Short Note

Biological specimens and scanning electron microscopy

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THE study of biological specimens under scanning electron microscope requires pre-investigation procedures such as fixation, dehydration and conduction. Generally, soft tissues are not able to sustain high vacuum created during the sample preparation and observation hence care has to be taken to maintain the topography of the plant/animal tissue in its natural state. In order to meet out the above requirements, the sample has to be properly fixed and dehydrated so that the cellular organization does not change. It is generally observed that the cellular features of the plant tissues undergo deformation and create artifacts when processed for conventional scanning electron microscopy (CSEM). In recent years, more advanced techniques have been brought forward in the field of microscopy where the delicate cellular organization can directly be observed without undergoing the cumbursome procedure. In order to achieve quick results, SEM has been provided a natural state of environment where the tissue could retain its original features, i.e. the establishment of environmental scanning electron microscope (ESEM). Here the specimen can be viewed without undergoing any sample preparation process. The data achieved by ESEM can reflect the characteristics of the specimens in its natural form.

Sample Preparation (Fresh Non-conductive Samples)

Specimens in an electron microscope are exposed to a high vacuum pressure generally in the range of 10^{-4} to 10^{-6} torr. If a wet specimen is introduced to such vacuum, its water contents evaporate and the specimen becomes dry. Therefore, the following precautions should be taken before observing the specimens under SEM-

a) The sample must be dry, non-volatile, should not emit gases and be non-contaminating.

b) The hydrous biological specimens do need extensive sample preparations like chemical drying, freeze-drying as well as cryo-fixation etc.

c) The sample must be conducted and coated with conducting material.

Chemical Fixation

A wide variety of chemical fixatives for biological tissues have been recommended for scanning electron microscopy (Hayat 1970, 1972, Haywood 1971, Hall 1978, Goldstein 1992, Johnson *et al.*, 1993). The fixatives commonly employed include:

a) 1.5% to 3.0% glutaraldehyde prepared in 0.5 or 1.0 M sodium phosphate or sodium cacodylate buffer (pH 7.2 to 7.4). Karnovsky's (1965) paraformaldehyde – glutaraldehyde fixative prepared using one of the buffers already indicated. The fixative has also been used at dilutions of one-half to one-fourth of the full strength mixture depending upon the osmolality of the particular tissue to be studied.

b) 1% or 2% osmium tetraoxide in 0.5 or 0.1 M sodium phosophate or sodium cacodylate buffer (pH 7.2 to 7.4).

c) A "cocktail" fixative containing both glutaraldehyde and osmium tetraoxide has been employed to retain structural details in biological specimens.

The mixture frequently used consists of cold 1.25% glutaraldehyde and 2% osmium tetraoxide in 0.1 M sodium cacodylate buffer (pH 7.2 to 7.4).

Dehydration

The tissues are dehydrated through a graded series of ethanol (30%, 50%, 70%, 85%, 95% for 5 to 10 minutes each, and then in absolute ethanol with several changes). The time taken for dehydration varies according to the nature and size of the sample, and finally critical point drying using liquid carbon dioxide is done (Anderson, 1951).

Critical Point Drying (CPD)

For CPD the specimens are transferred to the drying chamber with just enough absolute alcohol or acetone to prevent air-drying prior to CPD. The cover of the specimen chamber is secured and liquid carbon dioxide is admitted to the chamber from tank through a siphon. Use of liquid carbon dioxide is controlled so as to change from gaseous phase without causing any damage on the surface morphology of the specimen. Specimens dried by this method are mounted on the stubs using double sided sticking tape and conducted by silver paint, finally coated by Au or Au/Pd alloy.

Investigation of Samples Under CSEM

a) *Samples prepared under standard method* – It has been possible to observe the features of the delicate tissues such as epidermis with stomata, trichomes and glands etc. It has been possible that the features of different plant cuticles could be resolved at any level (Figs. 1-3).

b) *Samples not prepared by standard method* – The sample not prepared carefully cause certain problems during observations, such as charging effect which create bright flashes on the screen (CRT) and also cause burst areas in the sample. It also creates deflection in the incident beam allowing only low energy secondary electrons which do not allow formation of a proper image (Figs 4-6). Sometimes the cellular deformities may also be caused in the specimens due to moisture trapped in the cells (Figs. 7-9).

The conventional SEMs (CSEM) have superior resolution, depth of field and microanalytical capabilities and also have number of limitations. Almost all of these limitations derive from high vacuum a CSEM must maintain in the specimen chamber. The column requires a high vacuum in order to generate and focus the electron beam. The sample requires a high vacuum to permit the use of available secondary electron detectors. To overcome these difficulties Environmental Scanning Electron Microscope (ESEM) has been developed in recent years. The benefits of ESEM over the CSEM are as follows :–

The gas ionization in the sample chamber eliminates the charging artifacts typically seen with non-conductive samples. ESEM can image wet, dirty, oily, out-gassing samples. The contamination does not damage the instrument or degrade image quality, the secondary detector is insensitive to heat. It can acquire electron images from samples as hot as 1500°C. It can be incandescent, fluorescent as well as cathode luminescent sample without interference. Delicate sample can directly be seen under ESEM without

PLATE 1 →

Images formed under standard sample preparation method:

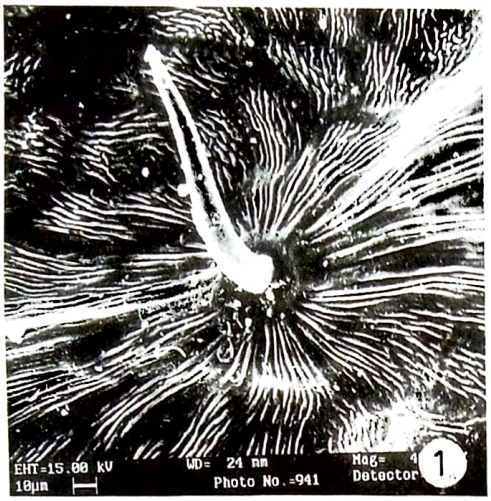
1. Granulated single trichome with ridges and other striated epidermal cells.
2. Finger–like projected structures of epidermal cells.
3. Stomatal complex with epidermal cells.

Deformation of cellular structure and charging effect in the samples due to trapped moisture:

4. Trichomes and glands on the epidermal surface, brightness effect due to charging of sample.
5. Single ornamented trichome, the base and surface of trichome is not clear because of charging effect.
6. Cellular deformity due to shrinkage of epidermal cells, trichome.
7-9. Cellular details are not clear due to high electron charging.

Sample investigated under ESEM (without fixation, dehydration and coating)

10. Ornamented trichome and epideremal cells.
11. Striated gland on the leaf surface.
12. Trichomes and glands on leaf surface.


EHT=15.00 kV
10µm
WD= 24 mm
Photo No.=941
Mag= 4
Detector
1

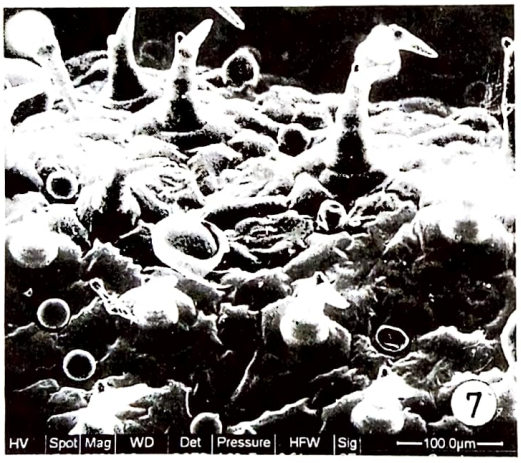

2


WD= 27 mm
Mag= 1.15 K X
3


HV Spot Mag WD Det Pressure HFW Sig
100.0µm
4


Mag WD Det Pressure HFW Sig
5


10µm
Mag= 574 X
6


HV Spot Mag WD Det Pressure HFW Sig
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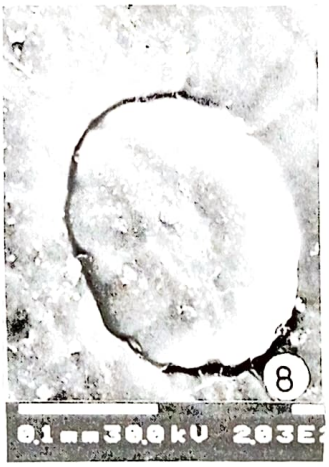
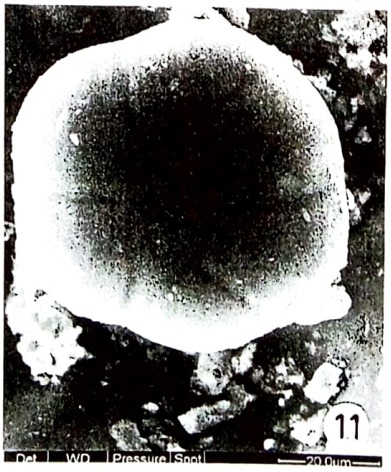

8


EHT=15.00 kV
20µm
Mag= 493 X
9


Spot Mag WD Det Pressure HFW Sig
50.0µm
10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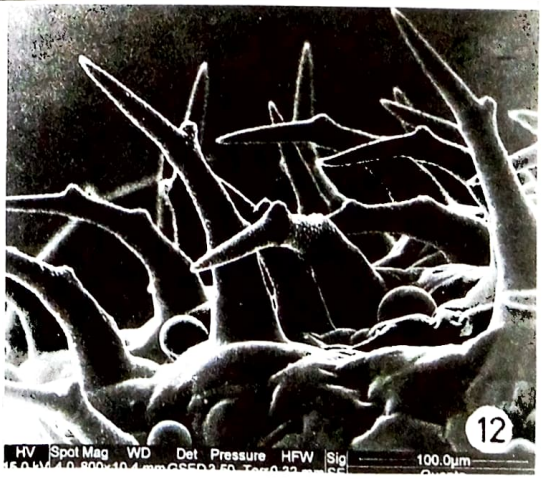
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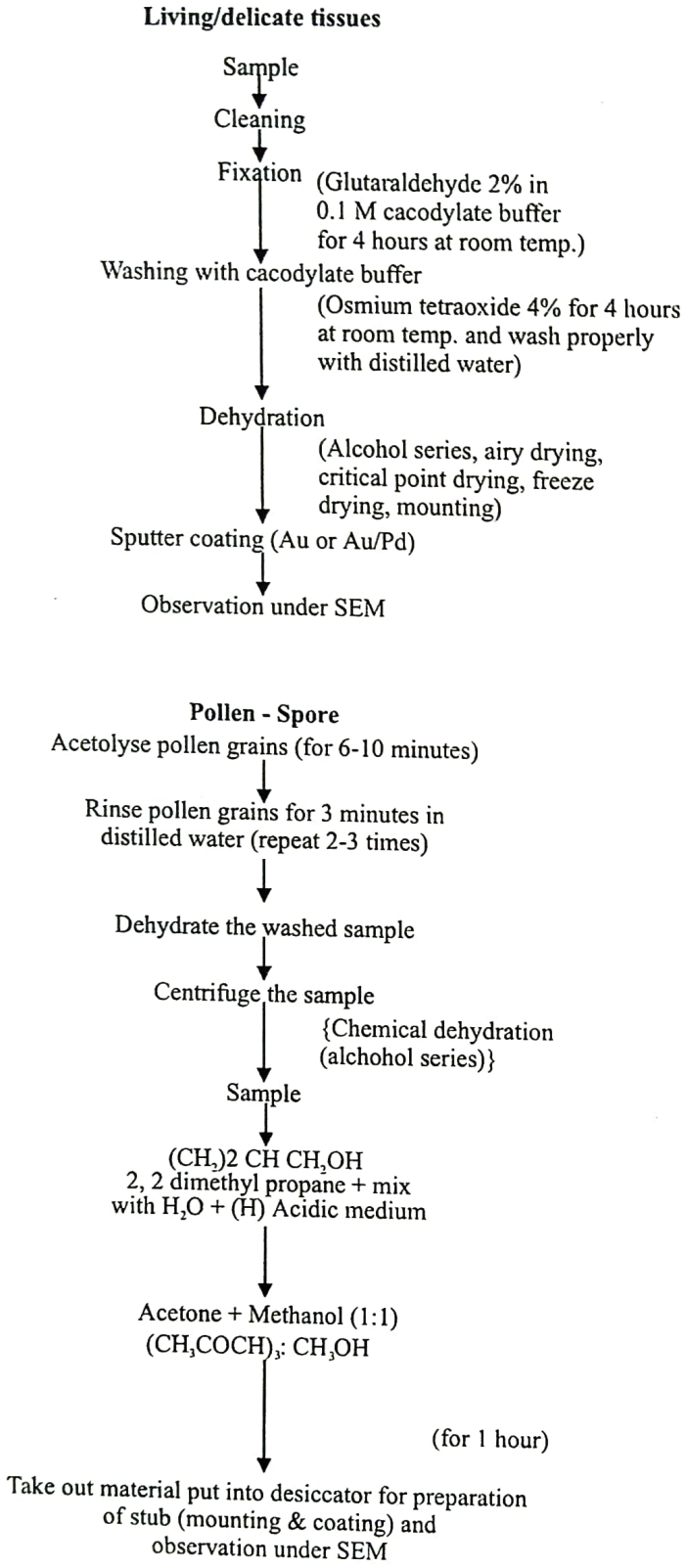
12

PLATE 1



coating and sample preparation (figs 10-12). Wet samples need not be dried before viewing in the ESEM. This is specially important for specimen that must remain hydrated in order to retain their structure (bacteria, algae, fungi, trichomes, glands, etc.).

Under such system ESEM also provides us instant results without undergoing elaborate sample preparation.

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