

Cytohystological Analyses of Mega-sporogenesis and Gametogenesis in Ovules of *Limonium* spp.

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[Abstract] *Limonium* spp. are known to have sexual and apomixis (asexual reproduction through seeds) reproductive modes. Here, we present dissection protocol developed for ovules of *Limonium* spp. using differential interference contrast (DIC) microscopy. This protocol permits better handling of ovules and offers certain advantages over earlier techniques particularly in larger ovules. This method also enables observation of meiosis and embryo sac development in intact ovules, and the readily detection of events distinguishing sexual and apomictic development.

[Background] To describe the events that occur during ovule development it is necessary to cytologically examine ovules. This study can involve microscopic observation of paraffin- or resin-embedded, sectioned material, or cleared organs. The first cytological investigations into ovule and embryo sac development in sexual and apomictic *Limonium* species were published in the pioneer works of D'Amato (1940; 1949). In these works, flowers were fixed using the Karpechenko's method, embedded in paraffin, sectioned and stained with Heidenhain's iron haematoxylin, which stains chromatin and chromosomes in the cell nuclei. Flower buds sectioning using these methods can result in preparations with poor quality, due to partial disruption structural integrity of individual cells. A more facile alternative is clearing formalin:acetic acid:ethyl alcohol fixed organs and staining with pure Mayer's hemalum (Wallis, 1957; Stelly *et al.*, 1984). This technique requires much less time and labor, particularly for species which usually only form a small ovule within the ovary, which is the case of *Limonium* spp. However, in both small and large ovules chloral hydrate worked better than methyl salicylate as a clearing solution, because in this latter fluid ovules become quite fragile and difficult to handle during experiments. Our approach with an enzymatic digestion of ovules helps to reveal the central mass of tissue, the nucellus and the two integuments it covers, particularly in large ovules. Examples of meiotic and ameiotic ovules and embryo sacs cleared in chloral hydrate were observed under differential interference contrast optics.

Materials and Reagents

1. Glass microscope slides (Belden, Hirschmann, catalog number: 8210101)

2. Diagnostic microscope multiwell slides (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 101432648 EPOXY)
3. Glass coverslips (24 x 50 mm) (Belden, Hirschmann, catalog number: 8000119)
4. Teasing needle (BioQuip Products, catalog number: 4751)
5. Micropipette tips (2-20 µl, 20-200 µl, 100-1,000 µl) (Eppendorf)
6. Six to eight months old *Limonium* plants in the flowering period
7. Formaldehyde (36% aqueous solution) (VWR, catalog number: 20909.290)
8. Glacial acetic acid (Thermo Fisher Scientific, Fisher Scientific, catalog number: 10304980)
9. Distilled H₂O
10. Ethanol (absolute 99.5%) (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP2818500)
11. Hematoxylin solution, Mayer's (Sigma-Aldrich, catalog number: MHS16)
12. Chloral hydrate (Sigma-Aldrich, catalog number: 15307)
13. Cellulase (Sigma-Aldrich, catalog number: C1184)
14. Cellulase 'Onozuka R-10' (Serva, catalog number: 16419)
15. Pectinase (Sigma-Aldrich, catalog number: 6287)
16. Citric acid-1-hydrate (Sigma-Aldrich, catalog number: 33114)
17. Sodium citrate dehydrate (AppliChem, catalog number: 131655)
18. FAA solution (see Recipes)
19. Dehydration solutions (see Recipes)
20. Hydration solutions(see Recipes)
21. 0.1% chloral hydrate (see Recipes)
22. Enzymatic mixture (see Recipes)
23. 1x EB (Enzyme buffer) (see Recipes)

Equipment

1. Forceps with extra fine tips (Rubis forceps [BioQuip Products, catalog number: 4524])
2. Glass watch glasses (O.D. 25 mm and 40 mm) (Sigma-Aldrich)
3. Stereo microscope (Leica, model: WILD M3Z LEITZ)
4. Fluorescence microscope (Carl Zeiss, model: Axioskop 2)
5. Differential interference contrast (DIC) optics
6. Micropipettes (2-20 µl, 20-200 µl, 100-1,000 µl) (Eppendorf)
7. AxioCam 289 MRc5 digital camera (Carl Zeiss)

Software

1. Adobe Photoshop 5.0

Procedure

1. Collect flower buds at different developmental stages ranging from 2 to 6 mm in length (Figure 1A, left – young stage; right – mature stage) using forceps. Flower buds are fixed in 1 ml FAA solution in a watch glass for 24 h at 4 °C. The fixed material may be stored in 1 ml 70% ethanol at -20 °C until use for 3 months. To this end, after fixation the flower buds should be dehydrated in a series of water/ethanol (v/v) solutions, 30%, 50%, 70%, shaking for 30 min each before storage.

Note: As the flowering season occurs during a short period of the year, storage can be useful to save flower buds for further use.

2. Dissect pistils from flower buds with forceps onto a microscope slide using a stereomicroscope. Make a sharp longitudinal incision in the ovary (Figure 1B, incision arrowed) with a needle to help subsequent ovule fixation in 1 ml FAA solution for 14-18 h, at 4 °C.

Note: Alternatively, big ovaries (> 5 mm) can be dissected directly in a drop (5 μ l) of FAA, and then proceed to step 3.

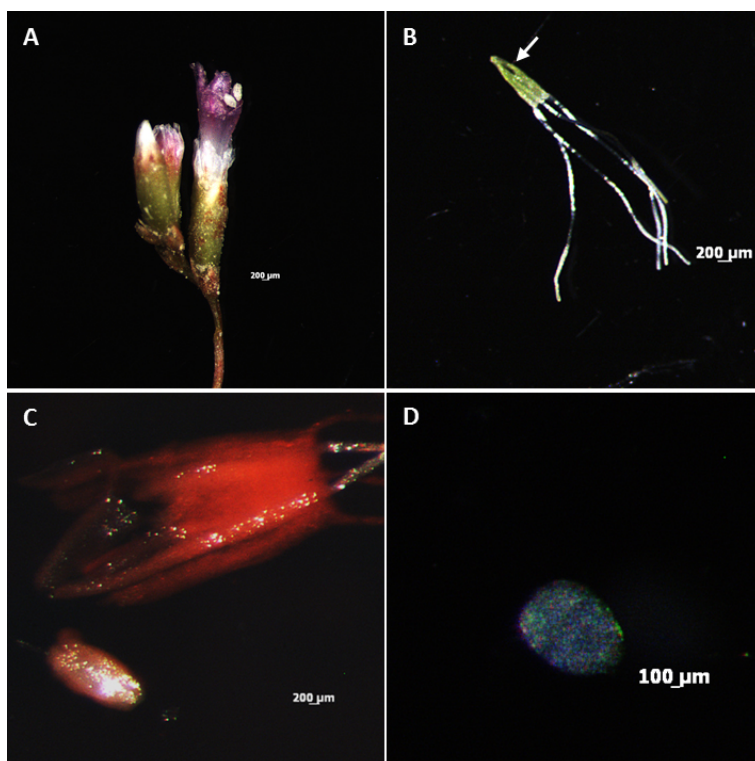


Figure 1. Dissection procedure and obtainment of digested ovules of *Limonium* spp. A. Flower buds at different developmental stages ranging from 2 to 6 mm (Figure 1A: left – young stage; right – mature stage). B. Pistil showing a longitudinal incision in the ovary (arrowed). C. Stained ovule immediately after dissection. D. Digested ovule mounted in a drop of chloral hydrate, in well of a multiwell slide.

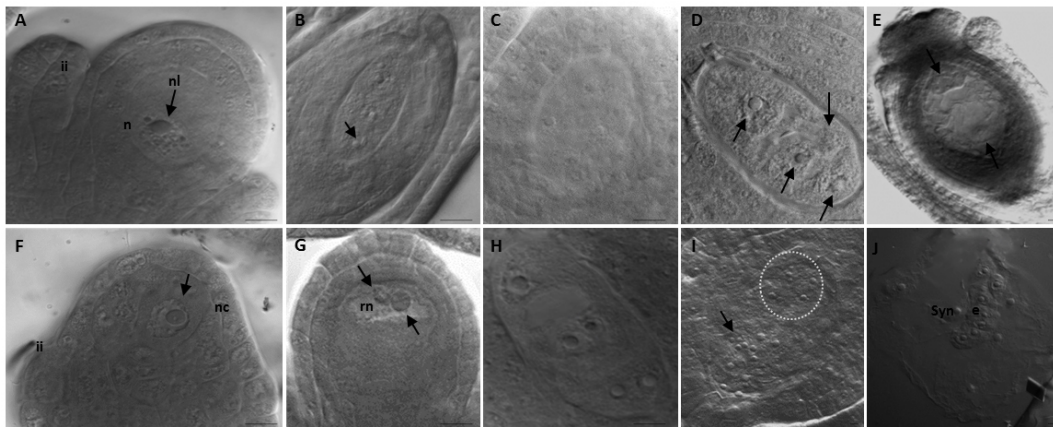


Figure 2. Examples of meiotic and ameiotic ovules and embryo sacs cleared in chloral hydrate and observed under differential interference contrast (DIC) optics. Figures A-E show sexual diploid ($2n = 2x = 16$) *Limonium ovalifolium* plants: accession numbers 2009I4SR (Sra. Remédios, Peniche), and 2009I1PL (Praia da Luz, Lagos), 2009I1PL (Praia da Luz, Lagos). Figures F-J were taken from apomictic tetraploid ($2n = 4x = 35, 36$) *Limonium multiflorum* plants: accession numbers 2009I4VF (Vale dos Frades, Lourinhã), 2009I9CR (Cabo Raso, Cascais), 2009I4VF (Vale dos Frades, Lourinhã). A. Megaspore mother cell at interphase with one nucleolus (arrowed). B. Coenomegaspore with four unreduced nuclei, each nucleus with two nucleoli (arrowed) (4-nucleated stage; tetrasporic development). C. Coenomegasporocyte at the beginning of gametogenesis with a cruciate nuclear arrangement. D. Megaspores in a bipolar 1 + 3 arrangement (arrowed) with the embryo sac showing extended vacuolization. E. Detail of an embryo sac of *Adoxa*-type with the egg cell and inferior polar nuclei arrowed. F. Megasporocyte within ovule showing one nucleolus (arrowed). G. Restitution nuclei after first meiotic division (meiotic diplospory). Nucleoli with distinct dimensions within the same nucleus are arrowed. H. Coenomegasporocyte at the beginning of gametogenesis. I. Detail of the egg cell apparatus (encircled by dashed lines) in a mature embryo sac; antipodal cells towards the chalazal pole are arrowed. J. Parthenogenetic embryo formation within a mature sac. Bars = 10 μm .

Note: Each image is oriented so that the micropyle is towards the top of the page and the chalaza is towards the bottom. Figures abbreviations: e – embryo; ii – integuments; n – nucleus; nl – nucleolus; nc – nucellus; rn – restitution nucleus; Syn – synergid.

3. Afterwards, hydrate pistils in watch glasses in a series of progressive 1 ml water/ethanol solutions, 50%, 75% and 100%, shaking for 30 min each.
4. Stain pistils with 1 ml pure Hemalum Mayer's solution for 24-48 h in a watch glass, and then place them in 1 ml 0.1% chloral hydrate in a new watch glass during 1-2 h, at room temperature. *Note: If the ovaries are small (< 3 mm) 24 h in the staining solution are required; for bigger ones 48 h may be needed.*
5. Thereafter, dissect ovules from ovaries (Figure 1C) onto a glass slide under a stereomicroscope. Then, place them in a watch glass for digestion with 500 μl of an enzyme mixture 2% (w/v)

cellulase, 2% (w/v) cellulase 'Onozuka R-10', and 2% (v/v) pectinase in 1x EB, for 10 (young stages) to 20 min (mature stages) at 37 °C, inside a box on a paper humidified with distilled water.

6. Remove the enzyme mixture by a micropipette and wash ovules in a watch glass, in 1 ml 1x EB solution for 3-5 min, at room temperature.
7. Finally, mount ovules in multiwell slides in a drop (5 µl) of chloral hydrate, under a coverslip (Figure 1D).
8. When the procedure is completed, cytohistological observations are made using a fluorescence microscope with DIC optics using 20x and 100x objectives (Figure 2).
9. Images are collected with a digital camera and if necessary further processed using Adobe Photoshop 5.0.

Data analysis

Female gametophytes of sexual and/or apomictic origin were determined in accessions from the ISA collections, and cytohistological observations were made under 20x and 100x magnification. For each species the development pattern ovules during megasporogenesis and gametogenesis were examined, and the number and frequency of tetrasporic versus diplosporous embryo sacs were evaluated (c. 700). In sexual species tetrasporic embryo sacs of *Gagea-ova*, *Adoxa*- and *Drusa*-types were found while apomictic species only produced diplosporous meiotically-unreduced eggs of the *Rudbeckia*-type. Details on numerical analysis of these ovules are published in Róis *et al.* (2016).

Recipes

1. FAA solution

Formaldehyde-acetic acid-ethyl alcohol in proportions of 1:1:18 (38.5% formaldehyde, 5% acetic acid, 70% ethanol)

For 100 ml mix:

5 ml of 38.5% formaldehyde

5 ml of glacial acetic acid

90 ml of 70% ethanol

Note: Use always a fresh solution. Do not store it.

2. Dehydration solutions

30% EtOH-70% H₂O

50% EtOH-50% H₂O

70% EtOH-30% H₂O

Note: Make fresh solutions every three months.

3. Hydration solutions

50% EtOH-50% H₂O

25% EtOH-75% H₂O

100% H₂O

Note: Make fresh solutions every three months.

4. 0.1% chloral hydrate

For 50 ml mix:

50 mg of chloral hydrate

50 ml of distilled water

Notes:

a. Make a fresh solution every three months.

b. Chloral hydrate is toxic if swallowed. Can be irritating to eyes and skin.

c. Store in a cool, dry, well ventilated place, in securely closed original container.

5. Enzymatic mixture

For 20 ml:

606 µl pectinase

0.12 g celulase 'Onozuka R10'

1.10 g celulase Sigma C-1184

Add 20 ml 1x PBS

Note: Store 500 µl aliquots at -20 °C.

6. 1x EB (enzyme buffer)

For 100 ml mix:

40 ml of 1 M citric acid-1-hydrate

60 ml of 1 M sodium citrate dehydrate

pH 4.8

Note: 1x EB buffer should be kept at 4 °C for 1-2 weeks. For longer periods of time, buffer should be stored at -20 °C.

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