

Investigation of Plant Surfaces with Environmental Scanning Electron Microscopy (ESEM®) – A Comparison with Conventional SEM

Dagmar KOLB, Edith STABENTHEINER*

Institute of Plant Physiology, Karl - Franzens University Graz, Schubertstraße 51,
A-8010 Graz, Austria

*corresponding author (e-mail: edith.stabentheiner@uni-graz.at)

Abstract. Environmental scanning electron microscopy (ESEM) enables the investigation of untreated and watercontaining material without preparation with the benefit of SEM (depth of focus and three dimensional imaging of surfaces with a high resolution). Conventional SEM (CSEM) usually requires time consuming fixation, drying and coating of samples. Their surface structures may be altered by this procedure. For comparison a large number of plant samples was observed with both methods. Using CSEM, secretion products or mucilaginous coatings may be removed and dynamic processes cannot be observed. However, the samples can be investigated several times. In contrast, ESEM allows the observation of watercontaining, native surfaces and this method is the only possibility to watch dynamic processes in the SEM. However, using ESEM the plant material is very sensitive to beam damages because of the lack of the protecting metall layer - necessary for non-conducting surfaces in CSEM and dehydration cannot be prevented completely. In summary, ESEM will not compete with CSEM but it will establish oneself as a valuable and essential supplement in studying plant surfaces.

Key words: environmental scanning electron microscopy (ESEM), conventional scanning electron microscopy (CSEM), plant surfaces

Introduction

Scanning electron microscopy (SEM) became an indispensable tool in studying plant surfaces. For conventional SEM (CSEM) biological samples usually have to be fixed, dehydrated and coated (ROBINSON & al. 1987, DYKSTRA 1992).

The environmental SEM (ESEM) allows the observation of many types of specimens without subjecting them to conventional preparation techniques (DANILATOS 1993). This is possible because of a pressure limiting aperture with high vacuum maintained in the beam-generating and - focusing part of the column ($\sim 10^{-4}$ Pa in the gun area), while low vacuum (up to 10^2 – 10^3 Pa) is tolerated in the specimen chamber (BOZZOLA & RUSSEL 1992, DYKSTRA 1992, DANILATOS 1993). The secondary electrons emitted from the sample collide with water molecules in the chamber so as to produce additional electrons and positive ions. The positive ions are attracted to the sample surface and eliminate charging artefacts. This ionization process results in a proportional cascade amplification

of the original SE signal which is detected by a special gaseous secondary electron detector (DANILATOS 1993, TAI & TANG 2001). As a consequence, unfixed and uncoated samples – even those containing considerable amount of water – can be investigated by SEM.

To compare CSEM and ESEM various plant samples were investigated on the one hand after fixation, drying and coating and on the other hand without any preparation at differing ESEM conditions.

Material and Methods

A large number of different plant samples was investigated using the following microscope conditions (KOLB 2002):

CSEM: conventional SEM with high vacuum ($\sim 10^{-4}$ Pa) in the chamber; sample preparation: chemical fixation (e.g., glutaraldehyde), dehydration, critical point drying with CO_2 as drying agent, sputtercoating with gold (ROBINSON & al. 1987).

ESEM: a) sample temperature 5°C (Peltier cooling stage); gaseous secondary electron detector; chamber pressure 133–930 Pa; relative humidity: up to 100 % (Table 1); no sample preparation. b) samples at room temperature (without cooling); gaseous secondary electron detector; chamber pressure 133–670 Pa; relative humidity < 20 % (Table 1); no sample preparation. c) large field gaseous secondary electron detector (pressure limiting aperture with wider diameter than a) and b); pressure in the chamber maximally 133 Pa; relative humidity < 10 % (Table 1); no sample preparation.

All samples (CSEM, ESEM) were mounted on aluminium stubs with double sided conductive tape and were investigated at different microscope conditions with a Philips XL30 ESEM using an acceleration voltage of 20 kV.

Table 1: Values show chamber pressure in Pa, corresponding relative humidity (%) at sample temperatures ($^\circ\text{C}$) from 0° to room temperature (25°C).

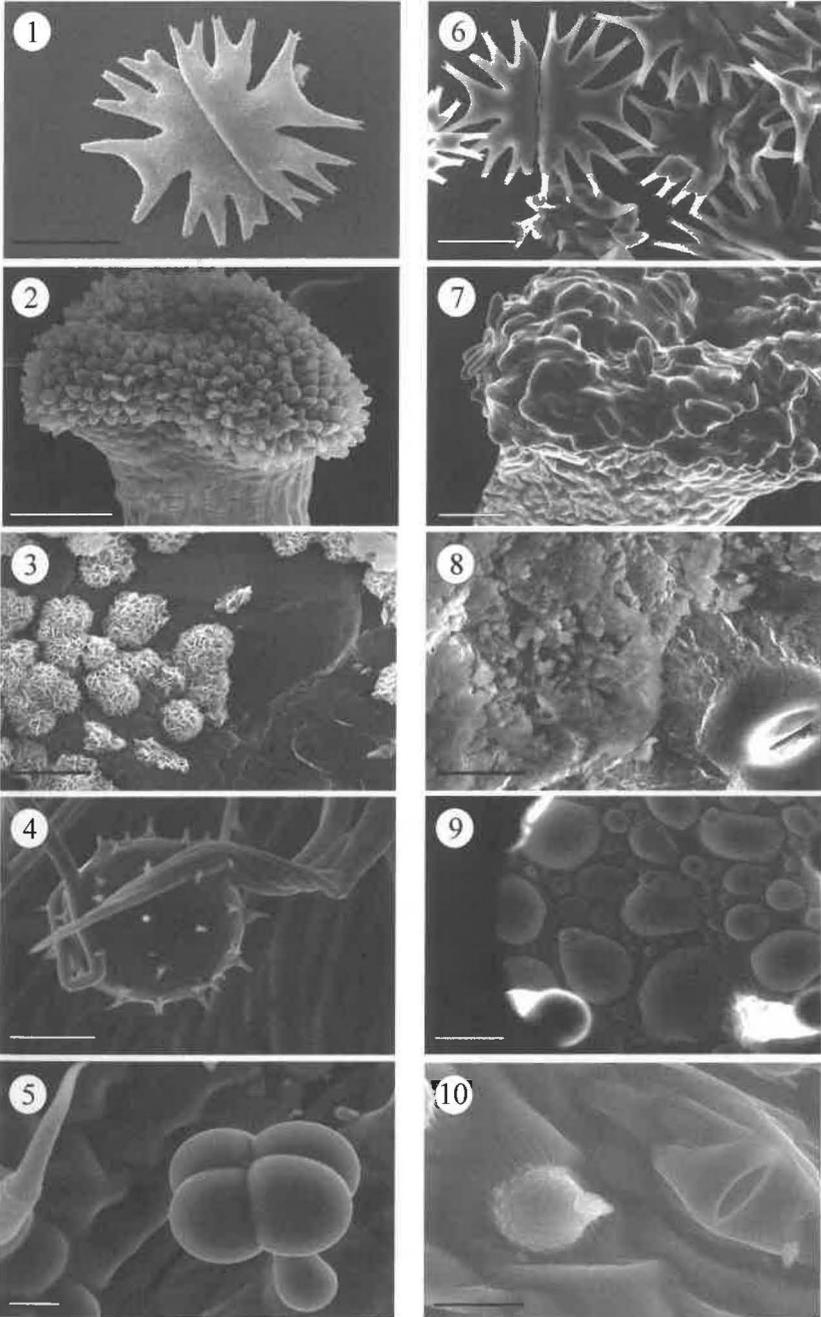
RH	100 %	80 %	60 %	40 %	20 %	10 %
Temperature						
0°	612	480	360	239	120	67
5°	865	692	519	346	173	93
10°	1224	971	732	492	239	120
15°	1702	1357	1024	678	346	173
20°	2328	1862	1397	931	466	239
25°	3152	2527	1889	1264	625	319

Results and Discussion

The environmental scanning electron microscope (ESEM) allows the observation of many types of specimens without subjecting them to conventional preparation techniques because it allows the introduction of a gaseous environment in the specimen chamber (DANILATOS 1993, TAI & TANG 2001). To compare CSEM and ESEM a great many different plant samples were investigated on the one hand after fixation, drying and coating and on the other hand without any preparation at differing ESEM conditions.

All samples – without any restrictions – could be investigated with conventional SEM (CSEM) using chemical fixation followed by dehydration, drying and coating procedure (Figs. 1–3). Leaves and shoots of plants are easy to handle during preparation. Very small samples, e.g. unicellular algae (Fig. 1, *Micrasterias sp.*) were attached to cover slips with poly-L-lysine prior to the preparation procedure (ROBINSON & al. 1987). Generally, no artefacts due to beam damage or due to insufficient

electrical (charging effects) and/or thermal conductivity occurred. Samples can be stored for a long time under appropriate conditions (dry and clean atmosphere) and can be investigated as often as necessary (CRANG 1988, DYKSTRA 1992).



Figs. 1–3 CSEM samples. Fig. 1: *Micrasterias* sp. bar = 50 μm ; Fig. 2: a stigma of *Setcreasea purpurea* with its stigmatic hairs bar = 200 μm ; Fig. 3: a preparation artefact of a calcareous crust surface of *Saxifraga kolenitiana* bar = 10 μm ; Figs. 4–10 ESEM samples. Fig. 4: a pollen grain interaction with stigmatic hairs bar = 50 μm ; Fig. 5: a turgescent trichome of *Lycopersicon esculentum* bar = 20 μm ; Fig. 6: a native state of *Micrasterias* sp. bar = 50 μm ; Fig. 7: a stigma of *Setcreasea purpurea* with mucus bar = 100 μm ; Fig. 8: the native state of a calcareous crust of *Saxifraga kolenitiana* bar = 20 μm ; Fig. 9: water droplets on the surface of *Drosera rotundifolia* bar = 100 μm ; Fig. 10: an example of surface dehydration and charging effects of *Melissa officinale* bar = 20 μm .

In contrast, ESEM allows the observation of biological samples in their natural state and without coating (Figs. 4–8; DANILATOS 1993, TAI & TANG 2001, YAXLEY & al. 2001). Typically, the first steps in sample preparation for CSEM are fixation and dehydration (ROBINSON & al. 1987). However, these steps often result in a removal of surface coatings (CRANG 1988). In Fig. 7 the mucilaginous coating on a stigma of *Setcreasea purpurea* can be observed investigating fresh samples (ESEM), whereas this coating is removed after preparation for CSEM (Fig. 2). However, the stigmatic hairs are hidden on the fresh surface and can only be investigated in detail after sample preparation. So both methods complement one another. A well known artefact due to dehydration is shrinkage of up to 40 % of the original volume (CRANG 1988). This can be clearly demonstrated here when comparing the sample with (Fig. 2, bar = 200 μm) and without (Fig. 7; bar = 100 μm) preparation. Only ESEM allows the investigation of the close interactions between pollen grains and stigmatic hairs of *Hibiscus* sp. (Fig. 4).

Besides the removal of coatings surface deposits can be modified. In Fig. 8 the native state of the calcareous crust on leaves of *Saxifraga kolenitiana* can be observed while in Fig. 3 the structure of the crust was altered due to sample preparation.

Investigating biological samples containing a considerable amount of water a cooling stage helps to control the temperature of the specimen and thus the relative humidity, which is a strong function of the temperature (Table 1; DANILATOS 1993). Cooling enables the maintenance of a high relative humidity on the sample surface (Table 1). As a consequence dehydration is prevented and even delicate plant structures can be observed in their native state for up to 60 minutes (Figs. 4–8). Different types of plant hairs can be easily observed without any preparation (Fig. 5). Even very sensitive algae as *Micrasterias* sp. (Fig. 6) can be investigated when sufficient water supply from a wet filter paper or agar is ensured. However, care has to be taken that chamber conditions are controlled in a way that humidity on the sample surface is high enough to stop dehydration but not too high to produce water droplets on the surface making it invisible (Fig. 9). The following conditions turned out to be optimal for the investigation of wet samples: 5°C sample temperature and approximately 640 Pa vapour pressure. These conditions are very similar to those found by TAI & TANG 2001. The possibility to control relative humidity enables the direct investigation of dynamic processes on plant surfaces, e.g. dehydration and rehydration cycles as it was done investigating the swelling behaviour of cellulose fibres (JENKINS & DONALD 1997).

However, if not undisturbed samples (e.g., whole leaves) but sliced samples are used dehydration can not be completely prevented. Shrinkage and charging are the consequence (Fig. 10).

ESEM without cooling results in quite low relative humidities on the sample surface (Table 1). Many plant structures, especially those equipped with thick cell walls and cuticles, can be investigated in this mode giving the same results as cooling the samples. Investigation time, however, is much shorter and dehydration occurs much faster.

In contrast to CSEM, wet samples can only be used once – for further investigations a new sample is necessary. The samples are more easily damaged by beam current and accelerating voltage since no coating is present that ensures sufficient thermal conductivity and stabilization of the surface (CRANG 1988). Another disadvantage of ESEM is a reduced field of view at lower magnifications

due to the pressure limiting aperture (Fig. 9). This can be overcome using the large field gaseous secondary electron detector (FEI). However, due to the wider diameter of the aperture the maximal chamber pressure is restricted to 133 Pa and as a consequence the relative humidity on the sample surface is rather low (Table 1). So the same restrictions for fresh samples as stated above goes for this mode of ESEM. The mentioned restrictions are not applied to dry and stable samples (e.g., wood, insects).

Conclusions

ESEM represents a step forward in the instrumentation of electron microscopy and it allows access to areas of research not previously possible (DANILATOS 1993). However, it will not compete with CSEM but it will establish oneself as a valuable and essential supplement in studying plant surfaces.

Literature

- BOZZOLA J. J. & L. D. RUSSEL 1992: *Electron Microscopy*. Jones and Bartlett Publishers, Boston-London.
- CRANG R.F.E. 1988: Artifacts in specimen preparation for scanning electron microscopy. In: *Crang R. F. E. & K. L. Klomparens* (eds.): *Artifacts in Biological Electron Microscopy*, Plenum Press, New York - London pp. 107-129.
- DANILATOS G. D. 1993: Introduction to the ESEM instrument. *Microscopy research and Technique* **25**: 54-361.
- DYKSTRA M. J. 1992: *Biological Electron Microscopy*. Plenum Press, New York-London.
- KOLB D. 2002: *Untersuchungen an frischem und fixiertem Pflanzenmaterial mit Hilfe des Rasterelektronenmikroskops*, Diplomarbeit, Karl - Franzens - Universität Graz.
- JENKINS L. M. & A. M. DONALD 1997: Use of the Environmental Scanning Electron Microscope for the Observation of the Swelling Behaviour of Cellulosic Fibres, *Scanning* **19**: 92-97.
- ROBINSON D. G., U. EHLERS, R. HERKEN, B. HERRMANN, F. MAYER & F.-W. SCHÜRMMANN 1987: *Methodes of Preparation for Electron Microscopy, An Introduction for the Biomedical Sciences*, Springer - Verlag Berlin Heidelberg New York.
- TAI S. S. W. & X. M. TANG 2001: Manipulating Biological Samples for Environmental Scanning Electron Microscopy Observation. *Scanning* **23**: 267-272.
- YAXLEY J. L., W. JABLONSKI, J.B. REID. 2001: Leaf and flower development in pea (*Pisum sativum* L.): mutants *cochleata* and *unifoliata*. *Annals of Botany* **88**: 225-234.