

Scanning Electron Microscope (SEM) Imaging to Determine Inflorescence Initiation and Development in Olive

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[Abstract] Here we present a protocol that describes how to image the structure of the olive axillary bud meristem with a scanning electron microscope (SEM) in order to characterize its identity and developmental stage. Briefly, the specimen is fixed with glutaraldehyde, saturated with ethanol, dried in a critical point dryer (CPD) system, dissected, coated with a conducting material and imaged with a scanning electron microscopy (SEM).

Keywords: SEM, Scanning Electron Microscopy, Olive, Flowering, Inflorescence, Meristem, Bud

[Background] The exact timing of flowering induction and inflorescence initiation in olive (*Olea europaea* L.) is in controversy (Haberman *et al.*, 2017). In olive, inflorescences emerge from lateral buds at the end of winter and flower in the spring. We have developed a protocol to better characterize the timing of inflorescence initiation in olive by imaging the meristem in the olive bud with a SEM at different times during the year. In these SEM images the meristem structure can be identified unambiguously, and the definition level of the meristem can be much higher than images of bud meristem sections presented in previous studies.

Materials and Reagents

1. Scalpel blade No. 11 (Sigma-Aldrich, catalog number: S2771)
2. Double-sided adhesive tape
3. Glass scintillation vials with screw caps, volume 20 ml (Sigma-Aldrich, catalog number: Z190535)
4. Pipette (BRAND, catalog number: 747760) or a similar instrument
5. Gold annular target for the sputter coater (Agar scientific, catalog number: AGB7370)
6. Olive (*Olea europaea* L.) buds
7. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S3264)
8. Sodium phosphate monobasic (NaH_2PO_4) (Sigma-Aldrich, catalog number: S3139)
9. 25% glutaraldehyde (Sigma-Aldrich, catalog number: G5882)
10. Ethanol absolute (Sigma-Aldrich, catalog number: 24102)

11. Optional: Technical grade ethanol (Sigma-Aldrich, catalog number: V0T0042)
12. 0.1 M phosphate buffer pH 7.2 (sodium phosphate buffer; see Recipes)
13. 5% glutaraldehyde solution (in 0.1 M phosphate buffer pH 7.2; see Recipes)

Equipment

1. Scalpel handle No. 3 (Sigma-Aldrich, catalog number: S2896)
2. Tweezers style #5 (Sigma-Aldrich, catalog number: T4537)
3. Critical point dryer system (BAL-TEC, model: CPD 030)
4. Stereo-microscope (Olympus, model: SZX12)
5. Sputter coater (E510 scanning electron microscope coating unit) (Polaron Instruments, model: E510)
6. Scanning electron microscope (JEOL, model: JSM-5410 LV)

Procedure

1. Using a scalpel, separate the axillary buds from the shoot. Leave a portion from the stem connected to the bud for gripping the bud with tweezers. Cut the opposite side to the bud of the stem piece vertically so that you can place the sample with the bud facing up (see Figure 1B).
2. Add 5-10 ml (enough to cover the buds) of the 5% glutaraldehyde solution (see Recipes) to a scintillation vial. Immerse the buds in the glutaraldehyde solution for 24 h at room temperature (fixative solution [Sabatini *et al.*, 1963]; see Figure 1C).
3. Use a pipette to remove the glutaraldehyde solution from the vial.
4. Wash the buds by adding phosphate buffer (see Recipes) to the buds. After 10 min, remove the buffer. Repeat this wash step 5 times.
5. Add 25% ethanol solution, suspend for 1 h and remove the solution.
6. Add 50% ethanol solution, suspend for 1 h and remove the solution.
7. Add 75% ethanol solution, suspend for 1 h (break point) and remove the solution.
Note: If desired the fixing procedure can be suspended at this step (75% ethanol solution). Buds can be stored in a 75% ethanol solution at 4 °C over-night or up to several weeks.
8. Add 95% ethanol solution, suspend for 1 h and remove the solution.
9. Add 100% ethanol solution, suspend for 1 h and remove the solution.
10. Add 100% ethanol solution.
11. Dry the buds in a Critical point dryer (CPD) instrument according to manufacturer's instructions (the drying procedure should take about 2 h).
12. Use tweezers to hold the dry bud by the stem piece. Using a stereo microscope, carefully remove the leaf primordia from the bud with a scalpel or tweezers (Style #5). Normally, after 4-5 pairs of leaf primordia are removed, the meristem is exposed.

Note: This is a crucial step, take the time to gently remove the leaf primordia without damaging the meristem.

13. Bond a piece of double-sided adhesive tape to a metal stub (the stub goes into the SEM; see Figures 1E and 1F). Use tweezers to bind the stem piece of the dissected bud to the metal stub, with the exposed meristem facing upwards (see Figure 1G).

14. Coat the specimen with gold (Au) or gold/palladium (Au/Pd) in a sputter coater instrument according to manufacturer's instructions (see Figures 1H and 1I).

**Coating parameters we used: vacuum 0.02 Mbar, sputtering voltage 2.4 KV, current 20 mA, coating time 150 sec.*

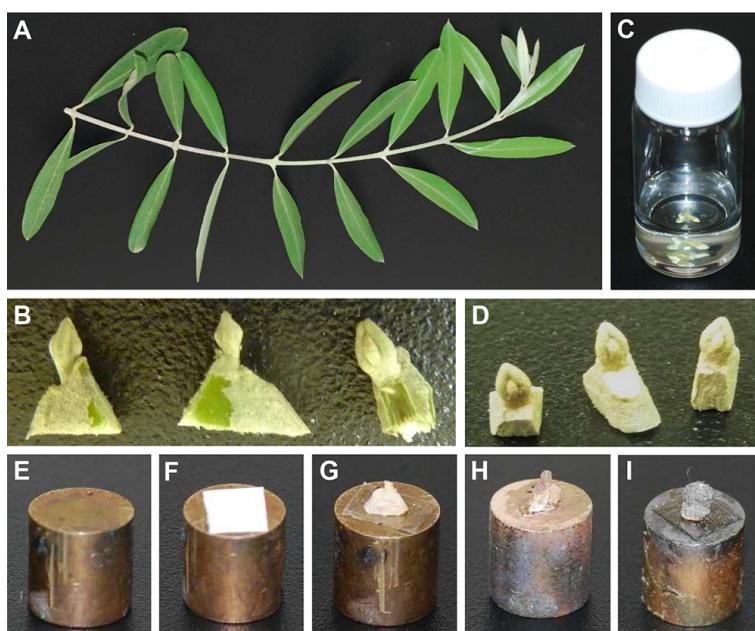


Figure 1. Preparation of the specimen for imaging. A. Picture of the olive shoot; B. Separated bud samples with attached stem pieces; C. Bud samples immersed in the fixative solution in a scintillation vial; D. Dried bud samples after drying in the CPD instrument; E. Metal stub used for the SEM imaging; F. Metal stub with a piece of double-sided adhesive tape; G. Dissected dried bud sample mounted on a metal stub; H. Dissected dried bud sample coated with gold; I. Dissected dried bud sample coated with gold-palladium.

15. Place the stub in the SEM and operate the SEM according to manufacturer's instructions to produce images of the meristem (see Figure 2).

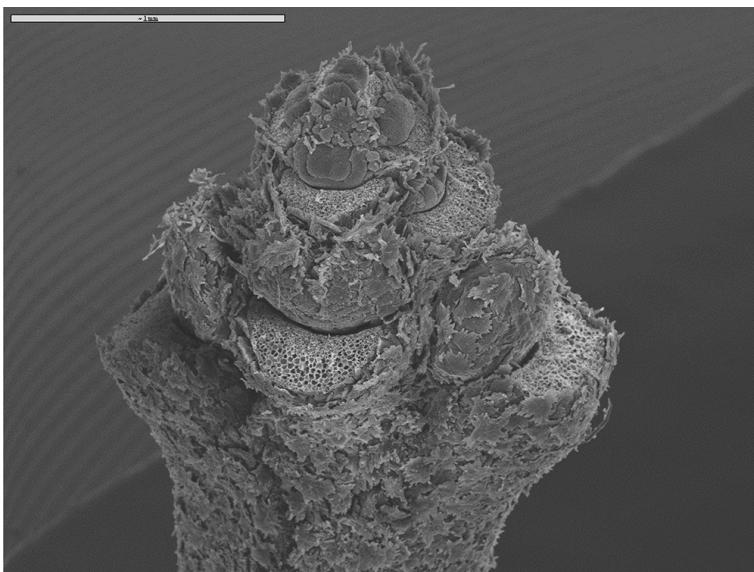


Figure 2. SEM image of a whole specimen. Taken under high vacuum condition at accelerating voltage of 20 kV. Bud was sampled on 23 February 2011. Scale bar = 1 mm.

Data analysis

Crop the images to zoom and center the area of interest in the image, make sure you retain an accurate scale bar. Create a figure from the images you produced, using the software of your choice (see examples for figures created with PowerPoint [Microsoft] in Figures 3 and 4).

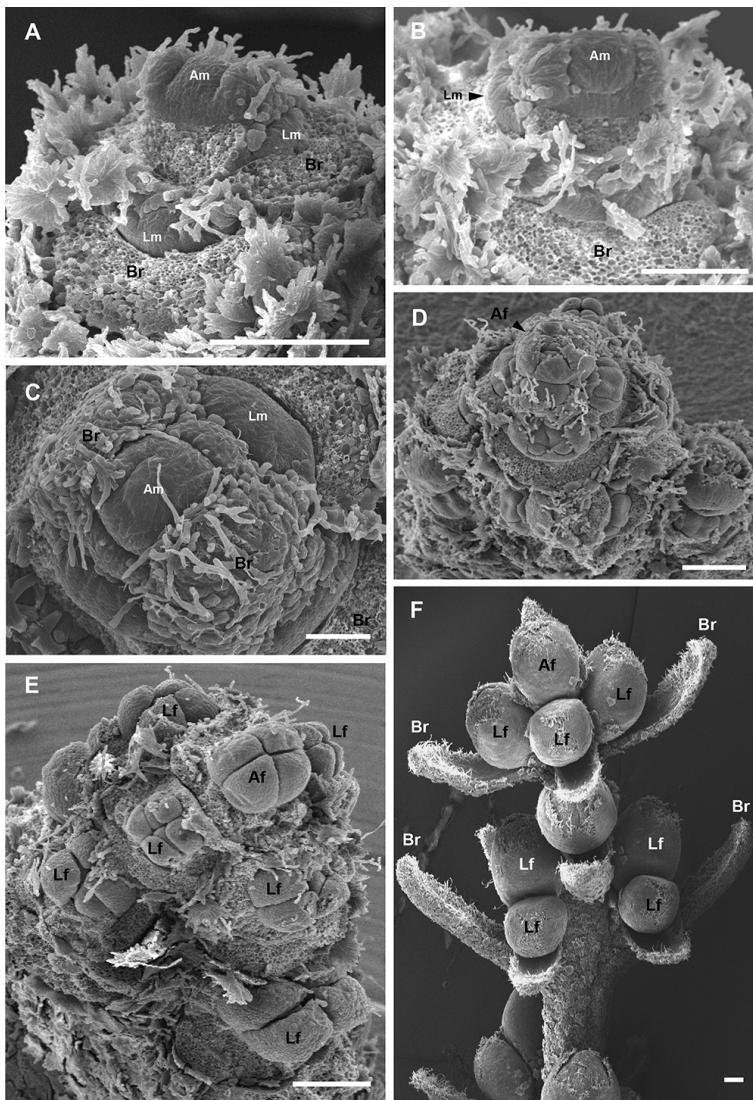


Figure 3. Development of inflorescence in *Barnea* olive. SEM images showing olive inflorescence at different stages of development. Images were produced from lateral buds sampled on 9 February 2011 (A), 16 February 2011 (B), 23 February 2011 (C-E) and 7 February 2010 (F). A-C. Initial stage of inflorescence development. The apical meristem (Am) do not initiate new leaf primordia, enlarge and bulge ('dome') subsequently becoming a terminal flower meristem. Lateral flower meristems (Lm) surrounding the Am begin bulging. Leaf primordia develop into bracts (Br). D-E. Subsequent stages of inflorescence meristem development. Apical flower meristem (Am) develops into apical flower (Af). Lateral flower meristems (Lm) differentiate into lateral flowers (Lf). F. Inflorescence before anthesis. Scale bar = 0.25 mm.

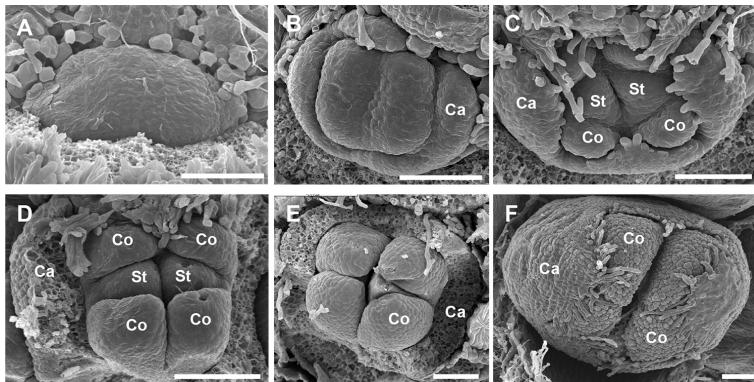


Figure 4. Development of Olive flowers. SEM images showing olive flowers at different stages of development. Images provided from *Barnea* olive lateral buds sampled on 23 February 2011. A. Initial bulging ('doming') and enlargement of the meristem; B. The periphery of the meristem differentiates into the calyx (Ca; sepals); C-E. Subsequently, the central floral meristem develops into the corolla (Co) and two stamens (St), the pistil is not seen. F. Flower bud before anthesis. Scale bar = 0.1 mm.

Notes

1. The diluted ethanol solutions can be prepared from cheaper technical grade ethanol (See Materials and Reagents #11).
2. This protocol can be implemented in other plant species as demonstrated in apple (*Malus domestica* Borkh.; Haberman *et al.*, 2016) and passion fruit (*Passiflora edulis* Sims; Nave *et al.*, 2010).

Recipes

1. 0.1 M sodium phosphate buffer pH 7.2 (1 L)
 - a. First produce 1 M stock solutions of Na_2HPO_4 (dibasic) and NaH_2PO_4 (monobasic). Dissolve 141.96 g of Na_2HPO_4 in distilled H_2O and complete the final volume to 1 L. Do the same for 119.98 g of NaH_2PO_4 .
 - b. Mix 68.4 ml of 1 M Na_2HPO_4 with 31.6 ml of 1 M NaH_2PO_4 and dilute to a final volume of 1 L. You get 1 L of 0.1 M sodium phosphate buffer pH 7.2.

Note: If you are using a hydrated sodium phosphate, make sure you adjust the amount of sodium phosphate for the production of the 1 M solutions.

2. 5% glutaraldehyde solution in phosphate buffer
Dilute your glutaraldehyde solution according to its initial dilution in your 0.1 M phosphate buffer (Recipe 1). If you are using a stock solution of 25% glutaraldehyde, for a final volume of 100 ml, mix 20 ml of the 25% glutaraldehyde solution with 80 ml of the phosphate buffer.

Note: Glutaraldehyde is toxic and a strong irritant, always wear gloves, work in a chemical hood, dispose of it properly.

Acknowledgments

The fixation method in the protocol was composed according to the book, Fixation for electron microscopy (Hayat, 1981).

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