A Comparison of Herbarium Dried, Critical Point Dried and Fresh Plant Material Using High Vacuum and Variable Pressure SEM

Frieda Christie

The Herbarium at the Royal Botanic Garden, Edinburgh (RBGE), houses some three million dried plant specimens collected from all over the world. Many of these are precious because of historical connections with people such as Charles Darwin and others are valuable because they may be the only remaining records of extinct species. Dating from the 17th century, the Herbarium collection provides botanists and taxonomists with an invaluable reference aid to plant classification.

In order to study plant microstructure, botanists require microscopes and RBGE has approximately 200 light microscopes many of which can be found in the Garden's Herbarium. The Garden also has electron microscope facilities and the microscope used during the present study was a LEO Supra 55VP. This field emission scanning electron microscope (SEM) is fitted with a variable pressure chamber which permits the scanning of fresh, un-processed samples.

Variable Pressure SEM

In conventional high vacuum SEM, the vacuum is maintained throughout the column from the gun region at the top of the microscope to the specimen chamber below. However, in variable pressure SEM a differential pumping system, in conjunction with pressure limiting apertures, maintains high vacuum in the gun region while it is progressively reduced down through the column until a partial pressure of ~1 mbar is achieved in the chamber (Figure 1). Nitrogen gas is bled into the chamber and when the primary electron beam scans across the sample, some electrons will collide with gas molecules. The resulting ionisation neutralises any build up of negative charge at the surface of the sample thus reducing charging artefacts so often seen in high vacuum SEM. The secondary electrons that scatter from the surface of the sample are too low in energy to produce ionisation but when they collide with gas molecules, photons are generated. It is the photon signal which is picked up

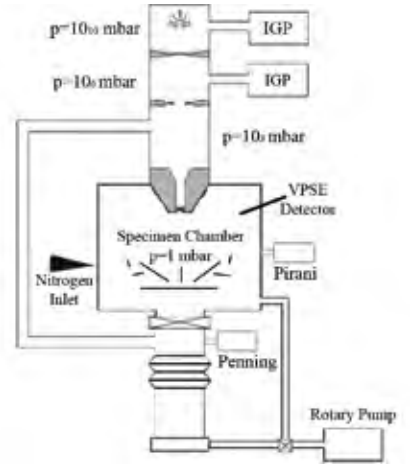

Figure 1: A simple diagram showing the pressure zones of the variable pressure SEM.



Figure 2a



Figure 2b



Figure 2c

Figure 2: Astilbe microphylla lower leaf surface. Figure 2a shows the herbarium sample where the trichomes have collapsed and the cells are distorted. There is also quite a lot of dirt on the surface. Figure 2b is the critical point dried sample and it can be seen that the trichomes have survived quite well and the stomata can be clearly seen in the background. Figure 2c shows the variable pressure sample and this material appears to be well preserved with no signs of shrinkage.



Figure 3a



Figure 3b

by the variable pressure secondary electron detector (VPSE) and amplified to produce an image (Stokes (2008)). The sample itself is cooled to ~-25°C on a peltier coolstage but is otherwise un-processed.

The study

The study compared the output from variable pressure using fresh samples with that of high vacuum SEM using herbarium and critical point dried (CPD) material. Species were selected from the Cultivated



Figure 3c

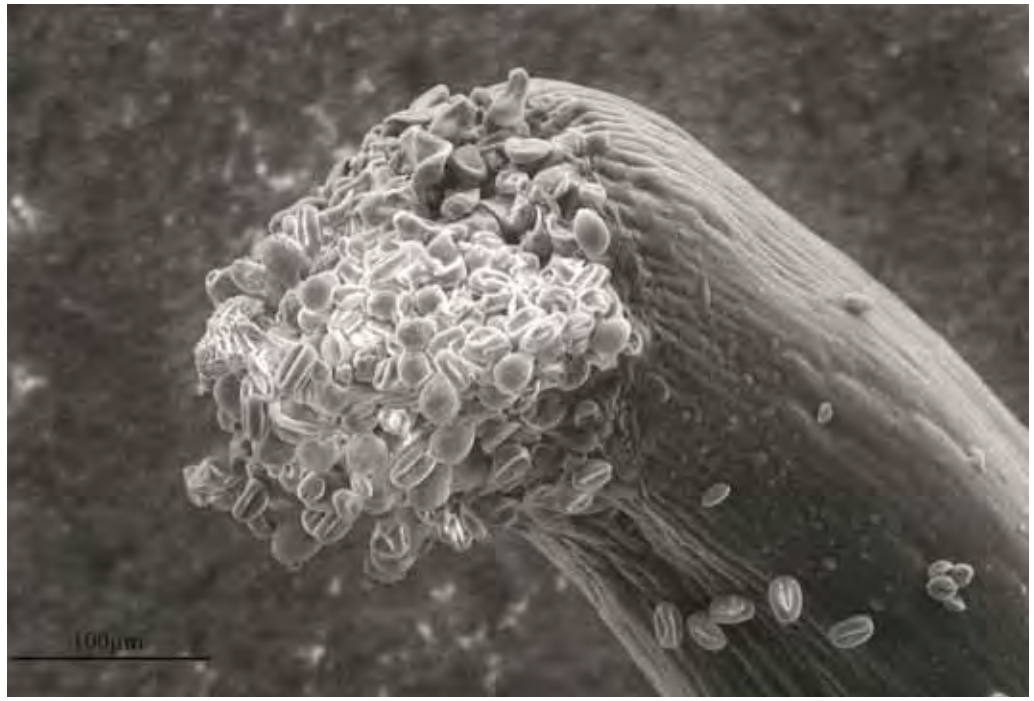

Figure 3d

Figure 3: Hypericum reptans anther and stigma. It is possible to re-hydrate and critical point dry herbarium material to obtain more information about it. However, if it has been severely damaged during the initial drying such as in Figure 3a, the additional effort may not be worthwhile. The variable pressure image in Figure 3b shows what the anther should look like. When samples are critical point dried, they retain their 3-dimensional appearance. This can make them difficult to mount as they sit proud of the conductive surface and may be prone to charging artefacts. In addition, the stigma in Figure 3c had a convoluted surface which made it difficult to sputter coat. Overall, poor conductivity led to charging artefacts as illustrated. It is possible to overcome charging by voltage reduction (changing the sample with respect to the beam or re-coating) although re-coating should be done with care to avoid the loss of surface detail. Figure 3d shows how well ionisation reduced charging on the fresh sample in variable pressure, producing a much improved image.



Figure 4a

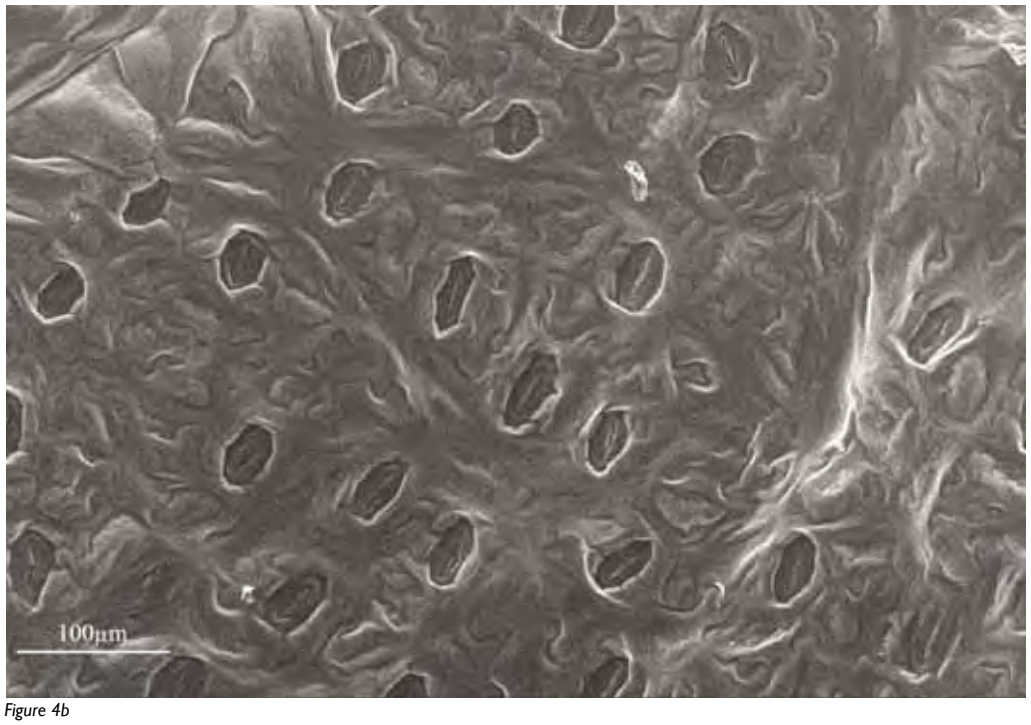

Figure 4b

Figure 4: Papavar Orientale lower leaf surface. Occasionally, samples swell when critical point dried. This is particularly true of things like pollen air sacks and is something that should be considered, particularly when looking at unfamiliar material. It was suspected that the 'bloated' appearance of the critical point dried material in Figure 4a might be an artefact. This was confirmed by comparing it with the fresh sample, Figure 4b, where the cells were not swollen.

List which is a list containing plant species with both herbarium samples and fresh material in the RBGE living collection. The main criteria were that the samples had to be delicate in order to test the effects of each method and there had to be living material available from the Edinburgh collection so that samples could be taken directly from the plant to the SEM. The first batch of material studied was from herbarium samples, that is plant material which had been pressed and dried at between 34 and 41°C. Samples were mounted on aluminium stubs using carbon adhesive discs and platinum sputter coated for 1 minute using an Emitech K575X sputter coater. The second batch comprised of fresh material which had been fixed overnight in formyl acetic alcohol (FAA); critical point dried using an Emitech K850 critical point dryer; mounted and sputter coated as before. The third batch was fresh material, mounted and taken directly to the microscope. Images were taken as 3 Mbyte TIFF files from dried material at 9 – 14 mm working distance at 3 – 5 kV and from fresh material at 4 – 5 mm working distance at 15 – 20 kV. Dried samples were retained after scanning but those scanned using variable pressure were disposed of once recorded.

Results Summary

Herbarium Material

Overall, the herbarium material was in a very poor condition. In many cases, it was dirty and delicate structures such as glands and fine hairs were destroyed. In some cases pollen collapsed; surfaces appeared shrivelled and it was often difficult to see details such as stomatal patterns. Only the toughest structures survived the herbarium drying process.

Critical Point Dried Material

CPD material was generally well preserved with glands and fine structures intact, although in some cases cells appeared swollen and distorted. The samples were cleaner than those of the herbarium material probably due to rinsing during the fixation and dehydration processes. Mounting critical point dried samples was sometimes problematic because, unlike flattened herbarium material, 3-dimensional structures can be difficult to anchor onto the stub. Sputter coating of both CPD and herbarium samples might also have been an issue as there is always the risk of introducing artefacts due to heat or over-coating.

Variable Pressure Material

Fresh material viewed in variable pressure mode was generally well preserved. There was no sign of shrinkage or distortion and delicate structures were intact. There was also less evidence of charging artefacts due to charge compensation at the surface.

Discussion

From a technical point of view, there are several considerations when choosing the mode of SEM scanning. Samples scanned under high vacuum are mounted on a stage plate that can be freely rotated and tilted. A full range of working distances and energy levels are available so the microscope can be set up to deliver first class resolution throughout the magnification range.

Variable pressure scanning is more restrictive in that the stage cannot be rotated because it is attached to a peltier cooling unit, required for botanical samples and there is only limited tilt. Also, variable pressure works best at higher energy levels, in the range of 20 – 25 kV, and shorter working distances are required. This limits the options for setting up the microscope according to sample type. The signal to noise ratio is generally lower in variable pressure because beam scattering in the residual gas atmosphere increases the level of background interference. To compensate for this, long dwell times and the use of look up tables may be needed to improve the quality of the final image.

When it comes to deciding which technique to use this may depend upon the availability of material. The vast herbarium collection at RBGE includes examples of precious plant specimens some dating back many years. Where this is the only material available it may yield some useful information even if scanned un-coated in high vacuum at low kV. Where possible, however, fresh material is obtained from the Garden's living collection. The choice is then whether to critical



Figure 5a



Figure 5b

point dry the sample or observe it directly using variable pressure. Again, the decision depends upon the availability of fresh material; how delicate it is and whether the highest resolution images are required. Where there is a limited supply, it is necessary to dry using CPD in order to preserve the material for future use. However, where material is abundant or particularly delicate and the highest resolution is not required, variable pressure is the preferred option.

This study has shown that while the variable pressure technique has certain limitations, it does offer a unique opportunity to visualise plant material in its natural state and provides a valuable tool for the study of plant microstructure.

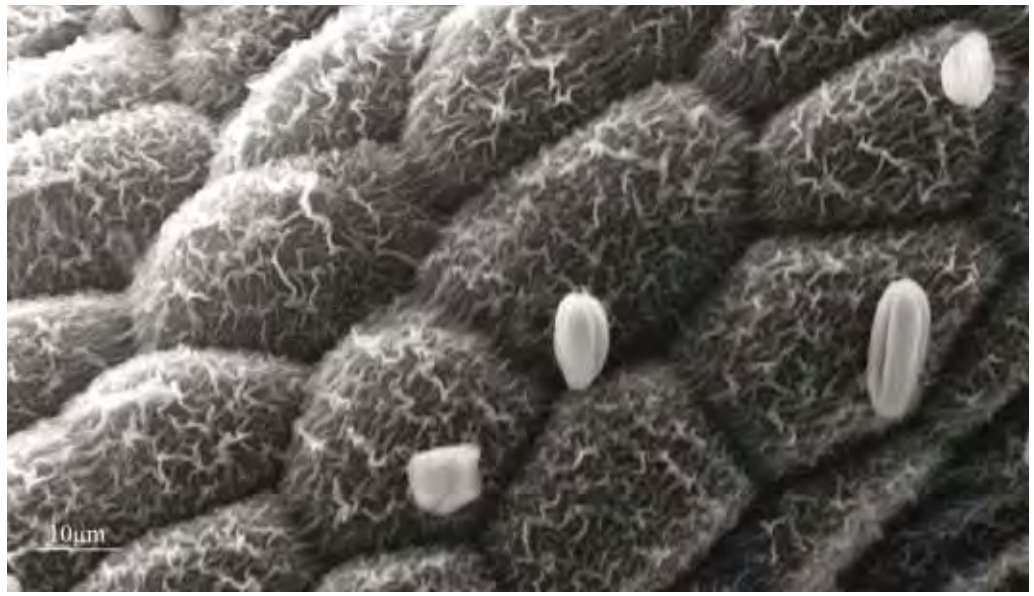

Figure 5c

Figure 5: Begonia tricornis anther. When looking at the Begonia tricornis anther it became obvious that neither herbarium Figure 5a, nor critical point drying Figure 5b, were suitable methods for preserving the delicate surface structure clearly seen in the variable pressure image Figure 5c.

Summary

The dried botanical specimens housed in the Herbarium at the Royal Botanic Garden, Edinburgh, provide a valuable reference collection for the identification and study of plants. However, the methods used to preserve this material often damage it so whenever possible, fresh samples are obtained from the Garden's extensive living collection. When prepared for SEM, fresh material is normally critical point dried although increasingly fresh, un-processed samples are scanned using the variable pressure facility on the Garden's LEO Supra 55VP SEM. I wished to compare the output from the SEM using material from the three sources and assess the value of variable pressure technology for the study of plant microstructure.

Reference

Stokes, Debbie J., Principles and Practice of Variable Pressure/Environmental Scanning Electron Microscopy (VP-ESEM), (2008) RMS John Wiley & Sons Ltd UK
See: www.rms.org.uk/books

Frieda Christie
Royal Botanic Garden, Edinburgh, UK
f.christie@rbge.org.uk



Frieda manages the Electron Microscope Suite and oversees the use of some 200 light microscopes at the Royal Botanic Garden, Edinburgh. Her SEM images have been published in a number of botanical journals and websites. They have also appeared in calendars and on the cover of the Journal of Microscopy.

In July 2000, East Lothian Council funded an exhibition of her work at North Berwick Museum as part of their Millennium Celebration of Science and Nature. The exhibition toured various venues throughout Scotland, including the Kelvingrove Art Gallery and Museum in Glasgow.

Frieda has won prizes in the RMS Micrograph Competition and Visions of Science competitions. She has also collaborated with local artists on several science and art projects.