and roots in a considerable number of species. Observations have been done using microscope OLYMPUS Inverted IX73, and LEICA DM500.

In this method, several steps required for the paraffin embedding method, such as fixation, were not implemented. The dissection process was conducted by the clamp-on hand sliding microtome (Allmikro; Haga metallwarenfabrik - Germany). This hand mini microtome needs a sharp razor to cut the plant materials. Since the hole to hold the plant material on the microtome was only 1.1 cm in diameter, the plant material should not exceed more than 1.1 cm wide, preferably less than that width.

1. Cassava corks, collected from the young stems of cassava plants, were used to embed the plant material. Before it can be used, fresh cassava corks should be dried under the sun or by drying it in a low heat oven to allow them to harden. Where the plant material is a thin leaf, then we used a half-sliced cassava cork, and inserted the piece of leaf between the sliced cork. Another alternative is using solid Styrofoam. If it is a thick leaf, then we cut the cassava cork so the cleft fits the piece of leaf. When the material is a cylindrical root, we create a small, deep hole on the center of the cork that fit with the size of the root, and then put the root into the hole carefully (Fig. 1a to 1c).
2. The cork has to be properly set to fit into the mini microtome's hole. The surface of the cork and plant's material should face straight upward (Fig. 1d).
3. Make several transversal slices, to produce material that is as thin as possible. Choose the most transparent, thinnest and intact slice of the plant material.
4. Fresh cross sections can be collected carefully from the razor blade using the tip of a small paint brush (Fig. 1e).
5. Dehydrate the cross sections in a graded alcohol series by putting them first into microtube contained 30 % alcohol. To promote faster dehydration, we can carefully circulate the liquid inside the microtube by sucking and blowing it using a long pipette. After 0.5-1 minutes, change the earlier liquid with 50 % alcohol and repeat the circulating technique. Conduct the next step with 70 % alcohol, followed by a final steo using 96 % alcohol. Very thin and soft cross sections make the dehydration process faster than in paraffin embedding dehydration, which may take an hour for each concentration [11] (Fig. 1f to 1g). When the sections are too thin and brittle, liquid circulation is not suggested since it could damage some of the soft tissues. Dehydration can be conducted by submerging the sections for a longer time.
6. After we get some clear and transparent sections, then suck all the 96 % alcohol and replace it with preservation liquid. Preservation liquid used here is a mixture between alcohol 70 % and glycerin (3:2) or (1:1). We can also put a stain, such as Safranin-O 1 % (diluted in alcohol 96 %) into the preservation liquid. This preservation liquid has been inspired by the 'Copenhagen' mixture that is usually used in the wet herbarium, with some modification. Write a specific label on the microtube using permanent and water resistant pen (Fig. 1h to 1i).
7. Anatomical observation under the microscope can be conducted by putting the selected cross sectioned sample on a slide with 1-2 drops of preservation liquid to avoid sample dehydration, then covering it with a cover glass.
8. Shortly after anatomical observation has been completed, the cross sectioned sample should be transferred again to the microtube contained preservation liquid for storage.

RESULTS AND DISCUSSION

This simple and quick technique allowed us to produce good quality sectioned samples (Fig. 2 and Fig. 3), at very low cost and without using too many commercial chemicals usually needed in the other methods. The use of cassava cork to replace paraffin as an embedding medium can reduce the cost of the process. In addition, cassava cork can be easily collected, especially in tropical developing countries where the embedding mediums are not easily available.

In this technique we found that leaves and roots from living plants as fresh material gave better results than liquid preserved materials (eg. leaves and roots stored in Formalin-Acetic Acid-Alcohol (FAA) or the wet herbarium). Plant material from FAA and the wet herbarium were easier to dry during the sectioning preparation due to rapid evaporation of the alcohols from their tissues. As the drying process started from the exposed surfaces and edges of plant material,

it made it harder and more difficult slice it properly. Therefore, fresh material from living plants would be the first priority to produce good quality of cross-sectioned samples. This alternative technique can be used effectively for both, thick or thin-leaved specimens.

The treatments during dehydration should be conducted very carefully since thin cross-sectioned samples were extremely brittle and vulnerable to any pressure. Our experiences showed that the thin cross-sectioned samples were usually ripped or perforated due to puncture by paintbrush hairs, especially during transfer from the razor blade to the microtube, or when selecting and picking up samples from the microtube. The use of a soft-haired paintbrush is suggested to minimize the risk of damage to samples in the post-sectioning process.

The preservation procedure demonstrated in this technique enables large numbers of cross-sectioned samples to be kept, without the need for numerous glass slides, as is required when preserving using permanent slides. This preservation technique allows the cross-sectioned samples preserved inside the microtube to be re-stained with another microscopic dyes for different purposes. Our experience showed that by using this technique there was no significant change in quality of the cross-sectioned samples after 3 years in proper storage. The relatively high concentration of glycerin in the preservative liquid functions to prevent sectioned samples becoming too brittle, and to maintain its elasticity during storage. Glycerin can also protect the sectioned samples from the effect of freezing. On the other hand, glycerin is prone to mold that easily grows at a relative humidity (RH) of 65 % or above, meaning that the microtubes should be stored in a refrigerator at 12-15 °C and should be maintained at RH less than 60 %.

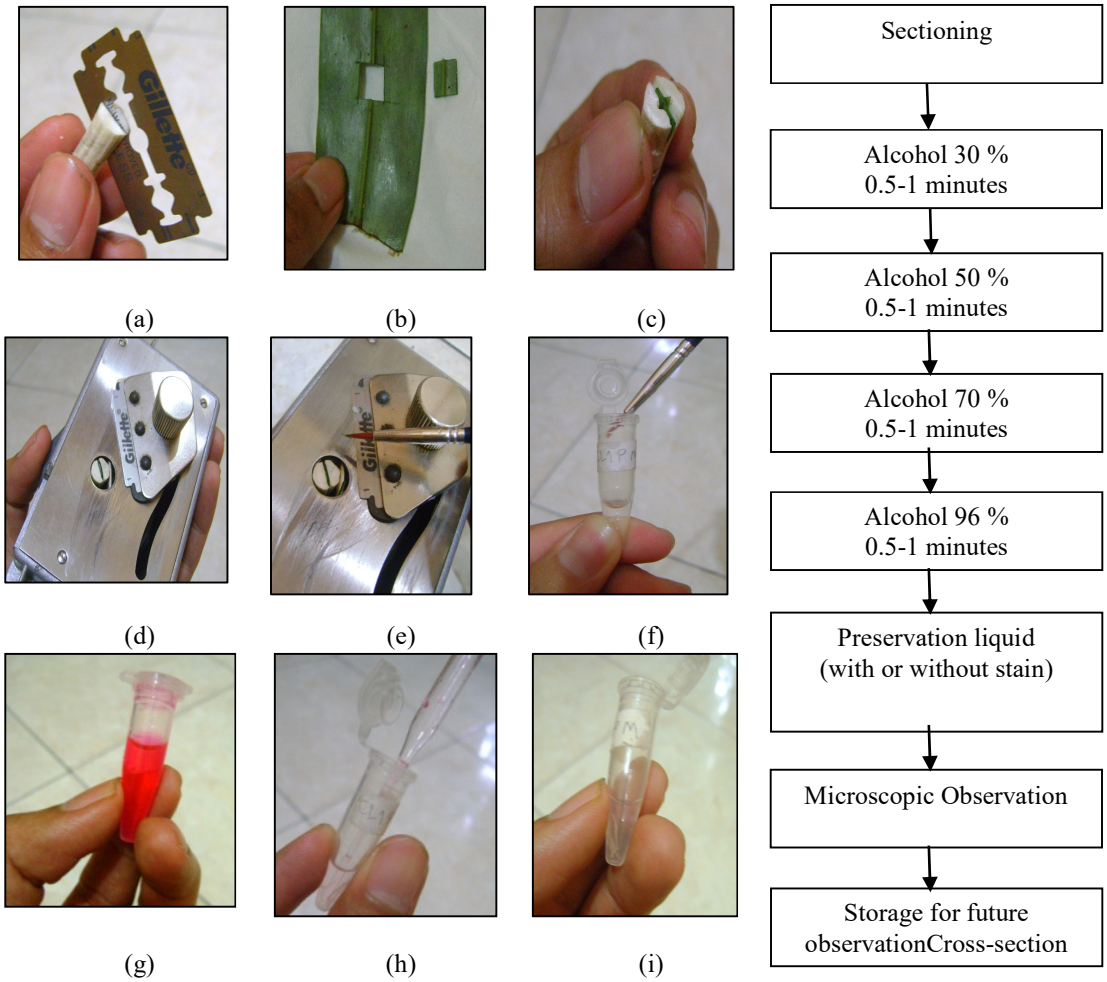

FIGURE 1. Procedure to prepare cross-sectioned samples

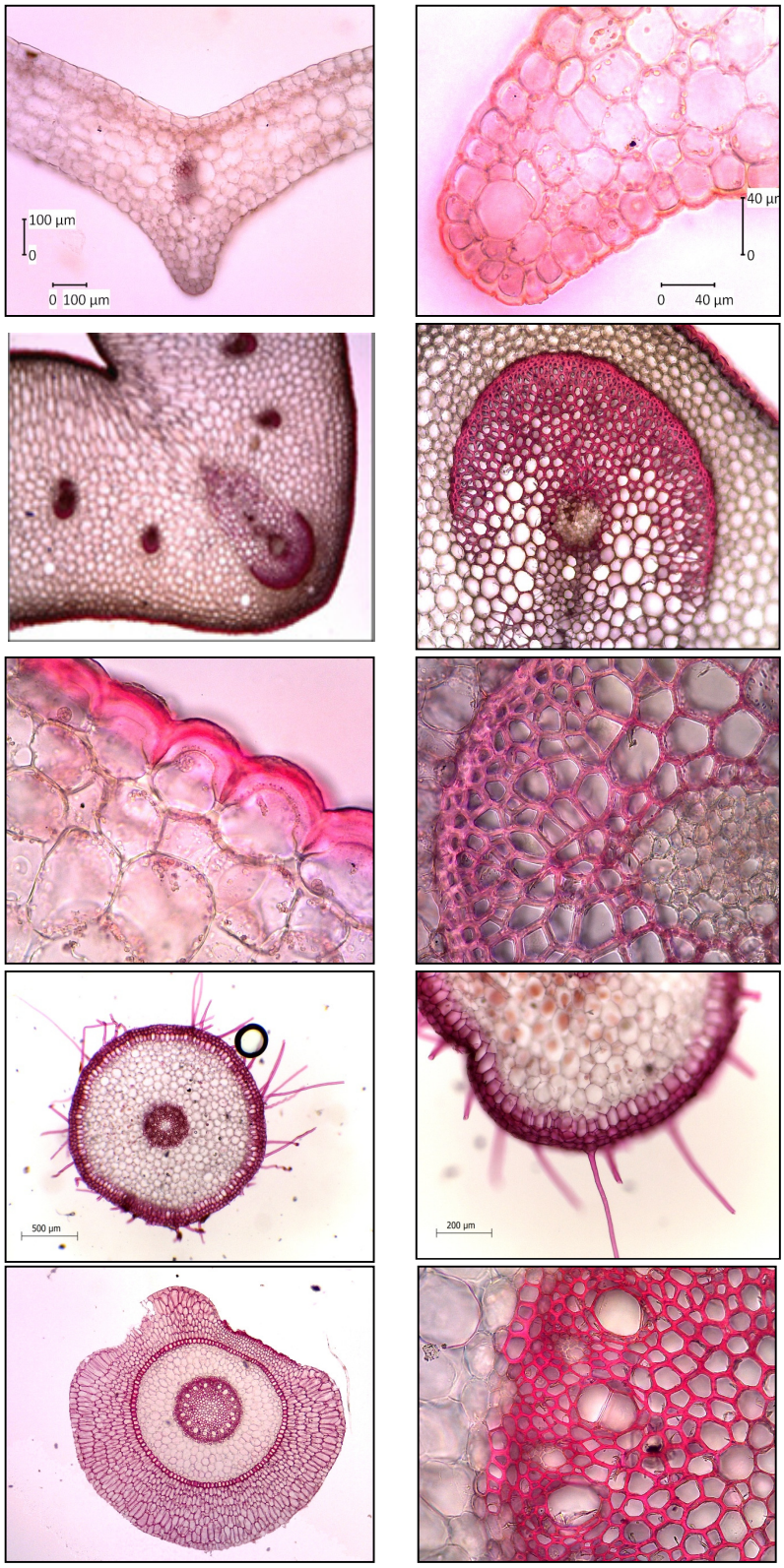

FIGURE 2. Cross sectioned samples (simple staining)

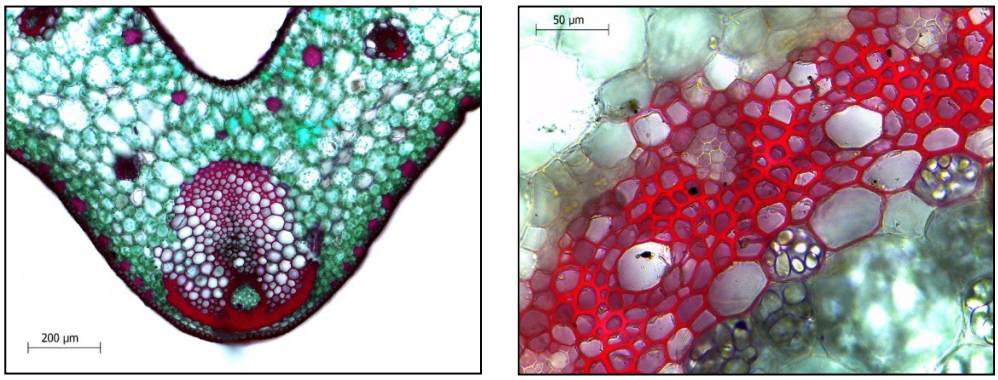

FIGURE 3. Cross-sectioned samples (double staining)

Compared to free-hand sectioning method, the method explained in this paper does not require high levels of skill or intensive practice, so it can be done easily by beginners and amateurs. However, having some practice and experience are suggested before doing anatomical work, in order to improve the quality of results. The stability to produce good quality cross-sectioned samples in great numbers, and the ability to dissect thin lamina are also strengths of this alternative method.

This technique can also be implemented in the field, since the mini microtome, chemicals, and all the equipment are portable. The simple and quick procedures could facilitate the practical use of sectioning and preserving processes, especially when during fieldwork with limited facilities. In conventional techniques, as in the Paraffin embedding method, at least 3 or 4 days are required in order to make a permanent slide, while this simple technique only requires 30-60 minutes per sample (including the sectioning step). This means that it would be a less consuming time procedure to prepare cross-sections of plant material, especially for an intensive anatomical project involving a large number of samples.

CONCLUSIONS

This alternative method provide a simple and faster procedure for preparing cross-section of leaves and roots in herbaceous plants, especially for orchids (Orchidaceae). This method was more practical than paraffin embedding method, and provides better cross-section results than free-hand sectioning methods.

ACKNOWLEDGMENTS

The author would like to express his gratitude to Dr. Dra. Nisyawati, M.S. for valuable discussion; to Byan Arasyi Arraniry, Reza Saputra, and Dimas Wahyu Prayogi for their helpful assistance; to Amy Hinsley for helpful comments that improved the manuscript; to Bioimaging laboratory, Department of Biology, Universitas Indonesia and Bio Imaging Center UI-Olympus (UOBC) for kindly facilitating the microscopic observation. I also thank to Hibah Pascasarjana Universitas Indonesia for the financial support.

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