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Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycete and fungal samples

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Abstract:	Common problems in the processing of biological samples for observations for scanning electron microscope (SEM) include cell collapse, treatment of samples from wet microenvironments and cell destruction. Using young floral tissues, oomycete cysts, and fungi spores (Agaricales) as examples, specific protocols to process delicate samples are described here that overcome some of the main challenges in sample treatment for image capture under the SEM. Floral meristems fixed with FAA (Formalin-Acetic-Alcohol) and processed with the Critical Point Dryer (CPD) did not display collapsed cellular walls or distorted organs. These results are crucial for the reconstruction of floral development. A similar CPD-based treatment of samples from wet microenvironments, such as the glutaraldehyde-fixed oomycete cysts, is optimal to test the differential growth of diagnostic characteristics (e.g. the cyst spines) on different types of substrates. Destruction of nurse cells attached to fungi spores was avoided after rehydration, dehydration, and the CPD treatment, an important step for further functional studies of these cells. The protocols detailed here represent low-cost and rapid alternatives for the acquisition of good-quality images to reconstruct growth processes and to study diagnostic characteristics.
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Title: Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycete and fungal samples (JoVE55031R1)

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Dear Editorial Board members,

We are submitting the reviewed manuscript JoVE55031R1. We would like to thank the anonymous reviewers for their valuable comments and suggestions. They helped to improve the content and the format of our paper considerably.

One of the major concerns of the reviewers was the content of sections 4, 5 and 6, describing the CPD, coating and SEM protocols. We do agree that most of the steps we had originally depend on the instrument used and could cause problems if performed in different models. We reworded those sections in order to remove instrument-specific instructions.

Additionally, the visualization part was modified and focused in one section (plants) as suggested by the editor. However, few steps from sections 2 and 3 were also included, since those include important modifications for oomycetes and fungi. We are open to modify this selection if required.

The paper was changed following the advice of the reviewers. Specific changes and explanations are detailed in the reply to each reviewer (see below in red). The changes in the manuscript are highlighted and the figures were changed directly on the TIF files.

Yours sincerely,

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TITLE:
Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycete and fungal samples

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KEYWORDS:
Agaricales, critical point dryer, cysts, formaldehyde, glutaraldehyde, *Phellorinia,* plant development, *Saprolegnia,* sputter coater

SHORT ABSTRACT:
Problems in the processing of biological samples for scanning electron microscopy observation include cell collapse, treatment of samples from wet microenvironments and cell destruction. Low-cost and relatively rapid protocols suited for preparing challenging samples such as floral meristems, oomycete cysts, and fungi (Agaricales) are compiled and detailed here.

LONG ABSTRACT:
Common problems in the processing of biological samples for observations with the scanning electron microscope (SEM) include cell collapse, treatment of samples from wet microenvironments and cell destruction. Using young floral tissues, oomycete cysts, and fungi spores (Agaricales) as examples, specific protocols to process delicate samples are described here that overcome some of the main challenges in sample treatment for image capture under the SEM.

Floral meristems fixed with FAA (Formalin-Acetic-Alcohol) and processed with the Critical Point Dryer (CPD) did not display collapsed cellular walls or distorted organs. These results are crucial for the reconstruction of floral development. A similar CPD-based treatment of samples from wet microenvironments, such as the glutaraldehyde-fixed oomycete cysts, is optimal to test the differential growth of diagnostic characteristics (*e.g.* the cyst spines) on different types of substrates. Destruction of nurse cells attached to fungi spores was avoided after rehydration, dehydration, and the CPD treatment, an important step for further functional studies of these cells.

The protocols detailed here represent low-cost and rapid alternatives for the acquisition of good-quality images to reconstruct growth processes and to study diagnostic characteristics.

INTRODUCTION:
In biology, the use of scanning electron microscopy (SEM) has been extended to studies of structural evolution, comparative morphology, organ development, and characterization of populations or species[1]. With its two-dimensional view of microscopic structures, areas such as micromorphology and systematics profited from SEM technique advances since the second half of the 20th century. For example, the introduction of the sputter coating methodology in the 1970's made possible observations of delicate materials such as shoot apices and flowers enhancing the imaging of non-conductive tissues[2-3]. SEM uses electrons ejected from the surface of the specimen to reproduce the topography in a high-vacuum environment[4].

Studies involving SEM are focused in both the inference of structural characters and the reconstruction of growth processes. New structural characters relevant to the taxonomy and systematics of a wide range of organisms have been discovered from SEM observations. For example, plant traits used for species diagnosis or supraspecific classifications, such as the vestured pits of wood[5], stigma diversity[6], nectary and floral morphology[7-8], trichome

details[9], and pollen grains[10-11], cannot be properly visualized without SEM. Successful observations with conventional SEM have been also achieved for long-time formalin-fixed organisms[12] and plant herbarium specimens[13].

On the other hand, studies of reconstruction of growth processes using SEM involve a wide range of topics, such as organ development[14], infections induced by bacteria[15], plant root physiology[16], parasite-host attachment mechanisms[17-18], drug effects on parasites[19], mycoparasitism and antibiosis[20-21], growth malformation[22], comparative development of wild and mutant individuals[23], and entire life cycles[24]. Although environmental scanning electron microscopes (ESEM)[25] may have important advantages for the observation of wet biological samples in growth processes, delicate material may still be compromised even in the low vacuum condition of the ESEM), and need to be processed adequately to avoid loss of valuable morphological observation.

In this paper, a review of specific protocols for SEM observation of three different types of samples is presented: floral meristems, oomycetes (*Saprolegnia*), and fungal material. These protocols compile the experience of our previous SEM-based studies[26-33], where specific difficulties and alternative solutions have been found. In the case of plant comparative developmental and structural studies, the use of SEM started in the 1970s[34-35], and since then, researchers discovered that certain floral features are more labile than previously thought[36]. Reconstruction of floral development involves the capture of all stages between young floral meristems and anthesis. To reach this aim, it is essential that the sample topography and the cell wall integrity are not compromised after the fixation and subsequent dehydration. Young floral meristems are particularly vulnerable to cell wall collapse (Figures 1a, 1b). Similarly, delicate structures such as nectaries, petals, stigmas and sporangia require effective and undamaging protocols. This review summarizes an optimal protocol to keep young and delicate tissues intact for SEM imaging.

In the case of the oomycetes (Straminopiles)—one of the most diverse and widespread groups of parasites, with hosts ranging from microbes and plants to invertebrates and vertebrates[37]— there are spores that grow and develop in a wet environment. This condition represents a challenge for SEM observation because the spores need an adequate substrate not suitable for standard SEM protocols. Among the oomycetes, species of *Saprolegnia* are of particular interest because they can cause severe reductions in aquacultures, fisheries, and amphibian populations[38]. Micromorphological characteristics, such as the hooked spines of cysts, have been found to be useful to identify species of *Saprolegnia,* which is fundamental to establish infection controls and potential treatments[39]. Here, there is an experimental protocol to compare the patterns of the spine growth of cysts on different substrates and to manipulate the sample for critical point dryer (CPD) preparation and subsequent SEM observation.

In a third case, there are interesting findings that came up after an inspection of the spores of the fungi *Phellorinia herculanea* f. *stellata* f. nova (Agaricales)[31]. Together with the spores, a group of unexpected nursery cells was identified under the SEM. With previous traditional protocols and untreated material, the nurse cells came out completely collapsed (Figure 1c). Further inferences about particular tissues associated to the spores can be made with the simple but crucial modifications to the standard approaches described here (Figure 1d).

In this review, there are detailed SEM protocols that can be used to deal with different problems associated with SEM observation in angiosperms, oomycetes, and Agaricales, such as cell collapse and meristematic tissue shrinking, non-optimal growth of cyst spines, and destruction of ephemeral tissues, respectively.

[place Figure 1 here]

PROTOCOL:

Note: This protocol includes six main sections, three devoted to specific organisms (sections 1–3), and three describing the procedures common to all (4–6). Asterisks (*) indicate steps modified by the experimenters.

1. Studies of developing and fully formed plant structures

1.1) Collection and fixation

1.1.1) If the plant material is collected in a place with no access to a fume cupboard, introduce and immerse the material in 70% ethanol in centrifuge tubes. Ideally, immerse the material after 48 h in FAA (steps 1.1.1-1.1.3) to avoid excessive dehydration in the ethanol. If a fume cupboard is accessible to the plant material, ignore this step and continue with 1.1.1.

1.1.2) Prepare the formalin-acetic-alcohol (FAA) fixative in a fume cupboard fitted with an aldehyde filter. Add 85 parts of 70% denatured ethanol, 10 parts of 60% formaldehyde solution, and 5 parts of glacial acetic acid. Prepare the FAA just before fixing the material, as its long term storage is not recommended[40].

1.1.3) Under the fume cupboard, pour the stock of FAA into individual wide-mouth and leak-proof plastic bottles. Use as many bottles as there are samples available, and create labels for sample identification.

1.1.4) Select the floral or vegetative meristems to fix, ensuring that they are not damaged by insects, fungi, or extreme weather conditions. Cut the branches, removing unwanted material, and deposit the sample **immediately** in the FAA solution.

1.1.5) After 72–96 h, pour the FAA into a plastic container for chemical disposal. Immediately, wash the samples three times with fresh 70% ethanol to remove any residual FAA. Fixed material can be stored indefinitely in 70% ethanol.

1.2) Dissection and dehydration

1.2.1) Dissect the fixed material in 70% ethanol under the stereomicroscope using ultra fine tweezers, needles, forceps, brushes, and micro-scalpels (the maximum size of the tissue should be around 1 cm^3, or 2 cm for flat material). Dissect the samples into a Petri dish covered with ethanol to prevent the tissues from drying. Use a Petri dish with the base covered with dry black silicon to better see the contrasting white tissues.

1.2.2) Put the dissected material in specimen containers for the critical point dryer (CPD, Figure 2a). At this point, immerse the containers into the Petri dish with 70% ethanol, and include the sample identification labels (made with paper and pencil). For more effective drying for further manipulation, avoid mixing the young and mature samples in the same container.*

1.2.3) Put the lids on the containers and deposit them in plastic centrifuge tubes with plenty of 70% ethanol. Store the tubes overnight if the material is not processed immediately.

1.2.4) Transfer the dissected material through the following ethanol series in hermetic jars or centrifuge tubes: 70%, 90%, 100%, and 100%. Leave the samples in each solution for 1 h at least. Keep the samples overnight in a 100% ethanol solution.

1.2.5) Transfer the containers with the material to the CPD (section 4).

1.3. Mounting and preparing plant tissues for SEM observation

1.3.1) Write the sample identification number underneath the SEM sample holders (*i.e.,* aluminium stubs). Cover the top of the stubs with double-sided tape. Place the stubs into a specimen holder (Figure 2b).

1.3.2) Under a stereomicroscope, carefully open the containers carrying the young and delicate samples already dried in the CPD. Bear in mind that after the CPD treatment, the samples become lighter and sensitive to electrostatics. Close the containers once the samples have been taken out to avoid dust or impurities.

1.3.3) Put the samples on the sticky surface of the stubs, planning ahead the desired position (once the samples touch the surface, it is very difficult to remove them). Do not try to carry a major dissection at this point; just remove unwanted tissue that is easy to pick up. For palynological studies, dissect the anthers and open them to expose the pollen on the stubs.

1.3.4) Put long samples (*e.g.,* 2 cm long) such as inflorescences in the horizontal position. When possible, orient samples of the same structure for polar, side, and bottom views. Leave enough space between samples on the stub.

1.3.5) If the samples cannot be processed immediately, keep them protected overnight in a hermetic container with silica gel to avoid rehydration (Figure 2c)*. Coat the samples using the sputter coater and transfer them to the SEM (sections 5 and 6).

[place Figure 2 here]

2. Study of cyst behaviour of *Saprolegnia* (Oomycetes) on different surfaces

2.1) Growing and fixing the cysts

2.1.1. Prepare peptone and glucose (PG-l) media[41] using D (+) glucose (6 g) and mycological peptone (3 g)*. Add up to 900 mL of tap water and autoclave 40 min at 121 °C. Pour 50 mL of the previously autoclaved solution A (NaH_2PO_4, 0.13M) and 50 mL of solution B (Na_2HPO_4, 0.13M).

2.1.2) From stock cultures of strains of *Saprolegnia parasitica* maintained on peptone, glucose, agar media (PGA, which is prepared as PG-l but adding 10 g of European bacteriological agar to the glucose and the peptone before autoclave), grow mycelia colonies in 0.5 mL of PG-l droplets for 24–48 h at 20 °C in Petri dishes. Induce sporulation by washing the mycelia with autoclaved tap water three times and incubating them for 15 h at 20 °C[42-43].

2.1.3) Collect the released secondary zoospores by gently pipetting the upper part of the suspension and pool them in 1 mL portions. Agitate vigorously the zoospores for 30 s by vortexing to produce secondary cysts[44].

2.1.4) To test the differential growth of the spines of the cysts, on separate Petri dishes (p60), put 0.5 mL of the secondary cyst suspension onto different surfaces (*i.e.,* carbon, gold, and copper TEM grids; salmon and hake fish scales (previously bleached); and glass cover slips)*. Incubate the cysts at 20 °C for 70 min, which favors the attachment of the cysts to the surface.

2.1.5) Remove the liquid and add 0.5 mL of 2% glutaraldehyde to each surface for the fixation of the cysts. Keep the samples at room temperature under a fume cupboard for 1 h.

2.1.6) Remove the glutaraldehyde and dehydrate the sample through an ethanol series (30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, and 100%), adding 5 mL of each ethanol solution for 15 min. Once in the last 100% ethanol solution, the sample can be stored for up to a month in a sealed Petri dish. At this stage, the samples are ready to be dried in the CPD.

2.1.7) Carefully transfer the grids and the scales from the Petri dish to a holder suitable for the CPD (Section 4). For this step, use a CPD grid holder or a stacking specimen holder, which keep samples separated from each other. Take the grids and scales with tweezers, keeping in mind that the cysts should be facing up on the grids all the time.*

2.2) Mounting and preparing cyst samples for SEM observation

2.2.1) Mount the grids and the scales on the aluminium stubs that were previously covered with double-sided carbon tape and labelled underneath.

2.2.2) Transfer the samples to the sputter coater (section 5).

2.2.3) Observe the samples under the SEM (section 6).

3) Study of herbarium fungal spores of *Phellorinia herculanea* under SEM

3.1) Rehydration and dehydration of spores

3.1.1) Wrap each sample carefully with filter paper, forming pencil-labeled envelopes ~0.5-1 cm^2, taking care not to crush them. Seal the filter paper with paper clips. Transfer the packed samples to a Petri dish and immerse them in 10 mL of water to rehydrate tissues around the spores.

3.1.2) Immediately put the samples in a microwave (600 W for approximately 20 s). Remove the material once the water starts to evaporate, and allow it to cool down at room temperature.

3.1.3) Pass the samples through the following ethanol series: 30%, 50%, 70%, 80%, 90%, 95%, 100%, and 100%. Depending on the amount of samples, use a beaker or centrifuge tubes for this step. Leave the samples for 15 min in each solution.

3.1.4) Place the samples to the CPD (section 4).

3.2) Mounting and preparing spores for SEM observation

3.2.1) Open the envelopes. Pour the spores on a previously-prepared stub with double-sided tape. Alternatively, collect the spores with the sticky surface of the stubs, taking care not to crush them*.

3.2.2) If the samples contain few spores, in addition to the previous step, cut a small piece of the envelope (~1 mm^2) and place it on a new stub*.

3.2.3) Place the tissues into the sputter coater (section 5).

3.2.4) Observe under the SEM (section 6).

4. Drying of material using a critical point dryer (CPD, Figure 2d)

4.1) Use the CPD in a ventilated area and verify that all the valves of the machine are closed. Check if the sample chamber is empty and clean.

4.2) Switch on the machine and verify that the temperature control system test takes place automatically. If the CPD has an external refrigeration bath system, check the water levels before switching it on.

4.3) Follow the manufacturer's instructions of the specific CPD used for the ethanol and CO_2 interchange. For safety, carry on this step under the supervision of someone trained for the use of the machine. Remember that it is exposed to rapid pressure changes, it could blow out violently.

4.4) Take out the samples and continue with the step 1.3 if working with plant tissues, step 2.2 if working with oomycetes cysts, and steps 3.2 if working with fungi spores.

5. Coating the samples with gold using the sputter coater (Figure 3a)

5.1) Check the sputter coater. Verify that the gold cathode target is in good condition. Use a lint-free cloth drenched with 90% ethanol to clean the walls of the vacuum chamber and the chamber lid if necessary.

5.2) Mark the sputter holder with numbers beside each stub hole for further identification of the samples under the microscope. Carefully, place the stubs loaded with the samples and secure them. Use a rotary planetary specimen stage to ensure a uniform coating on specimens with irregular surfaces.

5.3) Follow the manufacturer's instructions to adjust settings such as the working distance (*e.g*. 30 mm), operation gas pressure (*e.g.* 5 x 10^{-1} – 7 x 10^{-1} mbar), the sputtering time (*e.g.* 50 s), thickness of the gold layer (*e.g.* 12 nm) the current (*e.g.* 15 mA) and the voltage supply (*e.g.* 600 V)[45].

5.4) Remove the stubs and take them to the SEM (section 6). Alternatively, place the stubs into a sealed container with silica gel (Figure 2c).

[place Figure 3 here]

6. Observation under the scanning electron microscope (SEM, Figure 3b)

6.1 SEM start up

6.1.1) Follow the manufacturer's instructions to start and set the SEM, adjusting the sample height the objective aperture diameter (*e.g.* for plants 2 µm and for fungi and oomycetes 4 µm), the operating voltage (*e.g.* 15 kV).

6.1.2) Check the correct alignment of the electron beam system and set the axial alignment and the stigmators according to the manufacturers indications. Adjust the working distance in order to obtain an adequate depth of field.

6.2. Image capture

6.2.1) Get a focused image of the sample and use it as a starting point. Increase the magnification close to the maximum level and focus the image again. Choose areas with surface irregularities such as holes. Correct astigmatism and adjust the optimum contrast and brightness.

6.2.2) Capture the SEM Image with the high-resolution. Use the BSE detector if the image shows that the samples are charged. Otherwise, set the SE detector. Change the detectors following the manufacturer's instructions.

REPRESENTATIVE RESULTS:

Floral development and fixation of developing and fully formed plant structures

Using the FAA-CPD protocol described here, young and mature plant tissues are optimally fixed and dehydrated for SEM imaging. Processes such as floral development can be reconstructed because the topography and shape of the buds is not distorted by cell shrinking (Figures 1b, 1d, 4a–f). Structures with complex shapes can be successfully covered with a uniform layer of conductive material (the metal from the sputter coater), allowing the recovery of otherwise unreachable details (Figures 4g–l, 5e). Good quality images of samples coming from wet microenvironments, which used to be overcharged with the electron current if badly dehydrated and coated, can be achieved (Figures 4f, 5a, 5b). Important details such as the pollen wall surface (Figure 5b, 5c) and different types of indumenta (Figures 5d–g) can be studied in depth without artificial or undesirable particles or distorted shapes.

[place Figures 4 and 5 here]

Effect of different substrates on the spine growth of secondary cysts *Saprolegnia parasitica*

The spine growth pattern was different on different substrates. On glass, the spines were elongated and showed loops at their tips (Table 1, Figure 6a). On the carbon and gold grids the spines are shorter, curled and grow more abundantly around the cysts without forming loops (Figures 6b, 6d). Although on copper grids, the spines tended to curl without looping, some of them elongated (Figure 6c). On fish scales, the spines were curly, abundant around the surface and sometimes formed looped ends (Figure 6e–f).

[place Table 1 and Figure 6 here]

Unexpected observations in *Phellorinia herculanea* f. *stellata*

Apart from the unexpected finding of laticiferous hyphae on the exoperidium of *P. herculanea*, Calonge *et al.* [31] found globose and smooth nurse cells (8-13 µm) mixed with spores in the material dehydrated with CPD thanks to the rehydration step performed in the microwave. (Figure 1d). The structure and details of the walls of the nurse cells and the spores are well preserved despite their differential composition (Figures 1c–d).

Figure 1: Comparison of samples treated without (a, c) and with (b, d) the protocol FAA-ethanol-CPD.

(a–b) Floral buds of *Anacyclus clavatus,* mid-development. Bud treated with osmium tetroxide[46] (a) and bud treated with the FAA-CPD protocol (b). (c–d) Nurse cells with spores of *Phellorinia herculanea* f. *stellata.* Dried samples without any treatment (c) and with the protocol here described for Agaricales (d). Spores in orange. Scales: (a–b) 100 µm, (c–d) 50 µm. Photos were taken by Y. Ruiz-León.

Figure 2: Tools for sample manipulation and processing before SEM observation.

(a) Steel-made specimen container with holed walls for the ethanol/CO_2 interchange in the CPD chamber. (b) Steel stubs within a plastic specimen holder. (c) Glass container used to keep the samples protected from humidity and dust. At the base, there is a compartment for silica gel. (d) Critical Point Dryer. In the front, there are (from left to right) the manometer, the power switch, the temperature control system, and the temperature display. Usual working pressure for CO_2 – ethanol interchange is 60 bars (800 psi). In the top, there are four valves (inlet, drain, ventilation, and exhaust controls) flanking the central sample chamber. Photos were taken by Y. Ruiz-León and M.A. Bello.

Figure 3: Sputter coater (a) and scanning electron microscope (b).

(a) Front view of the vacuum chamber (left), gas valve, timer, vacuum, and current controls. (b) Side view of the SEM main components (from left to right): the vacuum column with the sample chamber, the computer screen with the controls, and the chamber's monitor. Photos were taken by Y. Ruiz-León.

Figure 4: Early (a, b), mid (c–e), and late floral development captured under the SEM.

(a) Raceme of *Caesalpinia spinosa* (Molina) Kuntze with several floral meristems. (b) Top view of a young raceme *Polygala violacea* Aubl. (c) Floral bud of *Krameria ixine* Loefl. during gynoecium differentiation. (d–e) Buds of *Erythrina* sp. (f) Side view of a flower of *Krameria ixine*. The anthers and style protrude. (g–i) *Hoya carnosa* (L.) R. Brown. (g) Flower at anthesis

(digital image taken with a Olympus SP-590UZ 26X). (h) Top view of the stamens and splitted carpels (yellow). (i) Side view of a pair of stamens (yellow and red) flanking two yellow carpels. Asterisks indicate stamens. g = gynoecium. Scales: (a,i) 1 mm, (b, d) 400 µm, (c) 200 µm, (e) 500 µm, (f, h) 2 mm, (g) 0.25 cm. Photos were taken by M.A. Bello.

Figure 5: SEM micrographs of sporangia and pollen (a, b), detailed surfaces (c–e), and indumenta (f–g).
(a) Sporangia of *Dryopteris* sp. (b) Pollen of *Prunus dulcis* D. A. Webb landing on stigma. (c) Pollen of *Nepenthes alata* Blanco. (d) Side view of an inflorescence of *Erigeron karvinskianus* DC. Image taken with BSE detector option. (e) Side view of the bryophyte *Peristoma* sp. (f) Glandular and no-glandular trichomes on the abaxial surface of a leaf of *Rosmarinus officinalis* L. (g) Top view of the leaf flat scales of *Olea europaea* L. Scales: (a) 400 µm, (b) 60 µm, (c) 5 µm, (d) 4 mm, (e) 600 µm, (f) 200 µm, (g) 600 µm. Photos were taken by Y. Ruiz-León.

Figure 6: Differential growth patterns of the spines of *Saprolegnia parasitica* in cysts immersed in liquid media.
(a) Glass. (b) Carbon. (c) Copper. (d) Gold. (e) Hake scale. (f) Salmon scale. Straight spines were developed on glass and copper, whereas curled spines were formed on carbon, gold, and fish scales. The hooked tips (white arrows) of the spines are observable in all spines growing on glass and in few spines on fish scales. The cyst walls are in yellow. Scale: 20 µm. Photos were taken by Y. Ruiz-León and S. Rezinciuc.

Table 1: Spine morphology using different types of substrate

DISCUSSION:
With respect to standard SEM protocols, the procedures presented here include relatively rapid, easy to follow, and low-cost methodologies. Depending on the amount of samples and on the ease of processing, it takes four to five days to acquire good quality images. Including adequate safety precautions for the CPD and SEM operation, the procedures are easy to handle. Particular caution should be taken with formalin and the glutaraldehyde (see steps 1.1.1 to 1.1.3 and 2.1.5 of the protocol). There are certain steps where, if necessary, the process can be stopped for a long time without damaging the samples or ruining previous steps (*e.g.,* steps 1.1.5, 1.2.3, and 1.3.5). In terms of cost, much of the equipment can be reused for several preparations (*e.g.,* CPD aluminum containers and stub holders), and the reagents are non-expensive chemicals available from all commercial suppliers.

Disadvantages of these procedures are the need for proper chemical waste disposal for the aldehydes and the ethanol and the lack of key supplies and the limitations of the technique. Materials such as the gold disk for the sputter coater and the CO_2 cylinder for the CPD should be checked well in advance. If they are in continuous use, the laboratory will require multiple stocks of them, which eventually elevates the costs. Because individual researchers often cannot justify the expenses derived from the maintenance of these types of supplies, or they simply do not need to use the SEM continuously, these procedures nowadays tend to be performed in laboratories with external electron microscopy services.

The observation technique of the SEM is limited to a high magnification study of surfaces. If internal tissues need to be observed, the samples should be cut in the proper way to explore the external appearance of the internal tissues. To explore internal aspects of the cells at high magnification, a transmission electron microscope (TEM) is required. Critical steps within the protocol are the proper fixation and dehydration of the samples before CPD treatment, where it is crucial to keep the tissues safe from shocking changes and direct contact with the air. Also, careful management of the pressure change in the CPD sample chamber and the amount of coating deposited on different types of samples is important.

Despite these limitations and special manipulations, the study of biological samples with SEM following the protocols described here allows for the resolution of some common problems, such as the cell wall and organ distortion (Figures 1, 4, 5), the restrictions on the observation and growth of structures coming from wet and liquid environments (Figures 5, 6), and the destruction of sensitive cells (Figures 1c, 1d).

The protocols presented here have allowed the reinterpretation and questioning of traditional taxonomic characteristics, such as the spine structure in cysts of *Saprolegnia*[47]. The differential growth pattern of the *Saprolegnia* spines on different surfaces demonstrates the lability of this feature and its limitations for species diagnosis. In addition to the capture of relevant characteristics for taxonomic studies, stages of organ development, and infectious diseases, functions of tissues never observed before have been explored thanks to these techniques. Now, this protocol can be extended to thousands of case studies waiting to be examined[33]. According to the peer-reviewed literature database Scopus, in the last the last five years there have been 7425 publications of papers dealing with SEM imaging of plants (4914), oomycetes (21) and fungi (2490). This fact suggests that research in oomycetes using the SEM imaging is still very scarce in comparison with plants and fungi. With ESEM, the numbers are even lower. There are 588 manuscripts in the same time span: 337 in plants, 1 in oomycetes and 250 in fungi.

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DISCLOSURES:
The authors have nothing to disclose.

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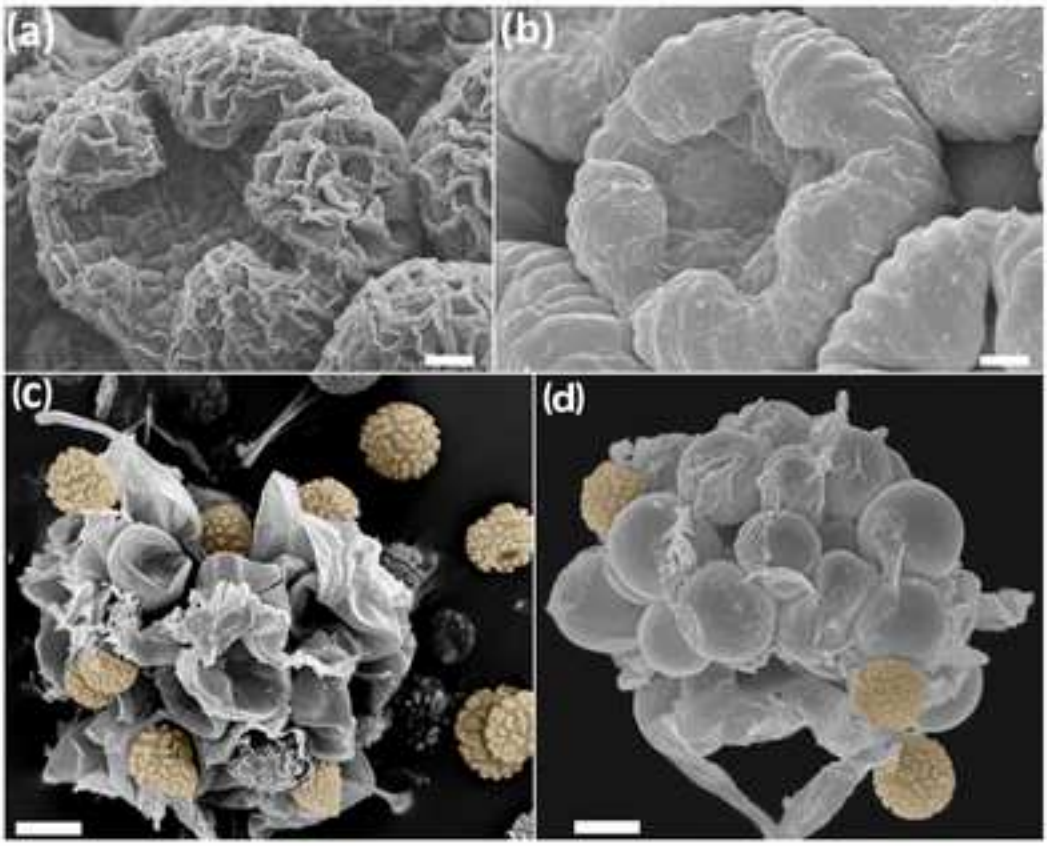

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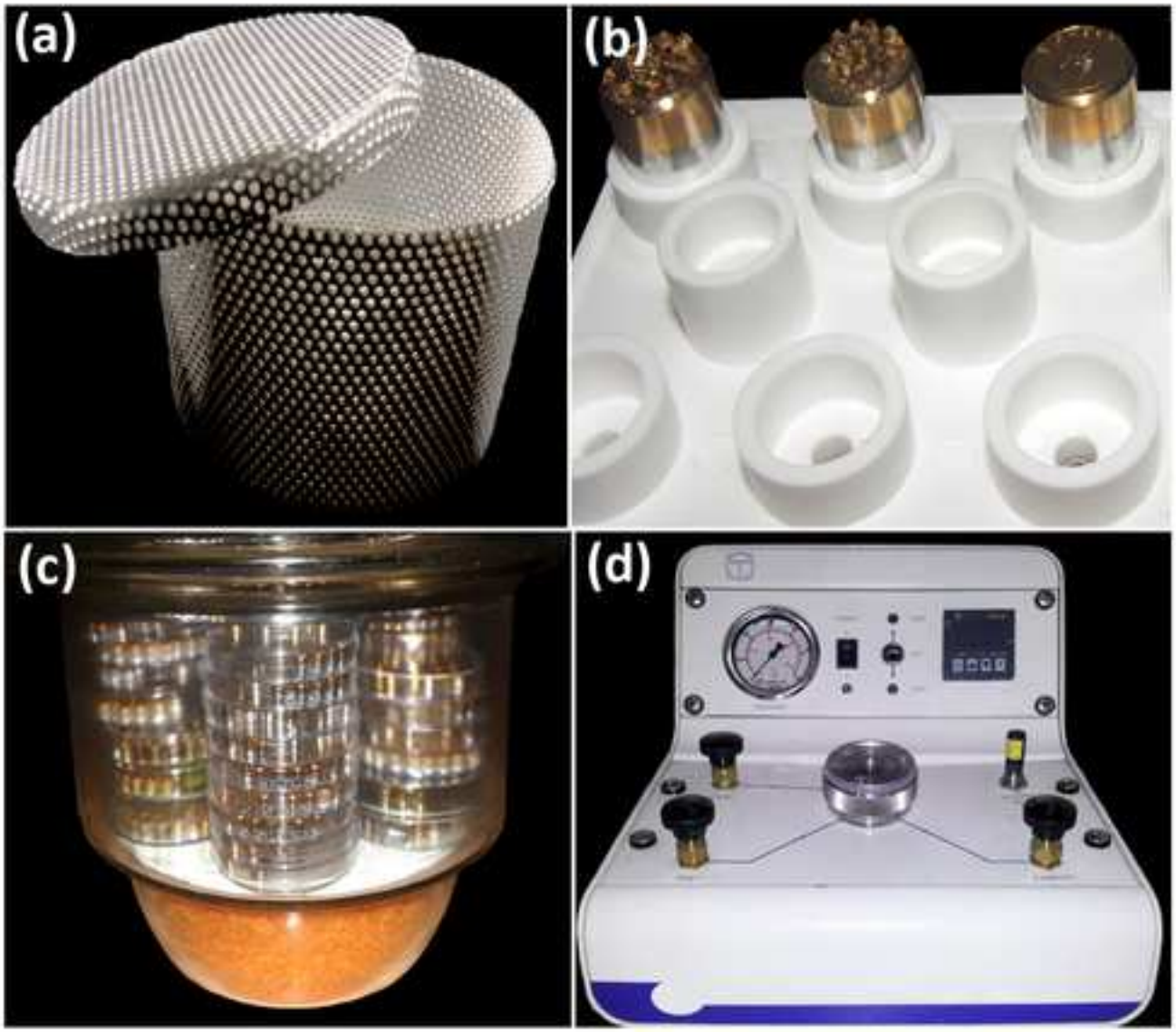

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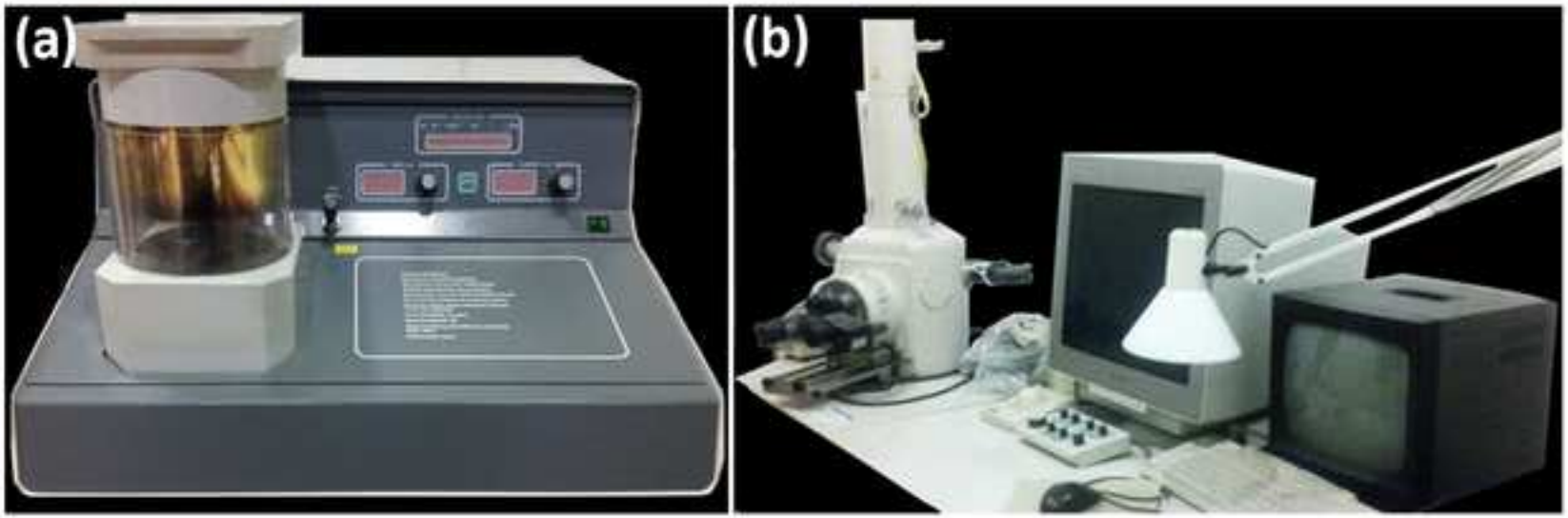

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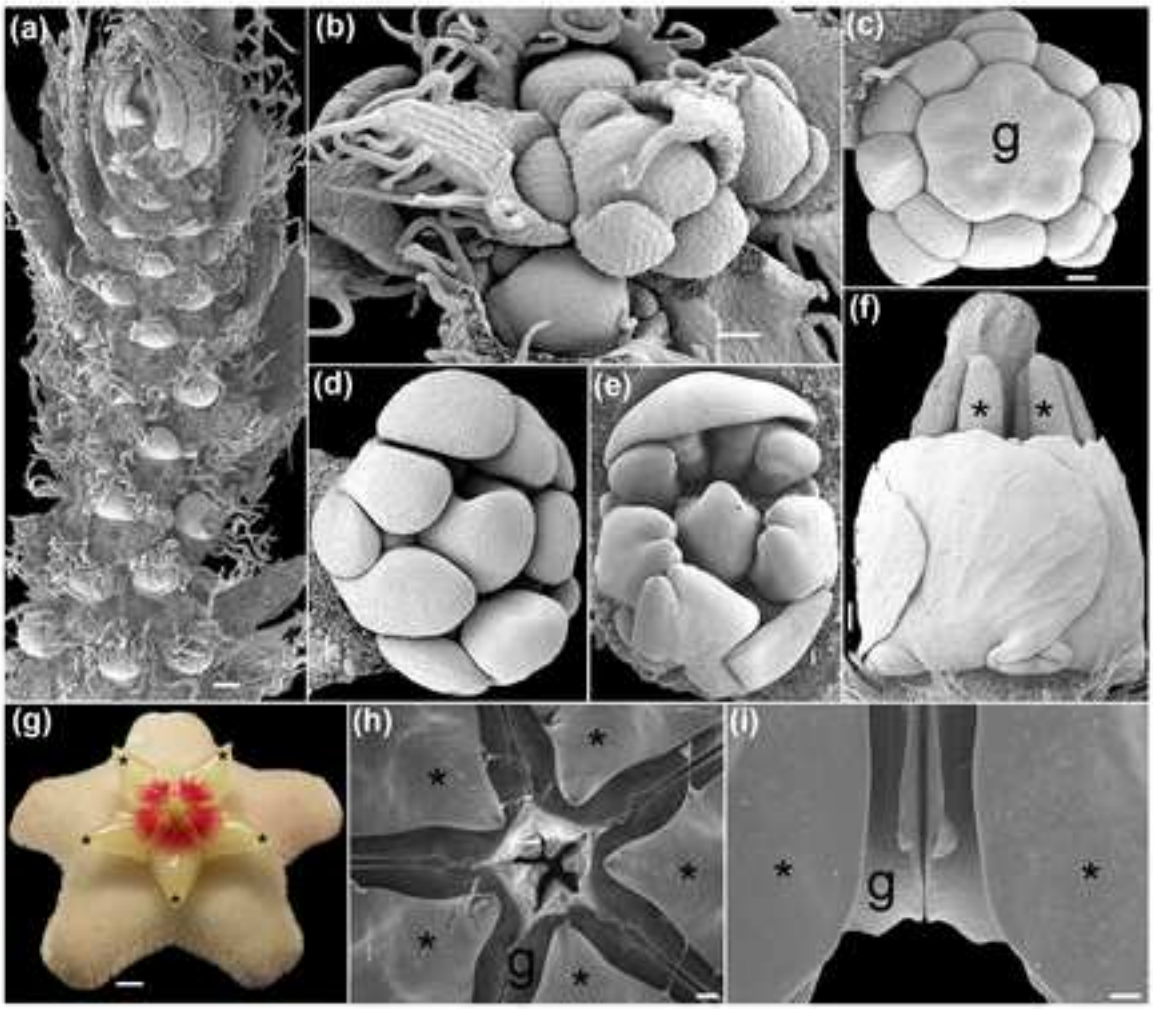

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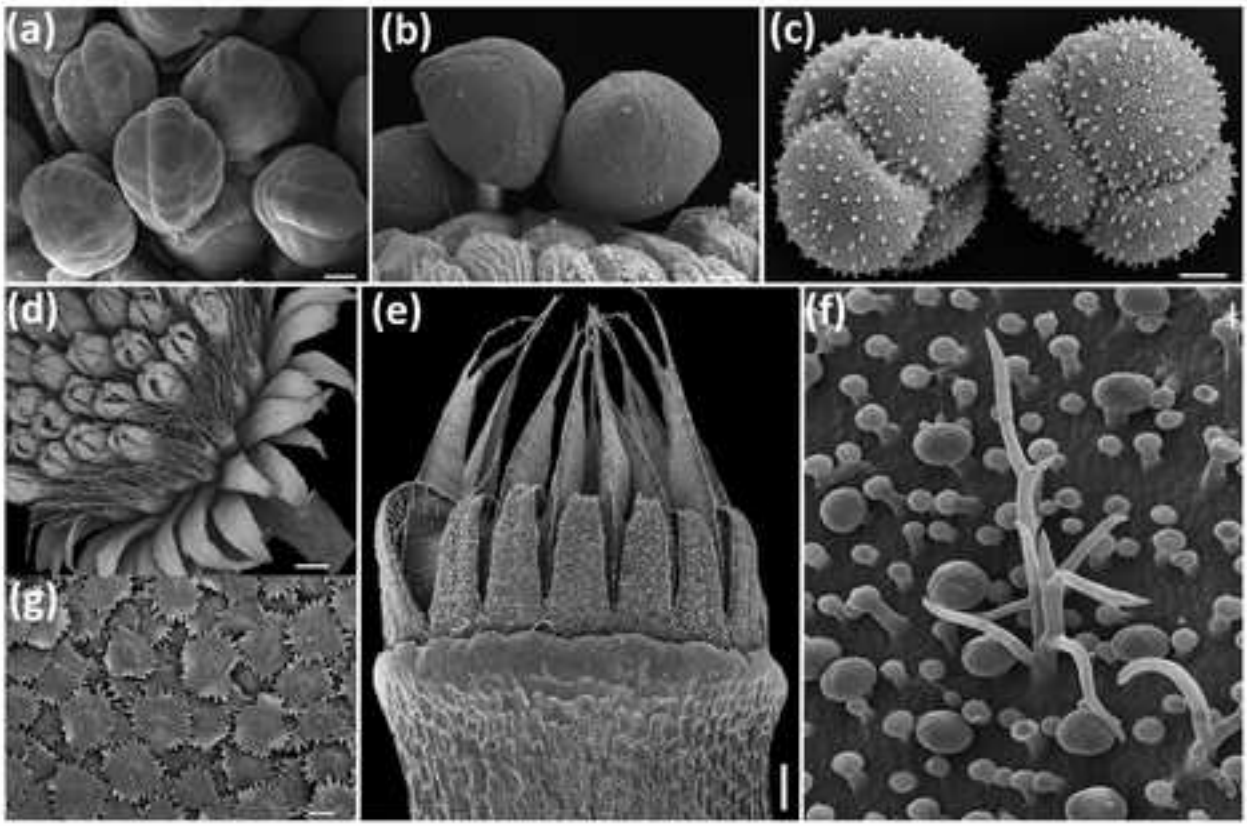

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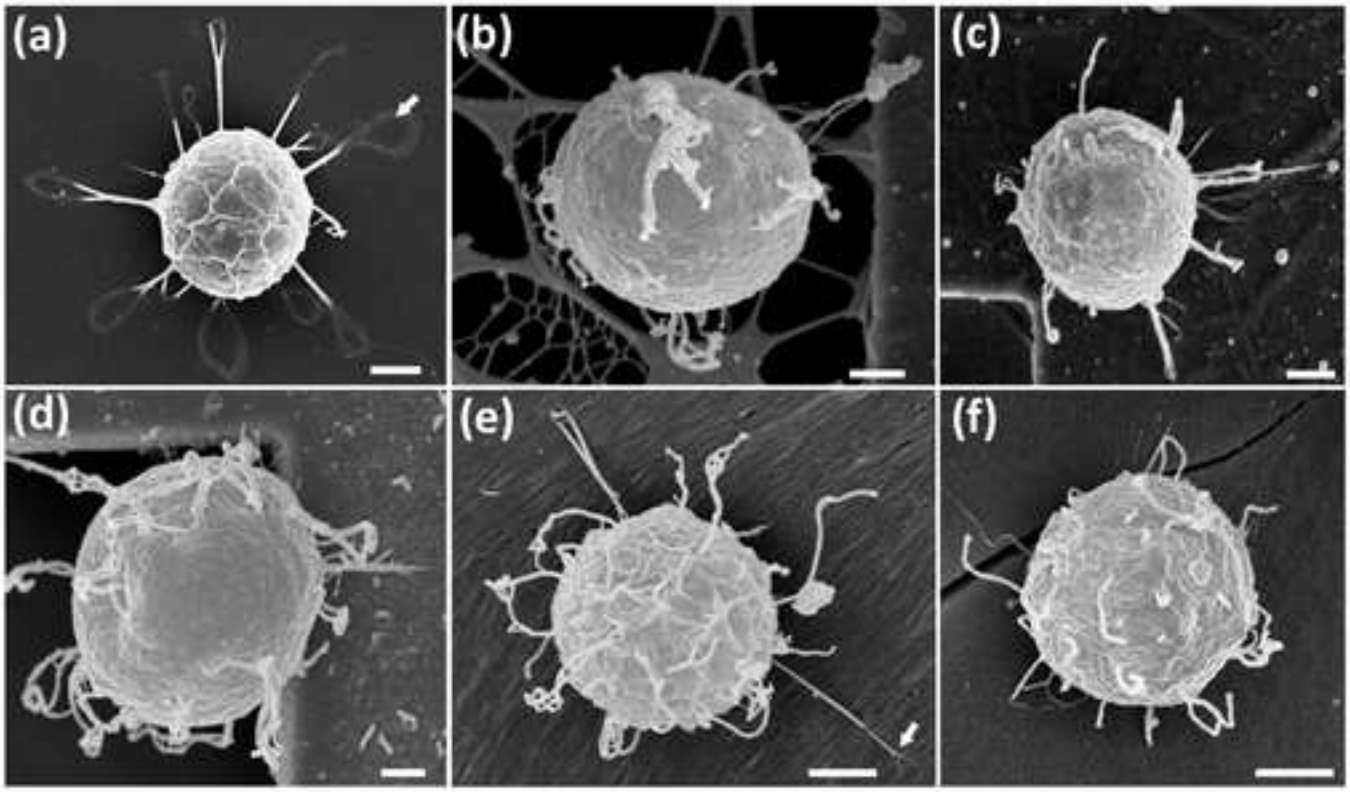

Table 1. Spine morphology using different types of substrate

Surface	Spine morphology	
	Elongation	Loop at the tip
Glass cover	Good elongation, spines easy to count	Present
Fish scales	Spines with curly shape not easy to count	Present
TEM grids	Short and curly spines not easy to count	Absent

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment Materials Table.xlsx

Name	Company
Acetic acid	No specific supplier
aluminium stubs	Ted Pella, Inc.
Centrifuge tubes	No specific supplier
Critical Point Dryer	Polaron Quatum Technologies
D (+) Glucose	Merck
Double sided sellotape	No specific supplier
Ethanol absolute	No specific supplier.
European bacteriological agar	Conda
Filter paper	No specific supplier
Forceps	No specific supplier
Formalin 4%	No specific supplier.
Glass cover slips	No specific supplier
Glass hermetic container	No specific supplier
Glutaraldehyde 25% DC 253857.1611 (L)	Dismadel S.L.
Mycological peptone	Conda
needles	No specific supplier
Petri dishes	No specific supplier
Plastic containers	No specific supplier
Sample holder with lid for the critical point dryer	Ted Pella, Inc.
scalpels	No specific supplier
Scanning Electron Microscope	Hitachi
Software for SEM	
Solution A: NaH_2PO_4	
Solution B: Na_2HPO_4	
Specimen holders	No specific supplier
Sputter coater	Balzers
Stereomicroscope	No specific supplier
Transmission Electron Microscope (TEM) grids	Electron Microscopy Sciences
Tweezers	No specific supplier

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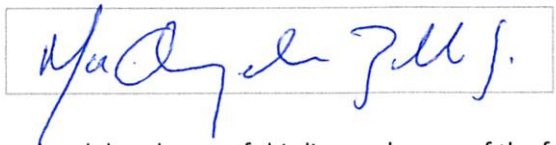
Signature:  Date: May 20th/2016

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Title: Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycete and fungal samples (JoVE55031R1)

Authors: Bello, M.A., Ruiz-León, Y., Sandoval-Sierra, J.V., Rezinciuc S. and Dieguez-Uribeondo, J.

Dear Editorial Board members,

We are submitting the reviewed manuscript JoVE55031R1. We would like to thank the anonymous reviewers for their valuable comments and suggestions. They helped to improve the content and the format of our paper considerably.

One of the major concerns of the reviewers was the content of sections 4, 5 and 6, describing the CPD, coating and SEM protocols. We do agree that most of the steps we had originally depend on the instrument used and could cause problems if performed in different models. We reworded those sections in order to remove instrument-specific instructions.

Additionally, the visualization part was modified and focused in one section (plants) as suggested by the editor. However, few steps from sections 2 and 3 were also included, since those include important modifications for oomycetes and fungi. We are open to modify this selection if required.

The paper was changed following the advice of the reviewers. Specific changes and explanations are detailed in the reply to each reviewer (see below in red). The changes in the manuscript are highlighted and the figures were changed directly on the TIF files.

Yours sincerely,

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Editorial comments

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55031_R1_060916.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

1. Formatting:
-Author names – Middle initials should be placed after the First name. Done.
-References – Please provide DOI where available. Done.

2. Grammar:
-Line 121 – "one of the most diverse and widespread group" Corrected.
-5.2 – "even coatings". Corrected.

3. Visualization: Protocol is highly discontinuous. In order to create a complete, linear narrative, please highlight preparation of one of the three types of samples (section 1, 2 OR 3) and make sure to highlight all steps that are essential to performing the protocol. For section 4, for example, substantial additional material should be highlighted (4.2, 4.4, 4.6-4.8, 4.10-4.12, 4.15) to form a complete narrative. Please re-evaluate the length of the protocol after adjusting the highlighting.

The visualization part is focused now in section 1 (Studies of developing and fully formed plant structures). Additionally we highlighted some parts in sections 2 (Study of cyst behaviour of *Saprolegnia* on different surfaces) and 3 (Study of herbarium fungal spores of *Phellorinia herculanea* under SEM) to illustrate the differences between plant, oomycetes and fungi procedures, particularly in the fixation stages. Because sections 4 (CPD treatment), 5 (coating) and 6 (SEM manipulation) are specific to the model of the instruments, we think it would be more interesting for the public to watch the samples manipulations rather than the instrument settings. However, we are open for further suggestions.

4. Additional detail is required:
-2.1.1 – What is the composition of PG-1 and PGA? If purchased, please include in the materials table. Are the mycelia removed from the agar and placed in petri dishes lacking media? Please clarify. Clarified in section 2.1.1.
-6.2.1, 6.2.2 – How are these actions performed? Manually or via software?

The protocol for section 6 was modified following the reviewers advice. All instructions in this section should follow the manufacturer instructions. Most of the instructions detailed in 6.2.1 and 6.2.2 are performed manually or with the software, but this can change in different SEM models.

5. Branding: Line 460 – thermopar. Done.

6. Results: Figure 2, 3 – Scale bars are not required. Removed.

Reviewers' comments

Editor's Note: We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

Reviewer #1:
Manuscript Summary:
N/A

Major Concerns:
N/A

Minor Concerns:
I just have some suggestions for improvement of manuscript, as following:
LONG ABSTRACT

Page 2, line 74. Change the pharse: Floral meristems fixed with FAA (formalin - acetic acid - ethanol) and processed with the…Done.

INTRODUCTION

Page 3, lines 108, 109 ...of wood (Jansen, 1998). This reference was not included in the reference, as well as was not used the same pattern of the other references. Done.

Page 3, line 130 - (Figure 1a, b). Done.

Page 4, line 151 (Figure 1c, d). Done.

PROTOCOL

Page 4, line 173. Prepare the formalin-acetic-alcohol (FAA) fixative in. Change for

Prepare the FAA fixative solution in Done.

Page 5, line 198. Change - 70% alcohol by 70% ethanol. Done.

line 198. Change put by place. Done.

line 199. Change Fig. by Figure. Done.

line 204. Change Put by place. Done.

line 217. Change Fig. by Figure. Done.

line 221. Change once by since. Done.

line 224. Change put by place. Done.

line 225. Change once by since. Done.

line 228. Change put by place. Done.

line 233. Change Fig. by Figure. Done.

Page 6, line 255. Change fume cupboard by fume hood. Done.

Line 275. Change once by since. Done.

Page 7, line 302. Change Rapidly by quickly. Done.

Page 9, lines 416 and 417. Change (Fig. 5 b,c) and (Fig. 5 d-g) by (Figure 5b, c) (Figure 5d-g). Done.

Page 10, line 436. Change Calonge et al (2011) by Calonge 30. Done.

Line 437. Change (Fig. 1 c,d) by (Figure 1c, d). Done.

Line 441. Change with (b,d) by (b, d). Done.

Line 465. Were pictures g-I captured under SEM? Please, check it. It is necessary to explain what the colors mean in these pictures. Done.

Page 11, line 480. Change: Branched and headed hairs by Glandular and no-glandular trichomes on Done.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
Dear authors,
I was recently invited to review the manuscript JoVE55031R1 entitled "Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycetes and fungi samples" and that you submitted for publication in JoVe. As I mentioned to the editor, I was not familiar with the format of the journal's article and based on my past experience, I think that the structure and the different sections of the text are overall well-written, and all the steps of the protocols are clearly explained. The materials needed are also relatively easy to come by and the experiments to replicate, and together with videos, I think it is perfectly suited for this journal and its format and can see myself using this paper as a reference for my class on microscopy.
However, my main comments or concerns are the use of some terms or the description of structures, which could be done in a more accurate or consistent and coherent manner, to help the reader better connect and remember more easily the different protocols and the importance of each step, results, and corresponding figures together. Thanks to the reviewer comments included in the PDF version we were able to improve the descriptions and their consistency along the manuscript.

I also felt that, at times, the distinction on what was original and what was modified in the protocols could have been easier, and that the improvements you made to the original protocols could also be more strongly emphasized since you have good images showing the importance and benefits of fixation, dehydration, and coating. These protocols are not novel, they represent adaptations to three groups of organisms and few steps represent our original contribution. Asterisks were added to the steps were particular adaptations to the protocol are made.

For these reasons, I recommend your manuscript for publication to the editor, pending mostly minor changes. I also made more detailed comments and suggestions embedded in an electronic copy of the MS, which I think may help you better understand my decision and to revise your manuscript, but I could not find out how to upload it and asked the editor for help.
If you have any questions or comments regarding my decision or revisions, please let me or the editor know, and I sincerely look forward to reading the revised or published version of your manuscript.

Abstract and Intro: Both sections are okay, and with the exception of a few things that could be more consistent like mentioning some of the examples of your wet samples (pollen grains…) in your intro, the contents and sequences are logical and pretty straightforward, and the goals clearly explained. We initially considered as a "wet" material a wide range of samples, thinking in the origin of the sample, e.g. in the case of pollen grains the interior of a young anther, a wet stigma, etc. Samples such as those showed in Fig. 5 are processed following the standard protocol here described in sections 1.1–1.3. Because this can be confusing with the oomycetes protocol, where we manipulate suspensions, we modified the figure legend in Fig. 5.

Protocols: this part lists very clearly and thoroughly all the steps and operations, and while I found the details on the fixation and handling of the specimens justified and helpful, the sections on the CPD, Sputter Coater, and SEM seem a bit redundant with basic instructions found in their operation manuals, and may not always be relevant if the readers have a different piece of equipment. However, depending on the video contents, they would be perfectly appropriate to show all the steps from the preparation to the observation of the samples and I guess it is up to the journal to decide.

We reduced the sections 4, 5 and 6 (CPD, Sputter Coater, and SEM respectively), in order to remove irrelevant steps for the users. We indicated in these sections the conditions we are used to work with as examples.

Representative results: this part explains briefly the benefits of the protocols and the observations they allowed for each type of tissue and organisms, with

proper references to the figures, and maybe could emphasize better on the new steps added to the protocols and their importance in general for preparing SEM samples. The results were reworded.

Figures: the quality and organization of the figures are very good and consistent with the text and the legends.
Discussion: I am not familiar with the format of the paper and the absence of references in the discussion and the emphasis on the benefits of fixing and dehydrating tissues properly could have been stronger, but all the points made are valid and mostly consistent with the goals of the paper. We included some references in the discussion.

Major Concerns:
None

Minor Concerns:
See above and on attached pdf, if made available

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The submitted manuscript "Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycetes and fungi samples" outlines chemical fixation protocols followed by critical point drying and scanning electron microscopy of floral buds and fungal cysts and spores. These samples are difficult to fix as they are hydrated and require delicate handling and preparation for SEM.

Major Concerns:
1. While there is a need for adapted protocols for plant and fungal material compared to mammalian tissue, unfortunately the methodology presented in this paper is overall not novel as critical point drying has been around since the

1960s and the methods presented are not innovative or gold-standard for this field since the development of environmental microscopes.

We are aware of both, that we are not proposing a novel methodology and the development of the environmental microscopes. The JoVE editors do not require in depth or novel results for publication in this journal, only representative results that demonstrate the efficacy of the protocol. Although hundreds of studies using the SEM in plants, oomycetes and fungi are published, the procedures are almost always barely explained and limited to instruments and reagent suppliers due to the usual editorial policies. When we look into the JoVE format, we thought that finally we could have a publication detailing our updated SEM protocols with the organisms we work with, together with a visual aid. This is in itself the innovation, the format how a routine protocol adapted to our organisms is delivered to the audience. We visualized this manuscript as a guide for laboratories where it can be helpful, rather than a gold-standard for SEM technology.

It is true that our protocols are useless for environmental microscopes. However, they could have an impact in most of the studies involving electron microscopy because the SEM associated techniques are more widespread and accessible to many laboratories. Looking through Scopus, the largest database of peer-reviewed literature, the number of published studies with SEM from 2011 to 2016 (plants 4914, oomycetes 21 and fungi 2490) exceed with difference those performed with ESEM (plants 337, oomycetes 1 and fungi 250).

2. FAA is not the best fixative for microscopy as the methanol in formalin prevents optimal penetration of the tissue and slows fixation. Formaldehyde under inert gas, freshly prepared paraformaldehyde or glutaraldehyde are the standard fixatives used in microscopy.

According to Johansen (1940)[1] and Feder and O' Brien (1968)[2], most botanical material can be fixed in FAA for morphological studies because of the rapid penetration of the material by the formaldehyde, which at the same time reacts slowly to the tissue components reducing the damaging effects of subsequent dehydration and inclusion. In the case of the glutaraldehyde, Feder and O'Brien

(1968) claim that this fixative penetrates more slowly in the material causing cellular shrinking and plasmolisis. However, Ruzin (1999)[3] states that glutaraldehyde is an excellent fixative when combined to plastic resins. In our experience with plant material for SEM examination, FAA is an appropriate fixative.

Of concern is the claim in the abstract that this is a "non-toxic" method when they use Formalin-Acetic-Alcohol (FAA) to fix their samples and also glutaraldehyde. These are known toxins and have multiple hazard classes associated with their MSDSs. I am uneasy about encouraging any use of FAA out of the fume hood (Page 4, Lines 166-168) even with a face mask as the vapours from the fixatives can lead to fixation of the eyeball and other mucous membranes. Beyond these safety and quality issues, there is a valid point in being able to use museum specimens that have been fixed in FAA for long term storage for SEM analysis where appropriate.

We acknowledge the reviewer this important comment about FAA safety. We eliminated the words “non-toxic” and also included an additional step with 70% ethanol (1.1.1) when there is not a fume cupboard available for the fixation. Also we rephrased the sentences were we claimed FAA was harmless.

3. As written, the paper does not flow easily from the introduction to the discussion.
For example, the use of multiple types of specimen holders for samples in suspension was interesting and may be of use for other samples in suspension. However, it was a bit unclear from the text why the experiment was being done. A second image showing a different holder giving a better result than glass could be useful for comparing the sample holders.

The aim of testing several substrates was to compare the differential growth of the spines of the *Saprolegnia* cysts on different surfaces. This is important for identification purposes because the same species can display different spine morphologies (Fig. 6) depending on the substrate where they are growing. The glass substrate was “ideal” from the researcher's perspective, because the

elongated and perfectly looped spines were relatively easier for counting and characterizing.

The study of herbarium fungal spores follows a protocol of rehydration followed by dehydration. Was there a specific reason why the samples were rehydrated and then dehydrated when the samples were already dehydrated?

We rehydrated the originally dehydrated sample to be able to see the NURSE CELLS. Interestingly the spores of *Phellorinia* (Agaricales) do not require this rehydration step, ethanol dehydration or the critical point drier for characterization under the SEM. This is important to know since in other organisms such as myxomycetes4 the spores need ethanol dehydration and the CPD treatment for their observation with SEM.

4. For sections 4 (Use of the Critical Point Dryer) and 5 (Use of the Scanning Electron Microscope) the method for these instruments is outlined. These are sophisticated instruments that vary in their use depending on the manufacturer and the model. A generic protocol as outlined in the paper may cause more problems than a simple referral to the manufacturer's guidelines and these sections should be removed. Tips on sample mounting for the CPD and getting the optimal image for these types of samples under the SEM could be useful.

Minor Concerns:
Abstract
Page 2, Line 60. The word "microphotography" could be replaced with micrography or something like sample preparation of difficult samples. Done
Page 2, Line 62. FAA is included but not the glutaraldehyde used in section 2. Please include glutaraldehyde in the abstract. Glutaraldehyde is included now in the abstract and in table 1.

Page 2, Line 66. Change "i.e." for "e.g." as diagnostic characteristics will vary depending on the sample. Done
Page 2, Line 71. Remove "non-toxic". This is not a "non-toxic" method. Removed
Page 2, Line 72. Dynamic processes are difficult to infer from fixed samples. Please attenuate the language. Done
Introduction

Overall the introduction is a repetitive and could be tightened up.

Protocols:

Protocol 1.

Page 4, Line 153. Replace "adult" with fully formed. Done

Page 4, Lines 166-168. Remove "If this step is *glutaraldehyde* without a fume hood, do it in an open area using a face mask and keeping the containers closed when not in use." This is potentially a dangerous suggestion. Removed

Page 4, Line 170. Usually samples are washed three times in the new solution (in this case 70% ethanol). This would be recommended to remove any residual FAA. Included in sección 1.1.4

Page 5, Line 203. "Avoid undesirable particles" - Do you mean dust? Please explain further. Yes, we ment dust

Protocol 2.

Page 5, Line 228. Please clarify that the secondary cyst suspension comes from the sporulation step in section 2.1.1.

Yes they are. Sections 2.1.1 and 2.1.2 were rephrased in order to clarify the initial treatment of these samples.

Page 5, Line 229. It is unclear why we need a selection. This is not outlined in the abstract or introduction, neither is the use of glutaraldehyde.

We used different substrates in order to test if it was a differential growth of the spines, and if so, how this growth was in each case. The use of different substrates is outlined in the long abstract (line 75 page 2) and the introduction (lines 147-149, page 3).

Protocol 3.

Page 6, Line 255. Why would you rehydrate before dehydrating already dehydrated spores?

The initial rehydration of the tissue (step 3.1.1) is to visualize the NURSE CELLS. Originally taxonomists were focused in spore ornamentation, so the rehydration process for surrounding cells was ignored for these organisms. This initial rehydration step allows us to visualize the otherwise collapsed nurse cells.

Page 6, Line 261. Is there a reference for rehydrating fungal spores using the microwave? Are these spores potentially viable?

No it isn't a previous reference. We haven't tried the viability of the spores after the microwave rehydration, but as you can appreciate in fig 1d, the spore wall ornamentation is maintained.

Protocol 4.
Remove the steps for the use of the CPD as each instrument is different depending on the manufacturer and model and not following the manufacturer's guidelines may lead to hazardous situations.
Sample preparation and handling for the CPD is useful for this section.

Protocol 5.
Again, remove the steps for the use of the sputter coater as each instrument is different depending on the manufacturer and model and it may lead to hazardous situations.
Coating with gold and using a rotary stage to ensure even coatings on the specimens is a good suggestion.

Protocol 6.
And again, remove the steps for the use of the SEM. These instruments can be very sophisticated and major (expensive) damage can occur if used inappropriately.
Methods for optimising imaging conditions can be suggested such as slight tilting of the stage (as long as the sample is far away from the pole piece) or use of different kVs. While the use of the back scatter detector (Page 9, line 412) is appropriate, in some instruments, this requires the insertion of the correct detector (by an approved person) prior to loading the sample and is not always a quick and easy way of changing the imaging conditions.

Following the manufacturers guidelines, our SEM allows us a relatively easy interchange between the back scatter detector and the secondary electrons detector (SE). Although sometimes the resultant image has not the best quality,

this change provides more information than a bad quality image taken with the SE. It is worth for unique but bad preserved samples. We rephrased the paragraph.

Representative Results

Page 10, Lines 422-423. "Wet surfaces, which can be overcharged with the electron current if badly dehydrated and coated, can also be well preserved". I thought this was the point of the paper.

These samples used to be difficult to handle for image capture under SEM. An adequate combined treatment of fixation, dehidration and CPD allows better results. We rephrased the paragraph.

Page 10, Lines 430-436. Are these results comparable to other samples that have been observed or is there an alternative sample where a different holder gives a more optimal result?

The protocol for observation of cysts under the SEM has been tested in *Achlya, Aphanomyces, Dyctyuchus, Leptolegnia, Protoachlya* (Saprolegniales), *Lagenidium giganteum* (Lagenidiales), *Phytophthora palmivora* and *Pythium aphanidermatum* (Peronosporales). However, *Saprolegnia* is the only genus where there are cysts with growing spines (Fig. 6). Structures similar to the spines of *Saprolegnia* occur in *Dyctyuchus*, but in this case the structures don't display differential growth on different substrates.

Figures

Figure 1.

Page 10, Line 449. Figure 1a) bud without treatment is as expected awful as is c) compared to d). Why does this need to be shown as this is the reason that CPD has been used since the 1960s?

We think it is good idea to show the samples processed with and without the proposed method in order to compare the results. In the particular case of the spores of this species (fig. 1c-d) the spores were observed without treatment and it was fine for taxonomists because the spore details were observed. However, with the here described protocol the surrounding cells were recovered, providing more information.

Figure 4.
Fig. 4g) is not an SEM image. Please adjust the figure legend. Done
Fig. 4h- i) Please remove the pseudo-colouring or put the non-pseudo-coloured image in beside the coloured image. It is difficult to see the nuances in the grayscale when this is done. Done
Page 11, Line 481. Replace microphotographs with micrographs. Done.
Page 11, Line 483-484. Pollen is not usually a structure from a wet microenvironment.

Pollen grains are difficult samples, particularly when they are attached to wet stigmatic surfaces. Inside the anthers, young stages of pollen development also occur under wet microenvironment. Nevertheless, we changed the heading of Fig. 4.

Figure 6.
Images are pixelated and pseudo-coloured. Remove the pseudocolouring and provide higher quality images. Color was removed and the photograph resolution improved.

Discussion
Page 11, Line 499. This is not a 'harmless' method. It is also only easy to follow if you have had appropriate and thorough training on the use of the CPD and SEM.

We do agree that any instrument for sample preparation requires certain training and a proper laboratory safety control. In several laboratories there are technitians in charge of the security and maintainance of these type of instruments, so they can provide the users the safety guidelines according to each institution. Once our protocol is optimized, it is relatively easy to follow. We reworded the first paragraph of the discussion mentioning safety with the CPD and the SEM and removed the adjective "harmless".

Page 11, Lines 501-502. These reagents are toxic enough to remove this whole point from the manuscript.

This is a method with evident reagent toxicity, but it is relatively less toxic than other protocols such as the one involving osmium tetroxide. The phrase was changed in the discussion.

Page 12, Line 535. This method cannot be used to look at 'dynamic' processes, just snapshots of differences between time points.

We do agree. This method provides the researcher the snapshots to reconstruct stages of structural growth rather than dynamic physiological processes. This part was reworded.

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