Flora 296 (2022) 152168

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

Salient ovule features of *Zamia furfuracea* L. fil.: Early stages of megagametophytogenesis

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ARTICLE INFO

Edited by Favio Gonzalez

Keywords:
Alveoli
Cycadales
Free nuclei
Megagametophyte
Ovule
Zamia

ABSTRACT

The anatomy of the megagametophyte of *Zamia furfuracea*, a cycad species with a short reproductive cycle, is described in the first stages of its ontogeny corresponding to the free nuclear and alveolation stages. The way in which the free nuclei are arranged centripetally to later organize themselves into a series of radially elongated hexahedral and short pyramidal trihedral or tetrahedral alveoli, forming rays that converge in the center of the megagametophyte, is noteworthy. In cross section, the hexagon-shaped alveoli together give the megagametophyte the appearance of a honeycomb. A three-layered jacket of transfer cells with dense cytoplasm and prominent nuclei is clearly recognizable and its anatomy is associated with nutrient translation that is compared to a tapetum like jacket. These two stages are continuous and occur within an interval of one week in the summer.

1. Introduction

The ontogeny of the ovule to seed of an angiosperm, referring to the time that takes place between the ovule initiation until embryo body development, can take month or less. In contrast, in gymnosperms, this process takes between one and three years. (Singh and Johri, 1972). According to Schnarf (1933), Singh (1961, 1978), Dehgan and Johnson (1983), Owens et al. (1997) and Hann-Chung and Yu-Fen (2001), the development cycle is long in temperate climate gymnosperms and short in tropical zones. It determines the time interval in which the different stages of the female reproductive cycle occur, which also are closely related to seasonality. Thus, ovule initiation occurs in autumn; megasporogenesis in winter; and pollination in spring upon completion of megasporogenesis. In Cycadales, the free nuclear stage of megagametophytogenesis coincides with pollination and there is an interval that occurs in the summer before fertilization with mature seeds containing an embryo present the following autumn (Singh and Johri, 1972; Norstog and Nicholls, 1997; Sanchez-Tinoco et al., 200). According to Singh and Johri (1972), the development of the ovules within a cone as well as in a population is usually synchronous.

The chronology of ovule and seed development in Zamiaceae has been studied in *Ceratozamia tenuis* (Dyer) D. W. Stev. & Vovides (Vovides et al., 2016), a cloud forest species. Its cycle is completed in two years. During autumn and winter, megasporogenesis and megagametophytogenesis occur. At the end of winter, the gametophyte is in the stage of free nuclei (coenocytic). Cellularization of the gametophyte ends in spring (Sánchez-Tinoco et al., 2000). In contrast, the reproductive cycle of *Zamia furfuracea*, a coastal dune species, develops in one year. Megasporogenesis and megagametophytogenesis occur in summer. The period of cell wall formation or cellularization extends from summer to early autumn (Sánchez-Tinoco et al., 2012; Sánchez-Tinoco et al., 2018). On the other hand, the ovule-to-seed development in *Dioon edule* Lindley, a species from low deciduous forest, has been estimated to take three years (Vovides and Iglesias, 1994).

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https://doi.org/10.1016/j.flora.2022.152168
Received 21 December 2021; Received in revised form 14 October 2022; Accepted 17 October 2022

The initiation of the ovules and the first stages of their development have not been studied in detail in many cycads, due to the difficulty of obtaining reproductive structures or megastrobili in their initial stages (Quisumbing, 1925; Chamberlain, 1935; Norstog, 1990). This is because the megastrobili differentiation takes place within the stem tissue (Stevenson, 2020), and by the time they emerge, a crown of cataphylls and petiole bases tightly surrounds them (Stevenson, 1988).

The classic literature on the first stages of ovule development in cycads dates from the beginning of the 19th century (Coulter and Chamberlain, 1903; Chamberlain, 1906; Smith, 1910). It is fragmented and sometimes contradictory in terms of species studied, morphogenesis, and anatomical descriptions. Histologically, isolated events of the early stages of ovule development such as megasporogenesis or megasporangium ontogeny and megagametophytogenesis in free nuclear stages, wall formation and archegonium development were described by Chamberlain (1906, 1919, 1935), Smith (1910), DeSloover (1964), Maheshwari and Singh (1967) and Singh, 1978. Their graphic illustrations and descriptions are valuable and provide starting points for more detailed studies on other species. Regarding megagametophytogenesis, it is a process that begins after the differentiation of the functional megaspore within the nucellar tissue. After innumerable karyokineses, further development and differentiation are followed by successive stages that later form the cellular gametophyte, archegonium, egg cell. After fertilization there will be an embryo within the megagametophyte, which supports germination and the seedling in its first years, both in cycads and other gymnosperms (Singh, 1978; Favre-Duchartre, 1957; Sánchez-Tinoco et al., 2000).

With the exception of *Gnetum* and *Welwitschia*, Maheshwari and Singh (1967) described the early stages of megagametophytogenesis in gymnosperms with a uniform pattern consisting of: (i) numerous synchronous nuclear divisions, the resulting number of free nuclei being constant for some species (Favre-Duchartre, 1957); (ii) a large central vacuole in the young gametophyte (Chamberlain, 1935); (iii) formation of anticlinal walls arranged in a centripetal direction that when completed, give the appearance of a honeycomb to the young gametophyte. Each cavity of the honeycomb is an alveolus (Maheshwari and Singh, 1967; Singh, 1978). In the alveoli, the nuclei are arranged in the periphery and by repeated periclinal divisions form rows of cells arranged in rays from the center to the periphery. However, according to Mauseth (1988), alveoli as the result of peripheral wall formations that form open boxes that continue to grow towards the central vacuole is a controversial issue.

Subsequently, by irregular divisions, the cellular gametophyte is formed (De Sloover, 1963). When cellularization is complete, cell differentiation occurs at the micropylar level, initiating the development of the archegonia (Chamberlain, 1919, 1935; Maheshwari and Singh, 1967).

To complement the aforementioned information, we developed the present study whose objective is to describe the anatomy of the ovule of *Zamia furfuracea* L. fil. in the early stages of megagametophyte ontogeny including free nuclei and alveolation.

2. Materials and methods

Previous records made in a natural population located in the ejido Ciénega del Sur, Municipality of Alvarado, Veracruz, Mexico, during the years 2004, 2005, 2017 and 2018 allowed the identification of outstanding events of the female reproductive chronology of *Z. furfuracea*. These events correspond to the emergence of megastrobili; free nuclei stage, wall formation, hardening and accumulation of nutrients in the gametophyte, lignification of the sclerotesta, archegonial development, dispersal and development of the suspensor.

These data were used to identify the first stages of megagametophytogenesis. Ovules were collected from plants in reproductive stages from that locality, planted in garden conditions in the city of Xalapa, Veracruz, Mexico, with annual repetitions in the month of June during the years 0.2020 to 2022. The ovules were collected when 1/3 of the length of the megastrobilus had emerged (Fig. 1A). Transverse sections of the megastrobilus (Fig. 1B) as well as some ovules were fixed in 37–40% formaldehyde, glacial acetic acid, 95% ethyl alcohol and water (10:5:50:35) (FAA) (Sass, 1958). These sections were washed repeatedly in the laboratory and preserved in glycerin, 96% ethyl alcohol and water (25:50:25) (GAA) (Engleman, pers. com.) and then measured under a calibrated stereomicroscope.

Some ovules were sectioned longitudinally while fresh, using razor blades, a technique that facilitates obtaining cutting fresh soft tissues. The thickness of these sectioned ranged from 10 μm at the edges of the cut to 100 μm in the thickest part. The ovules from the GAA were washed in running water and then embedded in wax. Serial sections were obtained in the longitudinal and transverse planes at 12 μm and 14 μm thickness using a rotary microtome, and stained with Safranine-Fast Green (Sass, 1958). The sections were studied under an Axiolab 5 Zeiss photomicroscope and image capture with Axiocam 208 color Zeiss and edited with Zen 3.2 software (blue edition).

3. Results

The free nuclei and alveolation stages in the ovules described here for *Zamia furfuracea* occur in the third and fourth week of June. The free nuclear stage was present during the third week when 1/3 of the megastrobilus had emerged (Fig. 1A) and measured on average 2–4 cm long and 1.5–2 cm in diameter. The alveolar stage was present during the fourth week when the ovulate strobilus had completely emerged with a peduncle 5–7 cm in length and 3–4 cm in diameter. The ovules, with a whitish integument, measured 2.0–4.0 mm in diameter and 4.0–5.0 mm in length (Figs. 1B,C). Longitudinal sections of the integument had mucilage channels with diameters ranging between 30 and 70 μm (Figs.3D). The integument is adnate to the nucellar tissue for approximately two-thirds of the length of the ovule (Figs. 2B,3B,D).

For both stages, the megastrobili had tightly packed megasporophylls (Figs. 1A,B). The ovules were orthotropous, unitegmic (Fig.2B) and the micropyle was oriented towards the megastrobilus axis (Fig 1B) with an already differentiated micropylar canal (Fig 2C).

3.1. Free nucleus stage

During the third week in June, the micropylar channel is 243 μm longitudinally and 88 μm in diameter (Fig. 2C). Externally, covering the integument there is a thin cuticle of 1.2 μm thick that covers a layer of superficially and anticlinally elongated cells (28 μm anticlinally and 14 μm periclinally). The cuticle extends into the integument and covers the nucellus. The nucellar tissue is formed by flattened cells surrounding the gametophyte each measuring 29 μm periclinally and 20 μm anticlinally with prominent nuclei (8 μm). Tannin cells (each 35 μm periclinally and 14 μm anticlinally) surround the micropylar channel and are seen as amber amorphous bodies containing precipitated particles (Figs. 2B,C). A wide pachychalaza is observed as a result of the adnation of the nucellus and the integument for approximately 1/3 of the length of the ovule (Figs. 2B, 3B,D).

In longitudinal sections, the nucellar tissue towards the micropylar end elongates forming a nucellar beak (Fig. 2C). In the central region, the megagametophyte, measuring approximately 700 μm anticlinally and 510 μm periclinally, is bounded by the megaspore wall 2 μm thick. At this time, free nuclear stage of megagametophytogenesis is taking place. There are three types of nuclei inside the young megagametophyte: 1) those that are more centrally placed in a coenocytic complex, 2) others that have presumably migrated towards the periphery, and 3) those nuclei that are organized centripetally towards the periphery forming rows adjacent to the wall of the megaspore. That latter contained up to six nuclei on average in a row, each nucleus measuring 8–9 μm diameter (Fig. 2D).

Fig. 1. *Zamia furfuracea.* A. Megastrobilus emerging in June. B. Cross section of megasporophyll showing two ovules each with a young megagametophyte (arrows) per megasporophyll. C. Ovule with whitish-yellowish sarcotesta.

3.2. Alveolation stage

One week after the free nuclear stage, when the size of the ovules has increased by 1 mm on average, the micropylar channel measures 353 μm in length and 94 μm in diameter and the megagametophyte has increased in size to 1.87 mm periclinally and 1.52 mm anticlinally. Longitudinal and transverse sections show 12 μm nuclei located at the periphery underlying the megaspore wall whose thickness averages 2 μm (Figs. 3F,G,I). There are six-sided polyhedric, hexahedral elongated cells or alveoli each with peripheral nucleus and radially elongated to 174 μm anticlinally and 92 μm periclinally; several elongated alveoli form rays that measure on average 750 μm (Figs. 3B,C,D,G). Also, there are other short pyramidal trihedral or tetrahedral alveoli formed by angular anticlinal walls that are formed at short distances that gives them the pyramidal shape measuring 40 μm. In cross section, each side of the hexahedral alveoli is 35 μm on average (Figs. 3I,J). The hexagonal shape of the alveoli at this stage gives a honeycomb-like appearance to the megagametophyte in cross section (Fig. J), which is surrounded by three layers of transfer cells with a dense cytoplasm and prominent nuclei (15 μm). This constitutes a jacket that is surrounded by tangentially elongated nucellus cells (100 μm and 20 μm) and these cells can become compacted in places (Fig. 3I).

4. Discussion

The relation between the duration of a short reproductive cycle and the different stages of its ontogeny, in a warm climate cycad species such as *Z. furfuracea*, facilitates the finding and description of the stages sequentially; in this case the free nuclear stage and the alveolation stage in the megagametophyte. This is a short process that takes about a week to complete. In contrast, it has taken several years of observation and repetition of collections even after considering previous studies regarding the reproductive chronology for *Z. furfuracea* (Sánchez-Tinoco et al., 2012, 2018).

4.1. Free nuclei stage

The arrangement pattern of centripetal nuclei within the megaspore wall described for cycads by Chamberlain (1906), Coulter and Chamberlain (1903), Lawson (1926), De Sloover (1963), Singh (1978), Maheshwari and Singh (1967) (Fig. 2A), is confirmed in the present research. Even though there is no information of the species studied when they refer to cycads, what is described here adds information in agreement with what Maheshwari and Singh (1967) refer to as a fairly uniform pattern in the ontogeny of the megagametophyte in its early stages. Here it was possible to observe the migration of nuclei from the center to the periphery, with some nuclei in mitosis, resulting the consequent arrangement of nuclei rows (Fig. 2D).

4.2. Alveolation stage

Schnarf (1933) described two types of alveoli, "true and false", which was later considered a misinterpretation by Maheshwari and Singh (1967) and Singh (1978). Maheshwari and Singh (1967) showed schemes of alveolation, based on previous surveys of some species within the gymnosperms. They also clarified the way in which at the end of the free nuclear stage, when the peripheral arrangement of the nuclei occurs, each nucleus is connected to six adjacent nuclei by microtubules, which subsequently, by anticlinal divisions of the wall in a centripetal direction, result in elongated six-sided cells or alveoli. With respect to cycads, De Sloover (1964) reported the occurrence of alveoli in *Encephalartos poggei* Asch.His-descriptions show that they are open internally, that the nuclei are located on the open inner face and as they grow are elongated. He also described “pyramidal cells” formed by the precocious closure of walls. For the free nuclear stage, DeSloover (1964) describes in detail the different layers of the megaspore membrane that

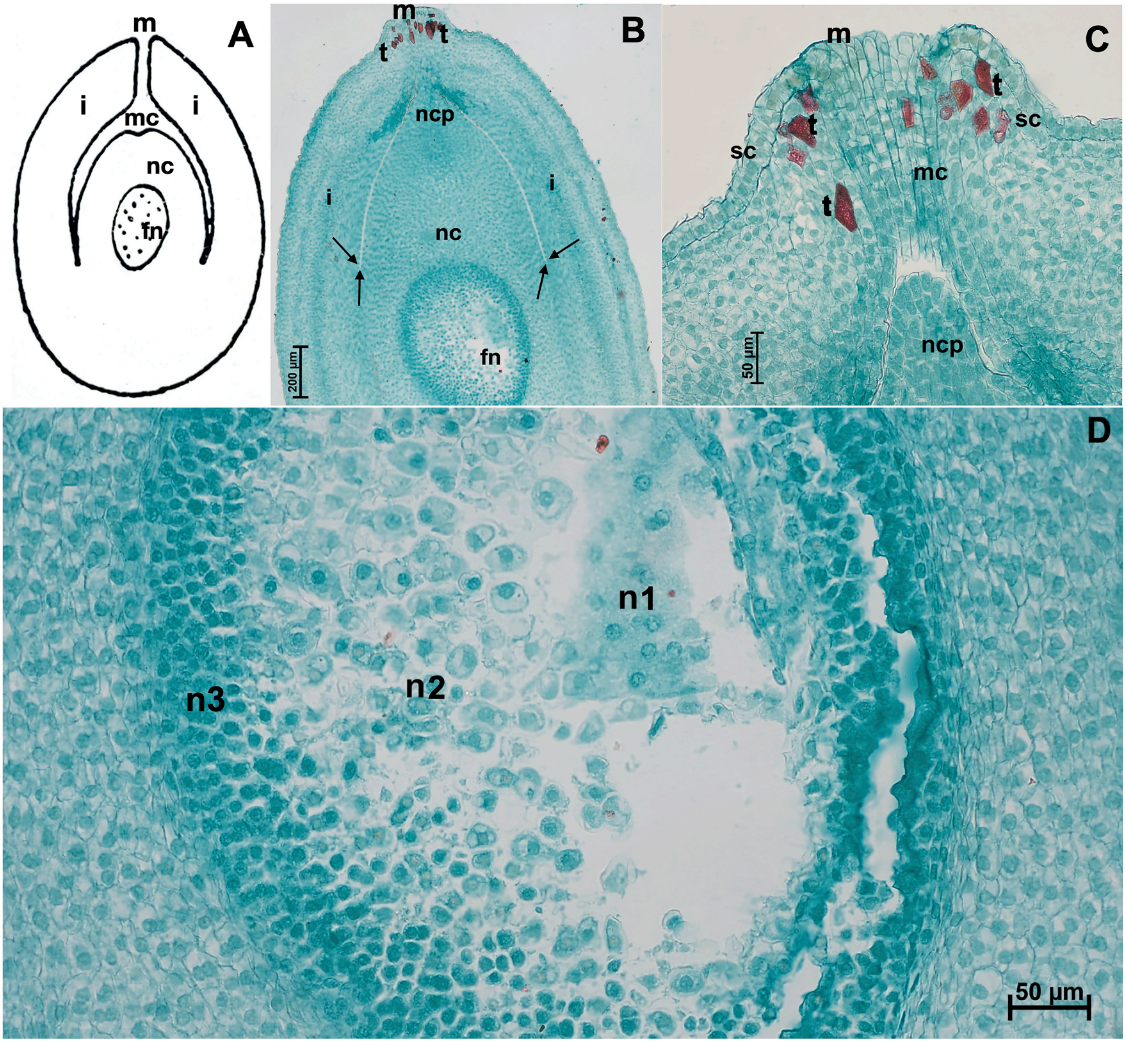

Fig. 2. A Schematic representation of ovule showing the formation of the female gametophyte (Modified from Chamberlain; 1919). B-D. *Zamia furfuracea*. B. Longitudinal section of the ovule during the free nuclei stage in the third week of June. C. Longitudinal section of the ovule micropylar end. D. Longitudinal section of the gametophyte in free nuclei stage stained with Safranin-Fast Green. Abbreviations: fn, free nuclei inside the young gametophyte; i, integument; m, micropyle; mc, micropylar channel; n1, nuclei in the coenocytic matrix; n2, nuclei migrating to the periphery; n3, nuclei forming files; nc, megasporangium or nucellus; ncp, nucellar beak; t, tannins as amorphous solid bodies. Arrows delimit the pachychalaza (fusion of tegument and nucellus).

encloses the gametophyte both in its free nuclear stage and during alveolation. Although as in several studies related to ovule ontogeny, it is emphasized that observations are needed with more detailed information, particularly on the early stages of megagametophytogenesis in cycads. The works of Swamy (1948), De Silva & Tambiah (1952) and Rao (1961), although contributing greatly to the knowledge of embryology, do not provide information on the development of the young gametophyte of cycads.

Fortunately, for anatomists studying cycads, the large size of most cells in cycad tissues allows histological studies in some cases without resorting to ultrastructural methods, simple microtome knife cuts or hand sections being the classical techniques used in the present investigation. From longitudinal sections made with razor blades on fresh ovules (Figs. 3B, C), the nuclei are described resting on the periphery of elongated hexahedral cells (alveoli) that form radial rows converging towards the center. In cross section, six sides are distinguished (Fig. 3J) and the hexagonal alveoli simulate the cavities of a honeycomb (Fig.3H), as depicted by Maheswari & Singh (1967)

In some sections, it was possible to follow the sequences of wall formation. Some walls close anticlinally forming angles that end at short distances and described here as short pyramidal trihedrons or tetrahedron alveoli as named by DeSloover (1964) and referred to Favre-Duchartre (1957) and Maheswari & Singh (1967) as pyramidal cells (Figs. 3E,G). As shown in Fig. 3A, both types of short pyramidal and long polyhedric alveoli together confer the structure described by Sokolowa (1890).

The three strata of cells with dense cytoplasm and conspicuous nuclei described here are characteristic of transfer cells as defined by Esau (1982). That is, parenchyma cells with wall invaginations that increase the surface area of the plasmalemma, specialized in short-distance solute translocation. The observations that these strata were differentiated during alveolation from among the nucellar tissue forming a jacket is comparable to the archegonial jacket whose cellular characteristics are similar. The participation of both in the nutrition of the young developing gametophyte seems probable. It is comparable to the description of DeSloover (1964) that refers to two layers of cells forming a kind of tapetum jacket that nourishes the gametophyte. The structure of the ovule in these two stages reveals that as the development of the megagametophyte progresses, the cells of the megasporangium or nucellus surrounding it become more compact, giving signs of its future reduction to a papyraceous layer when the megagametophyte continues to enlarge, the archegonium matures and the embryo develops. In the same way, the nucellar beak is reduced as the megagametophyte increases in size. This is not the case for the fusion between the nucellus and

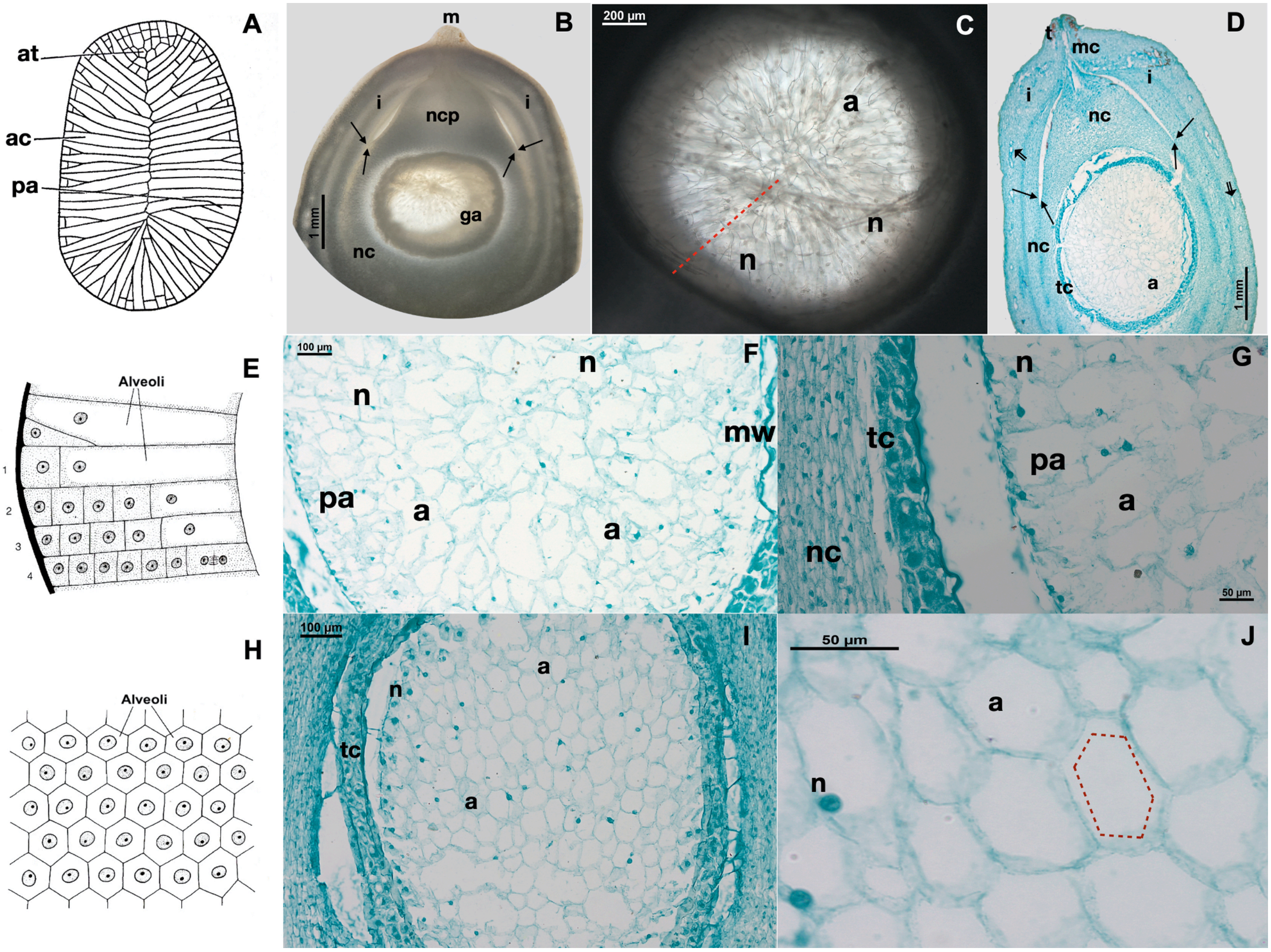

Fig. 3. A. Schematic representation of complete alveolation in an ovule of *Pinus sylvestris*, (Modified from Maheswari & Singh, 1966). B-D. *Z. furfuracea*. B. Thick longitudinal section of young ovule in the alveolation stage in the fourth week of June. Free-hand section. C. Thick longitudinal section of the young gametophyte showing radii of alveoli (dotted line represents radius). Free-hand section. D. Longitudinal section of the ovule during alveolation. E. Schematic representation of the alveolar partition sequence; precociously formed “pyramidal cell” in *Ginkgo biloba* (Modified from Favre-Duchartre, 1957), F. Longitudinal section of the gametophyte during alveolation showing alveoli in radial files in *Z. furfuracea*. G. Magnification of the alveolar gametophyte boundaries in longitudinal section, showing elongated hexahedral and pyramidal trihedral or tetrahedral alveoli in *Z. furfuracea*. H. Schematic representation of alveoli in cross section in *Ephedra helvetica* with the hexagon sides shown (modified from Maheshwari & Sing, 1966). I-J *Z. furfuracea*. I. Cross section of the young gametophyte during alveolation, the sides of the alveoli show the honeycomb shape. J. Greater magnification of the hexahedral alveoli in cross-section the faces of the alveoli show the hexagonal shape (hexagon in dotted lines). Fast Green staining. Abbreviations: a, alveolus; ac, alveolus reaching the center; at, alveolus in cross section; ga, gametophyte; i, integument; m, micropyle; mc, micropylar channel; mw, megaspore wall; n, nucleus; nc, nucellus; ncp, nucellar beak; pa, pyramidal alveolus; t, tannins; tc, transfer cells. Arrows, limits of the pachychalaza (fusion of tegument and nucellus): Bold arrows, mucilage channels.

integument by more than two-thirds of its length, named by Sánchez-Tinoco & Engleman (2004) and Sánchez-Tinoco et al. (2007) as pachychalaza in mature ovules and seeds; pachychalaza is a term introduced by Corner (1976, 1992) for dicotyledonous angiosperms, which refers to the vascularized zone of the chalazal end of the ovule where there is no difference between the nucellar tissue and the integument (Sánchez-Tinoco et al., 2007; Zumajo-Cardona et al., 2021). Both structures are presumably, congenitally fused. The stages described here, for ovules in their early stages and the newly emergence of the megastrobili, the expression of this adnation tends to clarify the congenital nature of the fusion.

5. Conclusion

The anatomical description of these two early stages in the development of the megagametophyte allows us to understand how the young gametophyte will later constitute a cellular structure that through cellular differentiation will give rise to the archegonia and the storage tissue for a developing embryo.

The stages of free nuclei and alveolation are sequential events that in a short reproductive cycle species such as *Z. furfuracea* are rapid, taking only one week.

During the free nuclear stage, nuclei are distinguished within the coenocyte as migrating towards the periphery and arranged in files towards the periphery. These later organize and form radial series of alveoli. Both elongated hexahedrons and short pyramidal trihedrons or tetrahedrons alveoli form radial series. The hexahedral alveoli give the gametophyte the appearance of a honeycomb in cross section. In longitudinal section, the short pyramidal alveoli are triangular in shape. Sheathing the young gametophyte, three layers of transfer cells differentiate during alveolation, constituting a kind of jacket comparable to the archegonial jacket associated with nutrient translocation from the nucellus.

Author contributions

MYS, APV and DWS planned and designed the research, collected the plant material, and wrote the final version of the manuscript. All authors

performed the experiments, analyzed the data, and read and approved the final manuscript.

Author statement

MYS, APV and DWS planned and designed the research, collected the plant material, and wrote the final version of the manuscript. All authors performed the experiments, analyzed the data, and read and approved the final manuscript.

Declaration of Competing Interest

None of the authors have any financial or personal relationships that may be perceived as influencing their work. Complete Elsevier's Declaration of Interests form. See below (after uploading your main file) for instructions.

Acknowledgments

The authors thank and acknowledge the generosity of Timothy Gregory, who supports both this research and the doctoral studies of the first co-author. A special thank you to Favio Gonzalez, Subject Editor, for his diligence in improving the content and text the manuscript. This work was funded in part by NSF Grants 7721495, BSR-8607049, EF-0629817 and DEB-2140319 to DWS.

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