The effect of drying step in the preparation of *Microlejeunea ulicina* for scanning electron microscopy observation

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Abstract: *Microlejeunea ulicina* is one of the leafy liverworts species found in Universitas Indonesia that has unique characteristics and its existence in Java Island has not been reported before. The ultrastructure analysis of *Microlejeunea ulicina* is important not only as the mean of identification but also for further exploration of their oil body which contains useful metabolites. Ultrastructure investigation can be accomplished by using Scanning Electron Microscopy (SEM). However, the optimization of the preparation steps for biological sample observation by SEM, including liverworts, is necessary to be carried out. Drying step plays the important role in maintaining the structure of the sample as close-to-native as possible. Thus, the aim of this study is to evaluate the effect of the drying step in the preparation of *Microlejeunea ulicina* for SEM observation. *Microlejeunea ulicina* were fixed with 2.5% glutaraldehyde, post-fixed with 4% osmium tetroxide, dehydrated with ethanol series, and then subjected to different drying method, i.e. air-drying and freeze-drying. According to the data obtained, samples dried by using freeze-drying method showed the more detailed structure in conjunction with the up-and-down contour, without any artefact covering the surface of the samples, while air-dried samples showed the flat surface hiding the real structure of the cell. Our data suggested the advantages of freeze-drying in preparing *Microlejeunea ulicina* for ultrastructure investigation using SEM. The results of this study can further be applied as the basic procedure for other biological samples observation by using SEM.

1. Introduction
Liverworts of *Marchantia* is one member of Marchantiaceae. It is composed of 37 species and 7 subspecies [1] and is distributed worldwide, including in Indonesia. *Microlejeunea ulicina* is one of the leafy liverworts species found in Universitas Indonesia that has the unique characteristics and its existence in Java Island has not been reported before. Previously, this genus was found in another part of Indonesia, except Java. Thus further study on *Microlejeunea ulicina* is of great important to give rise into Marchantiophyta diversity.

The ultrastructure analysis of *M. ulicina* is essential not only as the mean of identification but also for further exploration of their oil body which contains useful metabolites. The metabolites contained in the leafy liverwort’s oil body have previously been studied to be potential as the antimicrobial, antioxidant, etc as previously reported [2, 3]. Ultrastructure investigation can be accomplished by using Scanning Electron Microscopy (SEM) to study the characters of surface structure of the liverworts samples. These
characters are important for the identification of liverworts species. However, the optimization of the preparation steps for biological sample observation by SEM, including liverworts, is necessary to be carried out. A Drying step plays the important role in maintaining the structure of the sample as close-to-native as possible. Thus, in this study, we evaluated the effect of the drying step in the preparation of Microlejeunea for SEM observation.

2. Materials and Methods
In this study, the samples of *M. ulicina* were taken from Universitas of Indonesia Depok campus. After the removal of the contaminant by observing under optical microscopy, samples were fixed with 2.5% glutaraldehyde in Phosphate Buffer Saline (PBS), followed with post-fixation with 4% Osmium tetroxide and dehydrated using Ethanol series (70, 80, 90, and 100%), 10 minutes each.

All samples were then subjected to different drying method, i.e. air-drying and freeze-drying. Before freeze-drying, the samples were incubated at -80°C and finally inserted to the freeze-drier chamber. Both air-dried and freeze-dried samples were coated by using gold prior to SEM observation. The observation was carried out at CMM, BPPT by using the voltage of 80 kV.

3. Results and Discussions
In this study, the surface ultrastructure of *Microlejeunea ulicina* were assessed by the application of SEM which provide higher resolution and magnification as compared to the optical microscopy. Two different drying methods were applied, air-drying and freeze-drying (figure 1). According to the data obtained, samples dried by using freeze-drying method showed the more detailed structure in conjunction with the up-and-down contour, without any artifact covering the surface of the samples (figure 1.A). On the other hand, the air-dried samples showed the flat surface hiding the real structure of the cell (figure 1.B). Our data suggested the advantages of freeze-drying in preparing *M. ulicina* for ultrastructure investigation using SEM. Worthen and wickham [4] has shown that the improper drying may cause shrinkage.

Biological samples, including liverworts are wet with high moisture, while during the observation, the SEM chamber is set in the vacuum condition. Thus, the optimum drying is required to make the samples fit with the vacuum condition without any structural alterations. Our data suggested the importance of proper drying to maintain the structure of the biological samples particularly the leafy liverworts.

![Figure 1. Microlejeunea ulicina images observed by SEM. The samples were subjected to freeze-drying (A) and air-drying (B). Bars: 20 µm.](image_url)

To further investigate the ultrastructure of the surface, high magnification images were also depicted (figure 2). Fine structures of *M. ulicina* were clearly observed after treated with Freeze-drying compared with those treated with air-drying. Freeze-drying resulted in the more detailed structure and the cell surface was not covered by the artifact (figure 2.A), while air-dried sample showed the flat structure (figure 2.B). The ‘crater’ shape and the fiber with the diameter of around 1.33 um were observed only in the samples treated with freeze drying.
Based on the above results, it is clear that the drying step in biological sample preparation plays an important role in maintaining the structure of the samples. For the biological samples, the closer-to-native condition is the most favorable one to avoid the misinterpretation of the data. However, the preparation steps for biological samples observation by using electron microscopy is still challenging. The biological samples, including *M. ulicina*, are relatively soft, can easily be destroyed, and non-conductive. Thus, handling those samples requires optimization of each step. One of the common procedures applies a critical point drying (CPD) which removes liquids from a biological specimen by adjusting the temperature and pressure so that the liquid and gas phases of the sample are in equilibrium. Nevertheless, the negative effect of CPD on preserving biological sample structure has also been reported [5].

Drying of the samples to remove all of the water contained in the biological samples is essential to avoid any disturbance during SEM observation, which is carried out inside the vacuum chamber. Our results suggested the importance of the freeze-drying to maintain *M. ulicina* surface structure. Further optimization including the using the Hexamethyldisilazane [6] or ionic liquids [7] would also be beneficial to be applied for biological samples.

4. Conclusions
The fine structures of *M. ulicina* were clearly observed after treated with freeze-drying compared with those treated with air-drying. Freeze-drying resulted in the more detailed structure showing the up-and-down contour, and the cell surface was not covered by the artifact, while an air-dried sample showing the typical flat structure hiding the real structure of the cell.

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