Fast and simple biological sample preparation and observation procedure for scanning electron microscopy

C. R. Salgado Laurenti[a]* & M. C. Galíndez [a]

[a]Servicio de Microscopía Electrónica de Barrido, Secretaría General de Ciencia y Técnica, Universidad Nacional del Nordeste, Juan Bautista Cabral 2001, Campus Sargento Cabral, W3402 BKG Corrientes, Argentina

*Corresponding author, E-mail: meb.unne@hotmail.com.

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ABSTRACT

The aim of this work is to show options for preparing, observing, recording images of biological samples and provide recommendations on optimal procedures for each specimen. Some basic concepts are developed to take into account when preparing and observing biological samples of different origin. Some experiences carried out in the Scanning Electron Microscopy Service of UNNE are described. The procedures to observe, analyze and record morphological details of different biological sample types are explained. According to the fragility of the sample, some working conditions are recommended.

Keywords: animals; fungi; insect; Jeol 5800LV; plants.

Técnicas rápidas y simples de procesamiento y observación de muestras biológicas para microscopía electrónica de barrido

RESUMEN

El objetivo de este trabajo es mostrar opciones para preparar, observar, grabar imágenes de muestras biológicas y brindar recomendaciones sobre los procedimientos óptimos para cada espécimen. Se desarrollan algunos conceptos básicos a tener en cuenta a la hora de preparar y observar muestras biológicas de diferente origen. En el texto se describen algunas experiencias realizadas en el Servicio de Microscopia Electrónica de Barrido de la UNNE. Se describen los procedimientos para observar, analizar y registrar detalles morfológicos de diferentes tipos de muestras biológicas. Según la fragilidad de la muestra se recomiendan algunas condiciones de trabajo.

Palabras claves: animales; hongos; insectos; Jeol 5800LV; plantas.

INTRODUCTION

Scanning electron microcopy (here in after SEM) techniques are complementary to the ones of light microscopy. SEM has many applications in the study of a great variety of samples in life and material sciences. There are many reference texts that develop in vast detail the different aspects related to scanning (e.g. [1], [2],[3],[4]).

In Argentina, a wide background of biological sample preparation for light microscopy (e.g. [5]) and scanning electron microscopy (e.g. [6]) was published.

Since 2008 Argentina has a National Microscopy System (SNM for its initials in Spanish) (Resolution 556/08 and 631/09) that gathers information about available technologies and services attached to this system (https://www.argentina.gob.ar/ciencia/sistemasnacionales /microscopia). Nowadays, the Ministry of Science and Technology of the Nation has a total of 12 National Systems (SN for its initials in Spanish) (www.argentina.gob.ar/ciencia/sistemasnacionales).

Electron microscopy has certainly developed in our country in the past years. At present, there are 236 microscopy equipments, 23% corresponds to SEM (SEM, SEM dual beam), 12% to TEM (Transmission Electron Microscopy) and STEM (Scanning Transmission Electron Microscopy), 1% to EPMA (Electron Probe

Microanalysis) and 64% to high resolution CM (Confocal Microscopy).

The highest concentration of equipment is found in the central region of the country. The latest acquisitions were made in 2015 through an Equipment Modernization Project (PME for its initials in Spanish).

The technology park has mostly SEM with a traditional tungsten source, the most modern use lanthanum hexaboride (LaB_6) as a source and others have FEG (Field Emission Gun).

Universidad Nacional del Nordeste (UNNE) owns a SEM Jeol 5800LV. It was acquired in views of strengthening investigation projects in the university. The Service has been in operation since 1997 and currently has 148 users and several research projects are being developed.

The processing and observation of some experiences carried out in the laboratory of the SEM Service of UNNE were published [7], [8], [9], [10], [11].

The aim of this work is to show options for preparing, observing and recording images biological samples and provide recommendations on optimal procedures for each specimen.

MATERIALS AND METHODS

Samples used for this article were mainly plants and some invertebrate animals. Also an example of fungus was processed. Different treatments were applied, according to each sample properties. All micrographs were obtained with high vacuum mode in SEM JEOL 5800 LV (Table 1). Sample analysis and observations were also carried out taking into consideration their particularities, changing when necessary the working distance (WD) and accelerating voltage (kV).

Table 1. Technical characteristics of SEM JEOL 5800LV.

Origin and year of manufacture	Japan, 1997
Emission source	Tungsten
Detectors	Secondary Electrons (SE) and Backscattered Electrons (BSE)
Resolution	3.5 nm (WD 8 mm, Accv 30 kV, SE)
Magnification (Min - Max)	x18 - 300,000
Working distance (Min - Max)	8 – 48 mm
Accelerating voltage (Min – Max)	0.3 – 30 kV

Sample selection and preparation

Studied materials

To examine biological samples under a conventional SEM the standard procedure is the following: chemical fixation, dehydration/critical point drying, mounting on a stub and coating with a metal (e.g. gold, platinum, etc.) [12]. Biological samples are diverse, with the aim of showing procedures employed in various examples, Table 2 is presented.

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Table 2. Materials and processing methods applied.

Sample type: herbarium specimen		
Species	**Procedure**	**Estructure observed**
Plants *Cucurbita maxima* Duchesne	Mounted directly with double-sided tape. Hydration – Dehydration - Critical point drying – Mounting with double-sided tape.	Glandular and unglandular trichomes.
Sample type: fresh specimen		
Bacterium and Algae *Nostoc* sp. *Lyngbya* sp. *Microcoleus* sp. *Achnanthes* sp. *Nitzschia* sp.	Fixation – Dehydration - Critical point drying – Mounting with double-sided tape.	Cell.
Fungus *Penicillium* sp.		Spores.
Plants *Cucurbita maxima* Duchesne *Guazuma ulmifolia* Lam. var. *ulmifolia* *Hibiscus rosa-sinensis* L. *Hibiscus tiliaceus* L. *Sida rhombifolia* L.		Stomata, glandular and unglandular trichomes and leaf surfaces.
Craniolaria integrifolia Cham.		Pollen.
Ceiba speciosa (A. St.-Hil.) Ravenna *Borreria hispida* (L.) K. Schum.	Fixation - Freehand-sectioning - Critical point drying – Mounting with double-sided tape.	Anthers, Ubich body, endothecium, Xylem.
Solanum tuberosum L.	Fixation - Critical point drying - Mounting with double-sided tape.	Tissue and starch grains.
	Fixation – Extended on glass – Air drying.	Starch grains.
Adenoa cubensis (Britt. & Wilson) Arbo	Air drying – Mounting on aluminum foil with superficial tension.	Pollen sculpture exine.
Senecio pterophorus DC. *Merremia* sp.	Mecanical fracture - Air drying and superficial tension.	Pollen sporoderm - structure exine.
Animals *Apis mellifera* L. *Solenopsis* sp. *Varroa destructor* Anderson & Trueman	Dissection - Mounted directly with double-sided tape.	Insect parts (honeybee, ant) Complete insect (mite)
Tetragonisca fiebrigi Schwarz.	Fixated– Critical point drying - Mounting with double-sided tape.	Insect parts (stingless bee)
	Fixation - Air drying – Mounting with double-sided tape.	
	Mounted directly with double-sided tape.	Complete insect (stingless bee)
All samples	Sputter coating with gold.	

Fixation

Samples with fragile tissues were fixed using chemical methods, while the ones with hard tissues like exoskeleton or pollen grains, did not require fixation. For the purpose of preserving plant tissues as closely as possible to their natural state several processes are required. Formalin-Aceto-Alcohol (FAA) is the most used fixative in which samples are immersed to stop their life processes. In this case, the FAA used is in the following concentrations:

formaldehyde (5%), glacial acetic acid (5%), alcohol (50%) and distilled water (40%) [13], [14].

It is not necessary to fix the entire organism; in fact, it is better to choose a portion of the sample of interest such as a leaf, or part of a flower. If plant organs are large it is recommended to section them in small portions of 10mm or less to favour the uniform entrance of the fixative to all cells. If organs are smaller they can be immersed completely in the solution (flowers, fruits, leaves, fungus, larvae, etc.).

Substances used for fixation are dangerous and may be cancerous, while handling them the operator must be careful and take necessary measures following laboratory safety rules, such as wearing an appropriate mask and gloves.

Dehydration

In this process water in the specimen is replaced with a solvent using an alcohol and acetone battery in ascending order of concentration. Methanol, ethanol and acetone are the most commonly used dehydrating agents because they have a smaller surface tension and are miscible with liquid CO_2. The dehydration series used in this laboratory, with samples already fixed with a recommended method according to its nature, was: 70% alcohol, 70% acetone, 85% acetone, and 100% two washes of acetone, 15 minutes of immersion of the sample at each concentration [15].

Critical point drying (CPD)

The most reliable and common drying procedure for biological samples is CPD [16]. This is an established method of drying biological samples before imaging them in a conventional SEM. Prior to CPD samples are dehydrated. In order to preserve cell structures as close to the natural state as possible.

CPD is based on the principle that under certain temperature and pressure conditions, a fluid and its over-laying vapour will become indistinguishable (the critical point). At this point, the surface tension on a specimen originally in the fluid phase will be zero [3].

Sample is then placed in the chamber of the CPD apparatus (Denton Vacuum – Critical Point Drying Apparatus DCP-1) with enough 100% acetone to completely cover it. It is usually recommended to place the samples inside tissue cassettes, the same ones used for dehydration that allow liquids to permeate the material. Each sample must be identified with a proper label due to the fact that after CPD tissues may not look the same, changing specially its colour.

Then the chamber is purged with liquid CO_2 until all the acetone has been replaced with CO_2, this process depends on the fragility and size of the sample, extending or decreasing the purging time when necessary. The sample is now immersed in CO_2 and the chamber is then sealed.

Afterwards, the chamber is heated with a bucket with hot water between 70 and 80 °C. This event makes the chamber pressure rise up to 1300 PSI, reaching the critical point where liquid CO_2 converts to its gaseous state. The chamber should be vented slowly in an attempt to release CO_2 gas and prevent cells from collapsing. CPD is not a perfect technique and shrinkage can still be observed. These drying artefacts may be due to sample properties, a deficient fixation, and dehydration process itself.

Mechanical fracture

Usually, SEM observations allow viewing the external part of a sample although there are several methods to examine internal structures. The chosen method will depend on the origin and consistence of the specimen. For instance, the method of mechanical fracture can be employed to observe the structure of the rigid wall of pollen grains and spores. The applied procedure consists of concentrating pollen grains by centrifugation of the samples at 2500 rpm in a test tube. Using a glass rod, pollen grains in the bottom of the test tube are pressed between them in an attempt to break their wall. Afterwards grains are mounted as specified in Air Drying.

Freehand-sectioning

This technique consists of sectioning the chosen tissue with a sharp double-edged razor blade. It can be used rather for vegetal or animal tissue. It is recommended that instruments are new and are only used once, so as to assure a good sectioning and to avoid tissue artefacts. Size of the sample must be small to medium (no more than 10 mm). Another important factor is the skill and training of the person handling the specimen as it is a technique that improves with practice.

Hydration

Some herbarium specimens needed to be rehydrated and also washed as a way to partially recover their original structure. These types of samples are previously dehydrated in a plant dryer at 70°C or less. In these event, a dispersant substance was used to rehydrate samples, TritonX-100, which is widely used for processing biological samples for imaging with SEM [1], [17]. In 10 cc of distilled water, 2 or 3 drops of this non-ionic surfactant are placed. Afterwards, samples are immersed into the solution for at least a day and washed by shaking them in this substance. A previous control with a stereo microscope is made so as to appreciate if rehydration of the sample is acceptable before continuing with dehydration.

Air drying

Drying in air is not a process that can be accomplished with all samples. It is recommended only for hard tissue samples that resist air drying without losing its structure, for instance, pollen grains because they have a rigid wall. Pollen grains are immersed into a solution of water and alcohol in a proportion 1:1 so as to continue air dehydration by evaporation. An aliquot is taken using a capillary and this micro bead is placed on a portion of aluminium (8x8mm). Liquid evaporation must be slowly as a means to prevent pollen grains from collapsing [9]. The pollen grains adhere to the aluminium foil by surface tension and are then coated with gold.

Mounting Biological Samples

This SEM has stubs of different sizes: 12, 30, 50 and 75 mm. The most used in this Laboratory is the 50 mm one because it allows placing numerous samples together, benefiting the sputtering process and optimizing observation time. An aluminum disc is made and used to place the specimens, which is useful when samples need to be observed several times or stored. Samples can be mounted on aluminum discs using double-sided tape, carbon tape, manicure liquid, and aluminum foil. In an effort to accomplish an optimal observation there are several aspects to consider. To start with, samples should be the same size and thickness, and secondly its surface must be flat and be completely attached to the stub to obtain a suitable image. These are necessary conditions to prevent charge-up and give specimen surfaces uniform conductivity. Afterwards, this disc is coated and fixed to the stub to start observations with SEM.

Sputter coating

All biological samples need to be coated with a conductive material because they do not have metals in their composition. Coating prevents charge-up on the specimen surface and increases secondary electron emission. Samples were sputter-coated with gold (Au) using a Denton Vacuum Desk II. Another chosen substance for coating specimens are palladium gold (Au-pd) or platinum (Pt) [18].

Analysis conditions and observation

Working distance (WD)

This is the distance from the underside of the objective lens to the specimen surface [18]. WD has a direct influence on images, on one hand when WD is small an

image with high resolution can be obtained and the depth of field is smaller.

At the same time WD is selected according to the size of the specimen and the type of image required. If the sample analysed is of considerable size and a general image is needed, WD must be large to obtain a panoramic view. In contrast, when details are required, WD must be small so as to achieve a high edge definition image.

The graph shows that when WD increases the same happens with depth of field and an image with low edge definition is obtained (Fig. 1). WD is represented in millimetres, however, to indicate the relationship with depth of field and edge definition an arbitrary scale was used. As it might be seen, depth of field has an arithmetic progression while edge definition a geometric one.

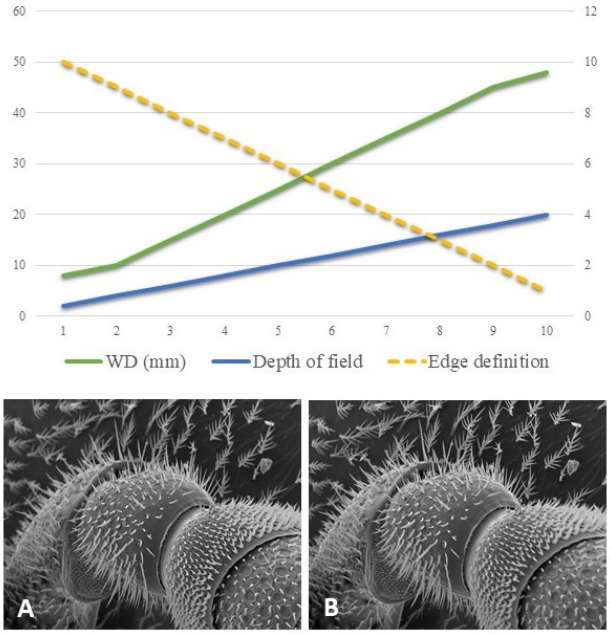

Fig. 1. Relation between different work conditions. Antenna of a moth. **A.** Image taken under the following conditions: small depth of field, short WD and high resolution. **B.** Image taken under the following conditions: great depth of field, large WD and low edge definition.

Accelerating voltage

Usually for biological samples observed in JEOL 5800 LV, an accelerating voltage of 15 kV is considered optimal specially for specimens with rigid walls, such as plant cells (impregnated of cellulose, lignine or sporopollenine). Voltages minor to 10 kV are generally used in bacterium and animal cells, to avoid damaging the specimen and to obtain an acceptable image. This SEM model can work with accelerating voltages between 0.3 and 30 kV. However, there are certain disadvantages in increasing the accelerating voltage, such as higher possibility of charge-up and specimen damage [18].

In addition, contrast and brightness increase with higher accelerating voltage. This is an important condition to achieve an optimum micrograph (Fig. 2).

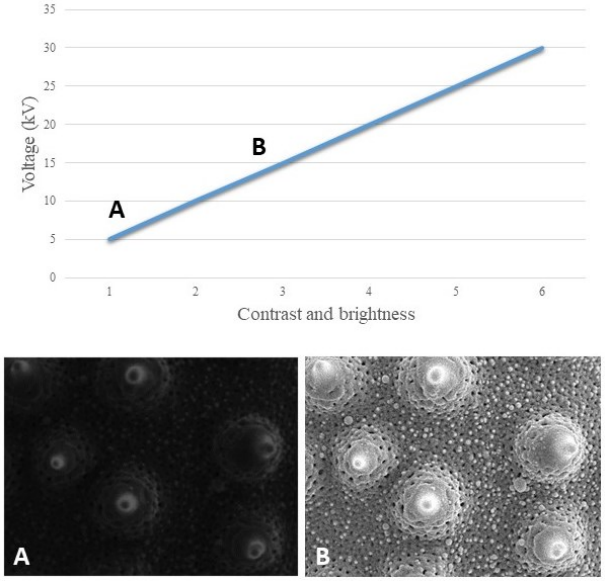

Fig. 2. Pollen wall. **A.** Insufficient contrast and brightness (5 kV). **B.** Optimum contrast and brightness (15 kV).

Secondary electron observation (SE) *and image capture*

From the interaction of the electron beam and the specimen, several signals are emitted, for instance: Secondary Electrons (SE), Backscattered Electrons (BSE), Auger Electrons (AE) and Characteristic X-ray (EDX) [18].

Most of biological samples are observed and images captures with Secondary Electrons. The contrast of SE images depends mainly on the tilt angle and topography of the specimen surface. Digital images are obtained using a Gatan DigiScan II Model 788 equipment.

To reach this stage, each sample was processed according to its characteristics and the steps are summarized in Fig. 3.

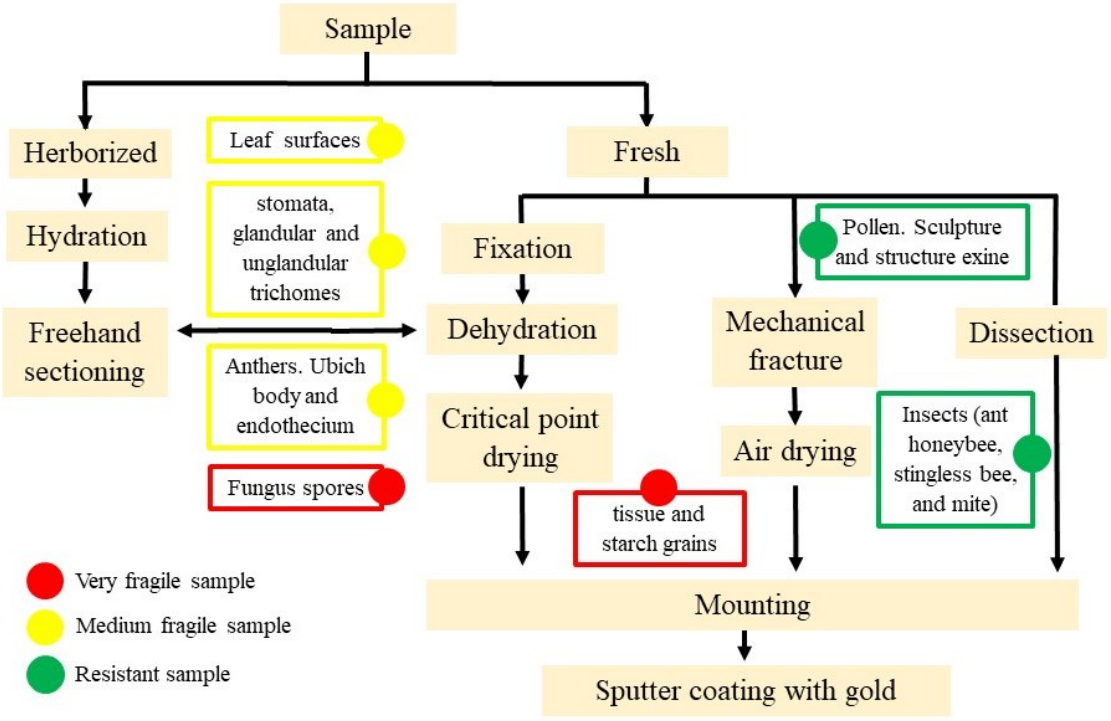

Fig. 3. Flowchart of processing sequence and level of complexity for different sample types

RESULTS AND DISCUSSION

SEM techniques are complemented by the classic light microscopy ones. Nanostructures can be observed due to high resolution present in scanning equipment. At the same time, an optimal tridimensional structure can be registered in multiple samples (e.g. fungus, pollen, trichomes, and insects). In this article the following results were obtained:

Bacterium and Algae

Most of these microorganisms are delicate and require adjustments in working conditions (minor to 10 kV), for example: *Nostoc* sp., *Lyngbya* sp., *Microcoleus* sp., however, others can tolerate harsher working conditions due to the resistance offered by the wall impregnated with calcium carbonate (between 10 kV and 20 kV), e.g.: *Achnanthes* sp., *Nitzschia* sp. [19].

Fungus specimens

These kinds of samples are fragile and working conditions must be very careful while handling and mounting the specimens. Conidiophores of *Penicillium* sp. were observed under low voltage (minor to 10 kV) in order to prevent conidium from moving away from the branches (Fig. 4).

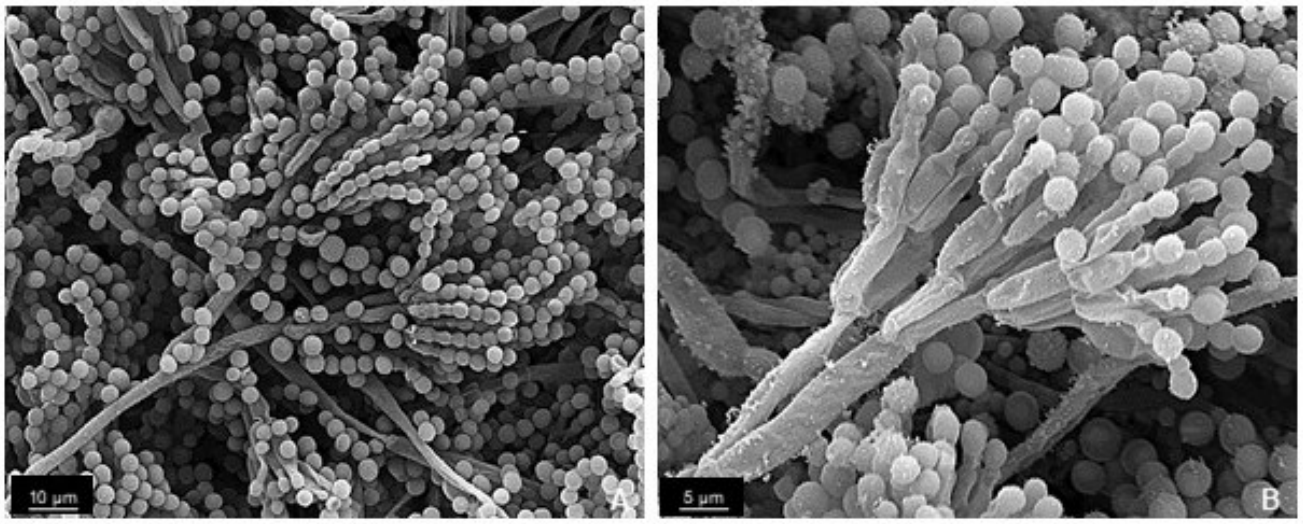

Fig. 4. Fungus specimen: *Penicillium* sp.: **A**, general view. **B**, detail of conidiophores.

Plant specimens

Vegetative samples

The optimal condition to analyse stomata, glandular and non-glandular trichomes, in leaf surfaces, is processing fresh material immediately fixated and dried with critical point drying. These structures were observed in several species belonging to different families: Malvaceae (*Guazuma ulmifolia* Lam., *Hibiscus rosa-sinensis* L., *Hibiscus tiliaceus* L., and *Sida rhombifolia* L.); and Martyniaceae (*Craniolaria integrifolia* Cham.). This method allows structure to remain similar to their live state, in different non-glandular trichomes: multiangulate (*Guazuma ulmifolia*), radiate (*Hibiscus rosa-sinensis*), stellate and T-shaped (*Hibiscus tiliaceus*), X-shaped (*Sida rhombifolia*), and conical (*Craniolaria integrifolia*) (Fig. 5. A - E).

Some tissues have lignin in their cell wall and other substances that allows their cells to keep its shape. For example, *Borreria hispida* Spruce ex K. Schum. (Rubiaceae) [20]. So as to observe these structures good free hand sectioning is needed. Afterwards samples are dried with critical point drying (Fig. 5. F).

When fresh material is not available, herbarium samples can be used. In this case material is rehydrated, as previously stated in material and methods. We carried out an experience with *Cucurbita maxima* Duchesne in Lam., leaves in which herbarium (Fig. 5. G), rehydrated (Fig. 5. H) and fresh samples were compared (Fig. 5. I). Fresh material is always the best option because when observed directly from herbarium, leaves present collapsed cells. However, some structures can be recovered with rehydration, for example glandular multicellular capitates trichomes.

In some particular cases other vegetative structures can be observed using different simple methods. For example, *Solanum tuberosum* L. stores starch grains in its underground organ (tubers). Free hand cuts of the organ are made to observe starch grains inside parenchymal tissue. This method needs critical point drying (Fig. 5. J). On the other hand, another simple method for observing starch grains is extending them in a thin glass and air drying (Fig. 5. K).

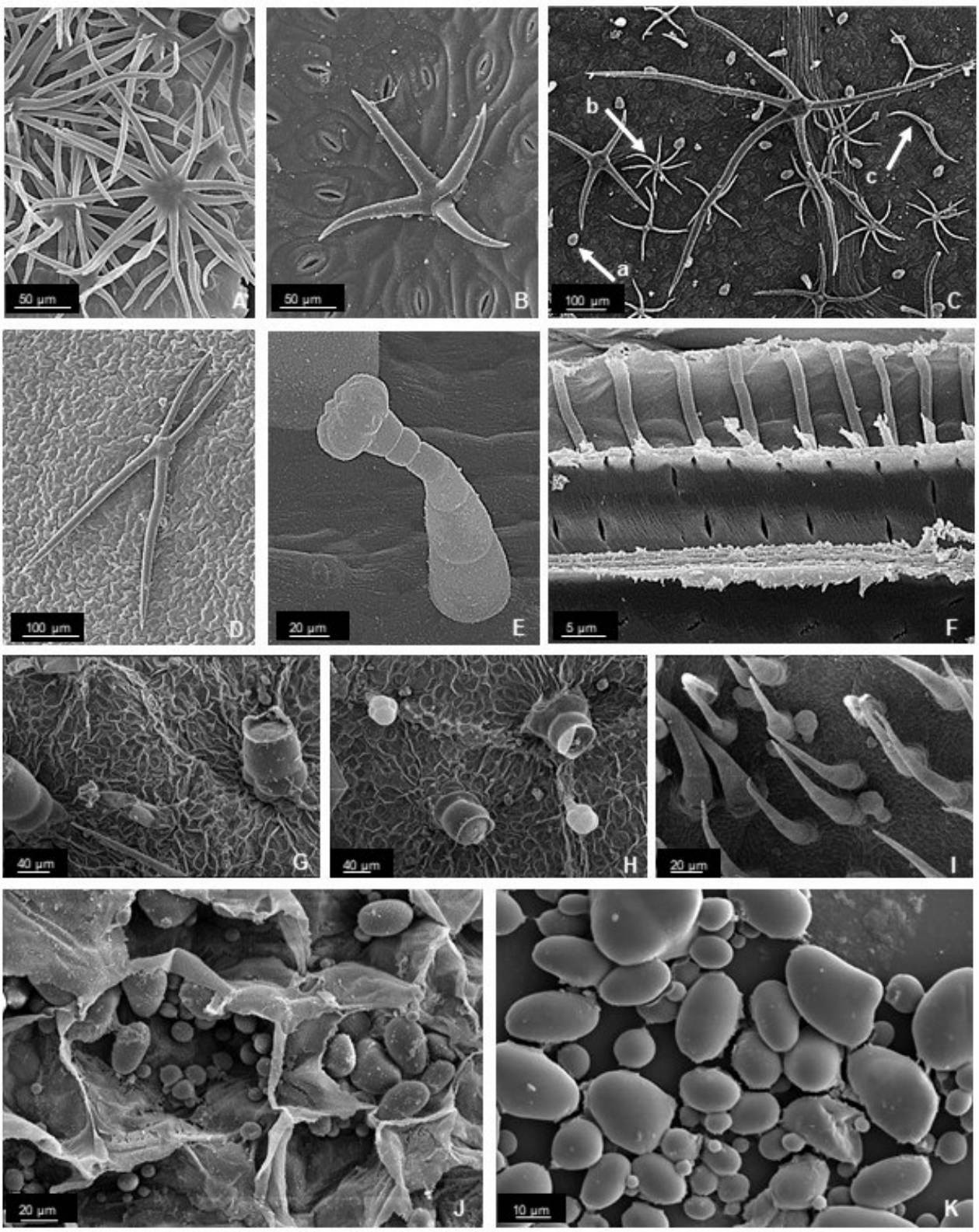

Fig. 5. Plant specimens. Vegetative samples. Micro-characters in leaves. *Guazuma ulmifolia*: **A**, multiangulate trichomes. *Hibiscus rosa-sinensis:* **B**, radiate trichomes. *Hibiscus tiliaceus*: **C**, glandular and non-glandular (strellate and T-shaped) trichomes. *Sida rhombifolia*: **D**, X-shaped trichomes. *Craniolaria integrifolia*: **E**, conical trichomes. *Borreria hispida*: **F**, xylem vessel. *Cucurbita maxima*: **G**, herbarium specimen mounted directly, **H**, rehydrated, **I**, critical point drying. *Solanum*

buberosum: **J**, critical point drying. **K**, extended on a slide and air drying. Abbreviations **a**, glandular trichome; **b**, stellate trichome; **c**, T-shaped trichome.

Reproductive samples

Freehand sectioned anthers, to observe Ubich bodies and endothecium of *Ceiba speciose* (A. St.-Hil.) Ravenna, were analysed following the usual methods: dehydration and critical point drying (Fig. 6. A and B). Ubich bodies are very small therefore short WD (8 mm) is needed to obtain details.

To observe pollen surface the optimal technique for many species is to wash with several alcohols, for example *Craniolaria integrifolia* but for some species (e.g. *Adenoa cubensis*) a more aggressive wash is needed to eliminate pollen kit and other substances covering the surface and preventing proper observation (Fig. 6. C and D).

Previous contributions [11] show that pollen grains can be mounted on aluminium and copper sheets; also two-sided tape and carbon tape. Different supports were analysed with the sample chamber working at high vacuum and under several work conditions in order to establish the best combination for image saving (acceleration voltage: 10kV, 15kV and 20kV, working distance 15 mm, 10 mm and 8 mm).

When the inner structure wants to be seen a fast solution is to break pollen grains, applying a mechanical force (Fig. 6. E and F).

Pollen samples can be extracted mainly from the anthers of flowers, also from fossil sediments, airborne pollen, and beekeeping pollen. Other samples that can be studied using these techniques are: small fruits and seeds.

We agree with other authors on the need to fix biological materials [5], [6], however, in some cases the fixation stage can be dispensed with (insects) and in others it is not necessary (pollen).

There are differences between authors regarding the concentrations and types of alcohol used to carry out the CPD process. Nevertheless, they all seem to be equally effective. Dehydration is not always carried out in the same way, [3] uses a series of increasing alcohol concentrations 30%, 50%, 70%, 80%, 90%, 95%, and 100% alcohol. Another authors [12] after fixation with 2.5% glutaraldehyde in 0.2 M cacodylate and 2% buffered osmium tetroxide, dehydrate the samples through a graded series of ethanol (10%, 20%, 30%, 50% and 70%—once for 10 min at each step), and then immerse in 100% acetone twice for 30 min each.

The most optimal supports were the metallic ones with respect to the tapes [11]. Other authors also recommend applying conductive paint to the four corners [18].

Gold coating is essential to obtain images of good quality and level of detail, especially in observation with the high vacuum mode. The coating layer must not be too thick, in order to allow the observation of nanostructures.

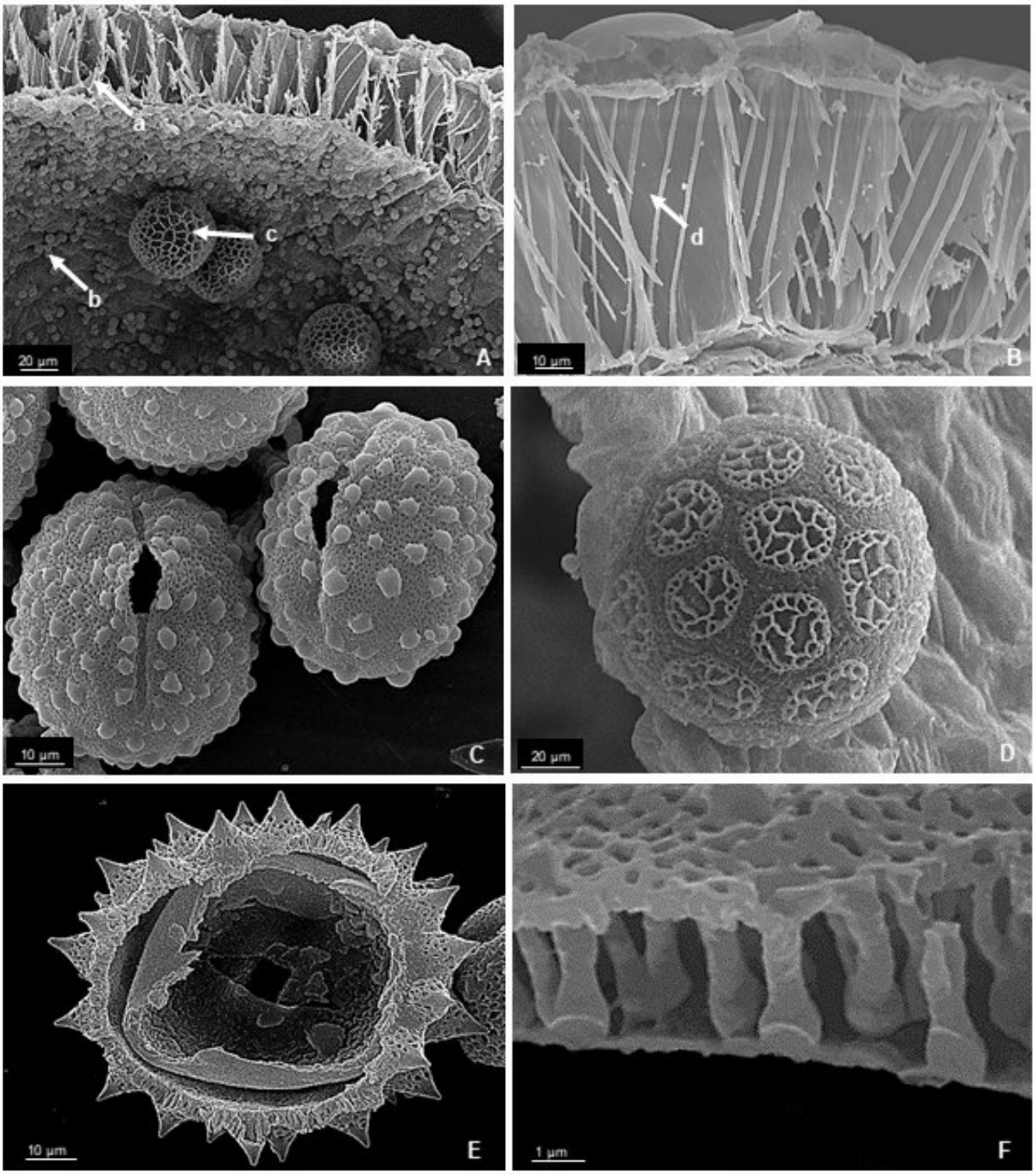

Fig. 6. Plant specimens. Reproductive samples. *Ceiba speciosa*: **A**, sectioned anthers. **B**, endothecium detail. Pollen grains: *Adenoa cubensis:* **C,** general view. *Craniolaria integrifolia*: **D**, general view. *Senecio pterophorus*: **E,** pollen grains in sections by mechanical fracture. *Merremia* sp.: **F**, pollen wall in sections by mechanical fracture. Abbreviations **a**, wall thickenings; **b**, Ubich bodies; **c**, pollen grains; d, endothecial thickenings.

Animal specimens

Insects such as: ant, butterfly, honeybee, mite, stingless bee and weevil; have hard exoskeleton. Due to this fact, samples do not require dehydration and critical point drying. Insects, or parts of insects, are directly placed in the aluminium foil with double sided tape (Fig. A, D-F). When samples have already been fixated it is best to dehydrate and dried with CPD, sometimes air drying is not recommended because micro-characters will not be seen as in the original state (Fig. 7. B and C). WD depends on images wanted, if general images of an insect are required WD must be long (Fig. 7. G) but if details are needed WD is short (Fig. 7. H and I).

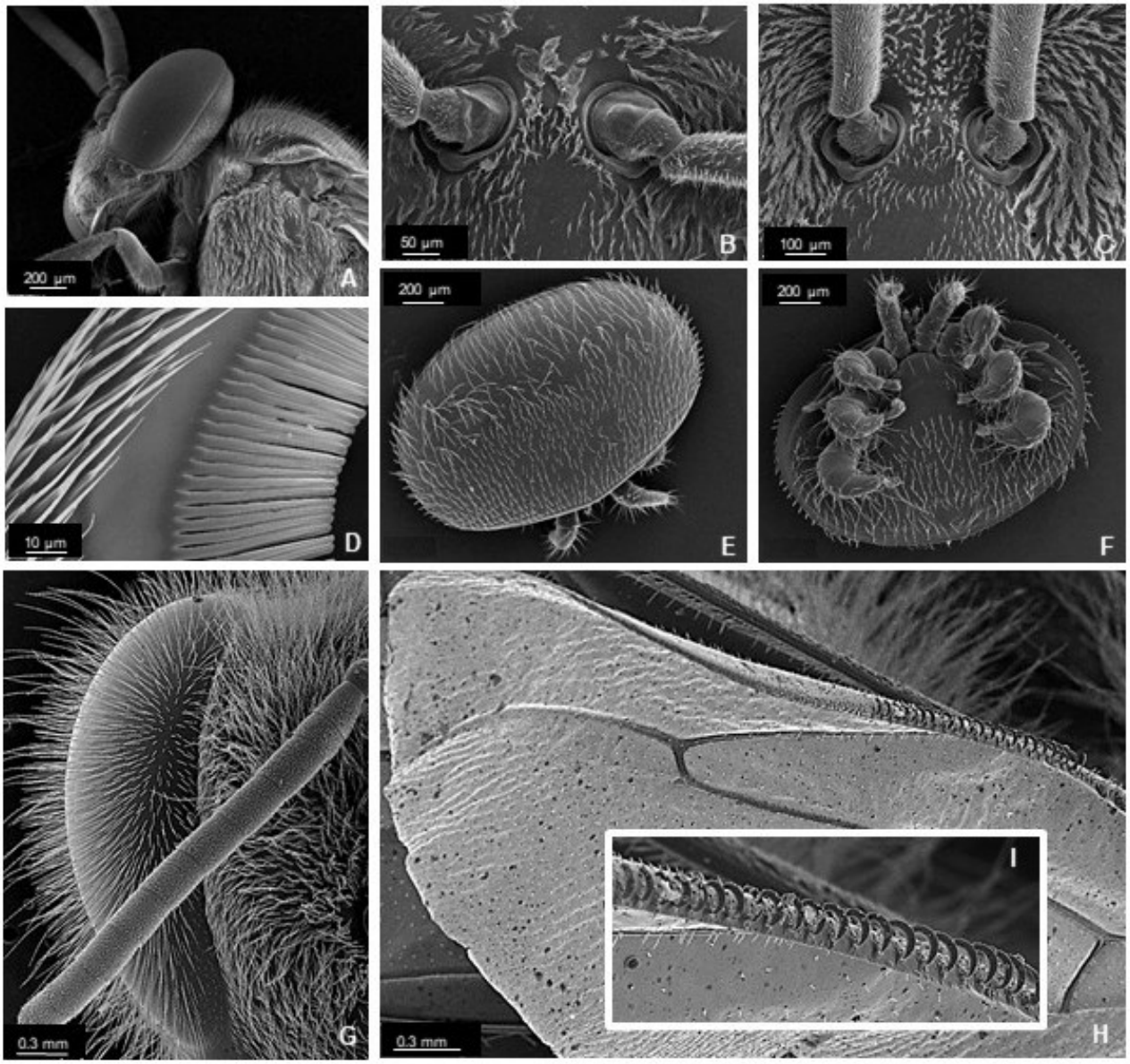

Fig. 7. Animal specimens. *Tetragonisca fiebrigi:* **A**, mounted directly. **B**, fixated and air drying. **C**, critical point drying. *Solenopsis* sp.: **D**, detail of antenna. *Varroa destructor*: complete insect mounted directly: **E**, dorsal view. **F**, ventral view. *Apis mellifera*: **G**, detail of eyes of specimen mounted directly, **H**, wing, **I**, detail of *hamuli* on the wing edge.

CONCLUSIONS

To deepen the study of biological samples, SEM techniques are an excellent tool and are complemented by optical microscopy techniques. Researchers can quickly and easily prepare different types of samples for observation. With this type of microscopy, important structures can be noticed to define taxonomic position based on morphological micro-characters, in all disciplines.

The following recommendations should be taken into account:

- Particular characteristics of their study material and some theoretical aspects related to the operation of the equipment needed to analyse their samples.
- It is recommended that the researcher has some basic knowledge regarding the operation of the SEM types and facilities (resolution, minimum and maximum WD, emission source, etc.) in the interest of obtaining the maximum benefit from the technological capabilities of the SEM.
- For the purpose of making CPD more efficient it is recommended to place in the chamber samples that share properties, size, fragility, and origin.
- It is very important to handle samples carefully to avoid artifacts, damage or marks from instruments (for instance, tweezers and needles).

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