

Replacing critical point drying with a low-cost chemical drying provides comparable surface image quality of glandular trichomes from leaves of *Millingtonia hortensis* L. f. in scanning electron micrograph

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Abstract

Sample preparation including the dehydration and drying of the samples is the most intricate part in the field of scanning electron microscopy. Till to date, most of the sample preparation protocols use critical point drying with the help of liquid carbon dioxide. Very few cases have been reported where samples were dried using some chemical reagents. In this work, we have explored the possibility of using hexamethyldisilazane, a chemical drying reagent, for the preparation of plant samples. As glandular trichomes are one of the most fragile and sensitive surface structures found on plants, we chose to use *Millingtonia hortensis* leaf samples as our study materials that contain abundantly glandular trichome. The results obtained by this new method are identical with the results obtained from the critical point drying method, in every aspect.

Introduction

Scanning electron microscopy (SEM) is widely used for generating a detailed image of surface morphology in plant samples. The steps required to prepare any tissue for SEM includes fixation, dehydration, critical-point drying (CPD), mounting and coating with gold or palladium for enhancing electrical conductivity of tissue samples (Bomblies et al. 2008). While hard structures usually present no problems and can be air-dried prior to coating with metal for SEM, soft plant tissues must be chemically fixed (hardened), carefully dehydrated and dried. Simple air drying, even of chemically hardened tissues, might results in nearly collapse and shrinkage (Nation 1983).

While fixation procedure is straight forward and does not require any expensive equipment, the dehydration needs to be carried out carefully ensuring preservation of cell structure and also to avoid tissue shrinkage (Pathan et al. 2010). The most commonly used dehydrating method for preparation of a biological sample is CPD using liquid carbon dioxide (CO₂). Liquid from the tissue can be removed by this method through avoidance of surface tension effects, where the transition from liquid to gas at the critical point takes place without an interface as the densities of liquid and gas are equal to this point (Meek 1976). Thus, CPD in general, is the method of choice for dehydration of biological specimens including plant tissues, despite longer sample preparation time (Shively and Miller 2009). The major disadvantage of CPD is its cost, as a specialized device is needed for using liquid CO₂ under vacuum and therefore cannot be afforded by small laboratories on an individual scale. Besides, CPD is not free from glitch; a minor change in the parameters during CPD may lead to the vacuum (Boyde 1980).

An alternative to CPD for preservation of plant tissue samples is the use of low-cost chemicals such as hexamethyldisilazane (HMDS) and Peldri II (Zimmer 1989). Although only scant information is available on chemical drying of plant tissues for SEM, Peldri II treatment in leaves shown complete removal of epicuticular wax, while CPD and HMDS treatment showed retention of the surface microstructure (Bray et al. 1983; Chissoe et al. 1994; Pathan et al. 2010). Use of HMDS in drying of biological samples is not something new particularly with animal tissues, where soft tissues to be prepared for SEM were dehydrated through a graded ethanol series, immersed in HMDS and air-dried without critical point drying

(Nation 1983). The reduced surface tension of HMDS added strength to the samples during drying and possibly reduce fracturing of collapsing of animal tissues (Nation 1983). Several studies were conducted with HMDS as a chemical drying reagent, but all of these studies used animal and human tissues (Braet et al. 1997; Shively and Miller 2009). Information on the HMDS as a chemical agent to dry plant tissues after fixation for SEM analysis is scant (Bray et al. 1993). Even, information available in such study did not promote HMDS as chemical drying agent for studying delicate surface structures under SEM (Bray et al. 1993). Apart from chemical drying, some researchers tend to use air drying method, which often resulted in producing tissue distortions (Zimmer 1989). In plants, glandular trichomes are considered as one of the most delicate surface structures (Muravnik et al. 2016). In this study, we report our findings of SEM analysis on leaf glandular trichomes of *Millingtonia hortensis*, a tree species of Bignoniaceae widely distributed throughout south-east Asia, with an aim to reassess the CPD, HMDS and air-drying methods.

Materials And Methods

Small leaves of *M. hortensis* were used as our study material. They were collected fresh from the greenhouse-grown plant at Komarov Botanical Institute in St. Petersburg, Russia. In India, leaf samples were collected fresh from field-grown plants at Indian Institute of Technology Kharagpur. During the collection of samples, the developmental stage of the leaves was taken care of and it was same in both the cases.

After collection, the samples were immediately put in the fixative solution containing 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.1M phosphate buffer and placed under vacuum until all the samples sink to the bottom of the vials. After the first round of fixation, half of the total number of samples were post-fixed in 2% (v/v) osmium tetroxide (OsO_4) (Sigma Aldrich) in 0.1M phosphate buffer at 4°C for overnight. The other half of the samples were subjected to dehydration by using graded ethanolic series in 30, 50, 70, 80 and 90% ethanol (each for 10 min) and finally two times in 95% ethanol (20 min each) in succession, at room temperature. These samples were then kept in a mixture of 95% ethanol and isoamyl acetate (1:1) for 10 min and finally in pure isoamyl acetate for 15 min. After removal of isoamyl acetate, the samples were placed on the sample holder for critical point drying in a Hitachi Critical Point Drier HCP-2 (Hitachi, Japan) according to the method as described by Muravnik et al. 2016. The samples which were kept overnight in OsO_4 for secondary fixation were treated in the same way (as described above) in the next day. Both types of samples (OsO_4 treated and untreated) were sputter-coated with a thin layer of gold and were viewed under a JEOL JSM-6390 (JEOL, Japan) scanning electron microscope at an accelerating voltage of 7 kV in the laboratory of Komarov Botanical Institute. The digital images were produced by using the control programme associated with the microscope (Muravnik et al. 2016).

For chemical drying, the same protocol was followed until isoamyl acetate stage and then the samples (both OsO_4 treated and untreated) were kept separately in HMDS for 5 min at room temperature. This was followed by drying of samples in a desiccator for 30 min and then sputter-coated with gold. The samples were viewed under a ZEISS EVO 60 (Carl ZEISS SMT, Germany) scanning electron microscope at an

accelerating voltage of 20 kV, in the laboratory of the Central Research Facility, Indian Institute of Technology Kharagpur. The control programme associated with the microscope was used to generate the digital images.

In case of air drying, samples were fixed and then were subjected to ethanolic dehydration as described before. But after the final round of dehydration with 95% ethanol, the samples were kept open for overnight to ensure complete evaporation of ethanol and proper air drying. The air-dried samples were viewed under ZEISS EVO 60 (Carl ZEISS SMT, Germany) scanning electron microscope at an accelerating voltage of 20 kV, after sputter coating with gold in the laboratory of the Central Research Facility, Indian Institute of Technology Kharagpur. The control programme associated with the microscope was used to generate the digital images.

Results And Discussion

Both CPD- (Fig. 1) and HMDS-treated (Fig. 2) samples showed an equal range of preservation in trichome architecture and morphology. Mechanism of action of HDMS on biological tissues is not clear. It is a reagent quite often used in gas chromatography for making silylation of non-volatile compounds such as sugars, amino acids and alcohols (Nation 1983). Perhaps the combined properties of low surface tension and cross-linking potential are likely to be the reasons for its suitability as chemical drying agents for biological tissues. However, in case of the air-dried sample, the occurrence of artifact due to tissue shrinkage has been observed. In all the cases both OsO_4 -treated and OsO_4 -untreated samples showed almost identical tissue fixation. But samples treated with OsO_4 as post-fixative agent showed slightly better tissue preservation and image contrast than those which were not post-fixed with OsO_4 . In case of air-drying as evident from Fig. 3, the samples were shown to be distorted. Further, drying induced artifacts were also evident from the images making the OsO_4 effect negligible. The images obtained from the air-dried samples are not comparable with CPD and HMDS based methods as the samples undergone structural distortion than the other two methods. As mentioned earlier, glandular trichomes are very hard to image due to their extremely delicate structure. Earlier attempts with chemical drying also failed to preserve the trichome structure (Zimmer 1989). However, in our method, upon using HMDS, fine structures of glandular trichomes in the young leaves were successfully preserved and no marked differences between images obtained through CPD (Fig. 1) and HMDS (Fig. 2) could be distinguished.

Besides, avoiding CPD means no need for investment in costly instrument by individual laboratories working on a small scale. Use of HMDS shall enable small laboratories to prepare samples in their labs and ensure a single trip to the SEM facility. However, there is a small point of concern that lies in the chemical property of HMDS. As it is a corrosive substance, it is always recommended to use gloves and masks while handling it inside the fume hood. Nevertheless, HMDS is not the only reagent used in SEM sample preparation; many other chemicals used for this purpose are corrosive such as isoamyl acetate, glutaraldehyde and OsO_4 . Besides, no special requirement needs to be fulfilled for handling and storage of HMDS. It can be kept normally at room temperature in an amber bottle and since the boiling point is

quite high (125°C), HMDS can withstand a good amount of temperature variation. On the other hand, air drying, despite being a low-cost method is unable to preserve the natural structure of trichomes. Therefore we conclude that HMDS can be considered as a substitute for air drying and CPD during preparation of plant samples for SEM analysis.

Conclusion

From the study, it can be concluded that the quality of preservation by using CPD and HMDS are identical. Further, preservation of the delicate structures like glandular trichome indicates that HMDS can be widely used in different ranges of plant materials. This establishment of HMDS mediated drying may provide enthusiasm to many plant biologists, as use of HMDS instead of CPD saves considerable time in the sample preparation.

Abbreviations

SEM: Scanning electron microscopy; CPD: Critical point drying; CO₂: Carbon dioxide; HMDS: Hexamethyldisilazane; OsO₄: Osmium tetroxide

Declarations

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Authors' contributions:

RB: conceived and conducted chemical drying experiment, analyzed and interpreted data and wrote the draft manuscript; SS: conducted critical point drying and air drying experiment and took part in manuscript writing; OK: conducted the critical point drying experiment along with SS; LM: analyzed and interpreted data obtained from critical point drying experiment; AM: supervised the research and finalize the manuscript. All authors are in agreement with the results obtained and approved the final version of the manuscript.

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The authors declare that they have no competing interests.

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Figures

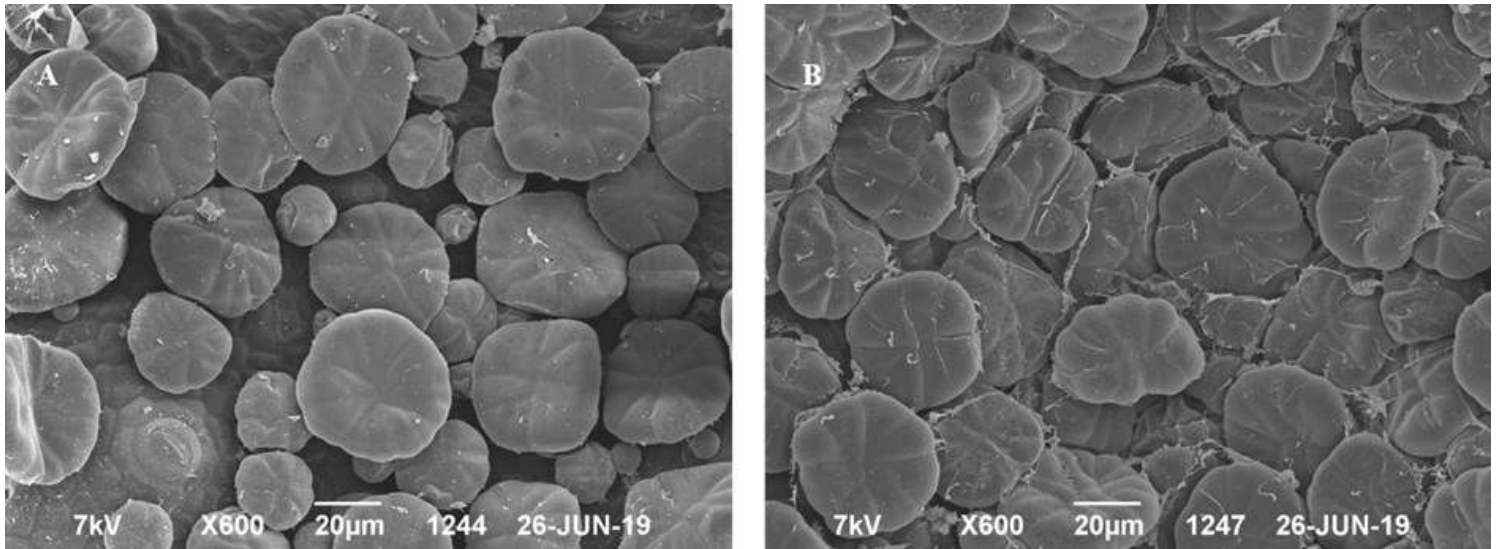


Figure 1

Scanning electron micrograph of *M. hortensis* leaf surface after critical point drying, (A) without OsO₄, (B) with OsO₄. Imaged at 7.00 kV with a magnification of X600. The scale bar represents 20 μm.

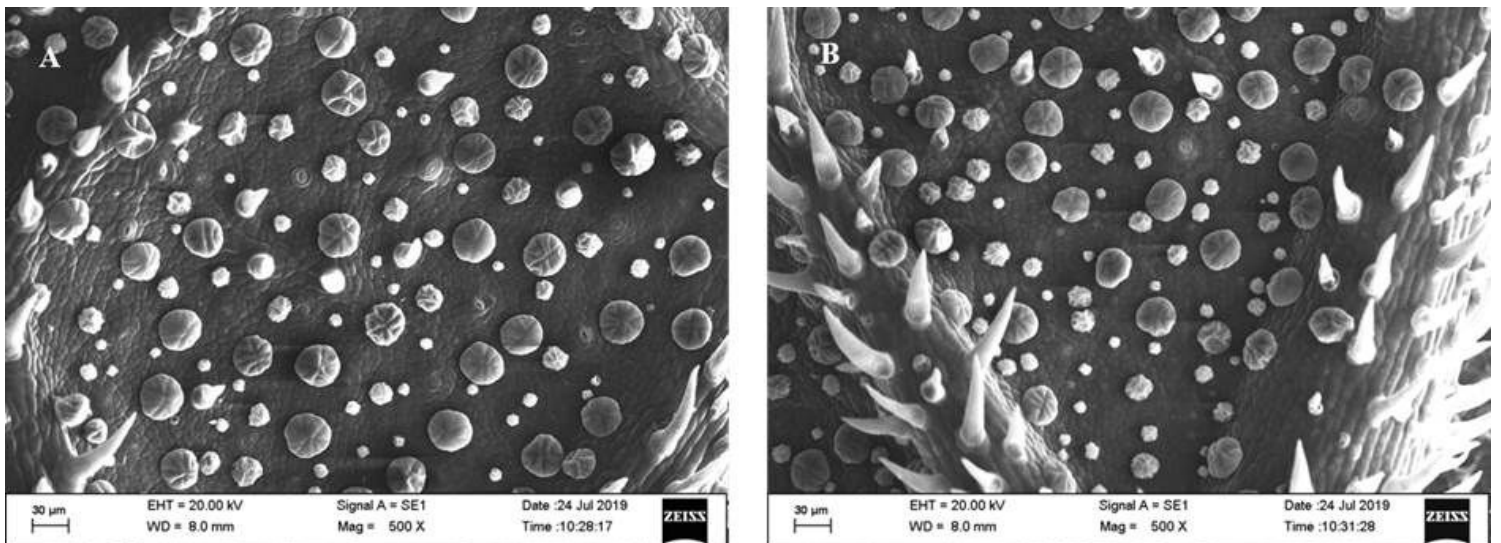


Figure 2

Scanning electron micrograph of *M. hortensis* leaf surface after HMDS based drying, (A) without OsO₄, (B) with OsO₄. Imaged at 20.00 kV with a magnification of X500. The scale bar represents 30 μ m.

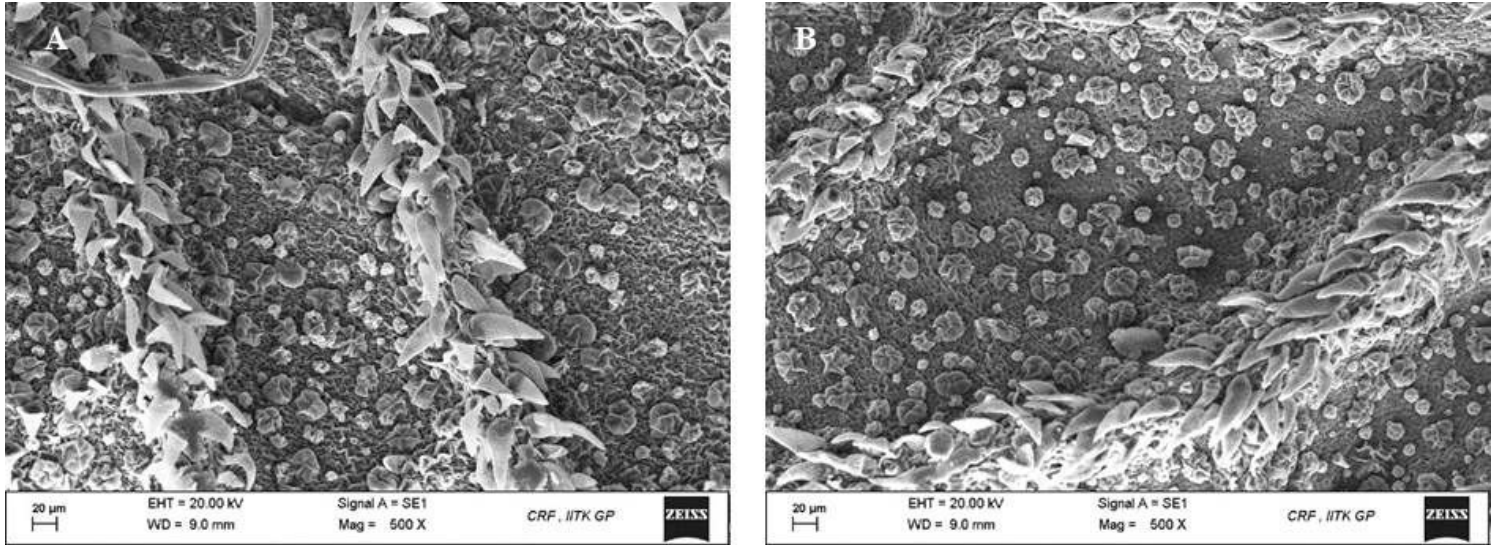


Figure 3

Scanning electron micrograph of *M. hortensis* leaf surface after air based drying, (A) without OsO₄, (B) with OsO₄. Imaged at 20.00 kV with a magnification of X500. The scale bar represents 30 μ m.