Flora 304 (2023) 152303

Contents lists available at ScienceDirect

Flora

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

Late stages of megagametophytogenesis: Archegonial development in Zamiaceae

M. Ydelia Sánchez-Tinoco [a,d], Dennis Wm. Stevenson [b,*], Andrew P. Vovides [c], Alejandro Alonso López [d], Lourdes G. Iglesias-Andreu [e], Edmundo García Moya [f], Galdy Hernández-Zárate [d]

[a] Laboratorio de Anatomía Vegetal, Instituto de Investigaciones Biológicas, Universidad Veracruzana, Xalapa, Veracruz, Mexico
[b] The New York Botanical Garden, Bronx, NY, United States
[c] Departamento de Biología Evolutiva, Instituto de Ecología, A.C., Mexico
[d] Colegio de Postgraduados, Campus Veracruz, Tepetates, Veracruz, Mexico
[e] Instituto de Biotecnología y Ecología Aplicada, Universidad Veracruzana, Xalapa, Veracruz, Mexico
[f] Colegio de Postgraduados, Campus Montecillo, Edo. de México, Mexico

ARTICLE INFO

Edited by Alessio Papini

Keywords:
Archegonium
Central cell growth
Central vacuole
Globose cells
Neck cells
Zamiaceae

ABSTRACT

The anatomy of the neck cells and the structure of the archegonium during the central cell growth as a stage in the ontogeny of the archegonium that occurs before fertilization is described and compared for *Ceratozamia tenuis* and *Zamia furfuracea*. Two neck cells are reported for both species with anatomical features that suggest they participate in the fertilization process. It is postulated that this stage is characterized by a profusely vacuolated cytoplasm from which a large central vacuole becomes prominent. This vacuole plays an important role in pushing the huge egg cell nucleus along with conspicuous smaller vacuoles of the central cell towards the micropylar region. It then presses and pushes the two globose neck cells with their respective subtending surface cells apart and towards the archegonial chamber. There are no significant differences between the development and structure of archegonial neck complexes in the two species studied.

1. Introduction

Because of their long evolutionary history, cycads have important features enabling the study of seed plant evolution (Norstog, 1972; Brenner and Stevenson, 2006). Among the ancestral characteristics, the presence of archegonia is an important feature of the ovule, with a decisive role in the evolution of plant life considering that the seed habit represented the liberation of fertilization from dependence of free water, as occurs the case of Cryptogams, because archegonia are enclosed within an ovule.

The archegonium constitutes the reproductive part of the megagametophyte where at the end of ontogeny the egg cell as the female reproductive cell or gamete is positioned (Singh, 1978). Studies on *Ceratozamia tenuis* (Dyer) D. W. Stev. & Vovides, *Dioon edule* Lindley, *Encephalartos poggei* Asch., *Zamia integrifolia* Ait., *Z. amblyphyllidia* D. W. Stev and *Z. furfuracea* L. fil., Sanchez-Tinoco et al. (2022); Maheswari and Singh (1967); De Sloover (1964); Norstog (1972); Zhang (2019), have provided relevant information in understanding some stages of megagametophytogenesis in cycads, complementing Chamberlain's (1935) summary of his valuable contribution to their reproductive biology and summarized in Fig. 1 from his work on *Dioon edule*. The development of the megagametophyte, megagametophytogenesis, begins with the first nuclear division of the functional haploid megaspore. Thereafter several sequential stages occur that are comprised of 1) numerous karyokinesis events resulting in a free nuclei stage, 2) formation of alveoli, and 3) subsequent cellularization through the formation of irregular walls. These stages result in the formation of the vegetative female gametophyte. Subsequently, the development of the reproductive part of the megagametophyte or archegonium occurs. Archegonia according to Chamberlain's (1906, 1910, 1935); Coulter and Chamberlain (1910)) scheme are initiated by the differentiation of a cell at the micropylar end of the vegetative gametophyte; however, Chamberlain did not study the alveolation process as later described by Maheshwari and Singh (1967); Singh and Johri (1972); Sánchez-Tinoco

* Corresponding author.
E-mail address: dws@nybg.org (D.Wm. Stevenson).

https://doi.org/10.1016/j.flora.2023.152303
Received 24 February 2023; Received in revised form 10 May 2023; Accepted 12 May 2023
Available online 16 May 2023

et al. (2022). As described by Singh and Johri (1972), the differentiation of the archegonia is initiated from some undivided and closed alveoli, situated at the micropylar end. The rest of the alveoli undergo a series of periclinal divisions so that the megagametophyte appears to be formed of a row of radiating cells (Sánchez-Tinoco et al., 2022). This arrangement of radial series of cells becomes lost in the mature gametophyte due to the formation of new walls in irregular directions (Sánchez-Tinoco et al., 2022).

The archegonium primordium originates from the differentiation of an alveolus at the closed micropylar end by an unequal periclinal division that gives rise to a large cell, the central cell, and another small cell, the neck cell initial. The neck cell initial. through an anticlinal division forms two neck cells (Fig. 1A,B). The central cell enlarges rapidly. This growth is the result of the expansion of a large central vacuole that pushes the cytoplasm towards the adjacent cell walls. Thus, its large size is not a product of cytoplasmic content but rather to the volume of the large vacuole (Chamberlain, 1906; Coulter and Chamberlain, 1910; Norstog and Nicholls, 1997). Chamberlain (1906) defined this stage in the development of the archegonium as comprised of the growth of the central cell that is also characterized by smaller surrounding peripheral cells, a large nucleus in the central cell, two differentiated neck cells. All of this is surrounded by a layer of transfer cells that form the archegonial jacket. Shortly before fertilization, the nucleus of the central cell divides giving rise to both the egg cell nucleus and the ventral canal nucleus as no cell wall is formed between these two nuclei (Gifford and Foster, 1989). The ventral canal nucleus is located near the neck of the archegonium and soon becomes disorganized, while the egg cell nucleus is located towards the center and enlarges. Chamberlain (1906, 1935) and Coulter and Chamberlain (1910) reported that this stage may take up to six months in *Dioon edule* before the nucleus divides to form the ventral canal nucleus and the egg cell nucleus.

Thus, the archegonium consists of a group of cells and nuclei from which a single functional gamete known as an egg cell is formed (Foster and Gifford, 1974). According to Norstog and Nicholls (1997), the number of archegonial neck cells is somewhat controversial in *Cycas, Ceratozamia, Encephalartos, Macrozamia*, and *Zamia*. Norstog (1972)

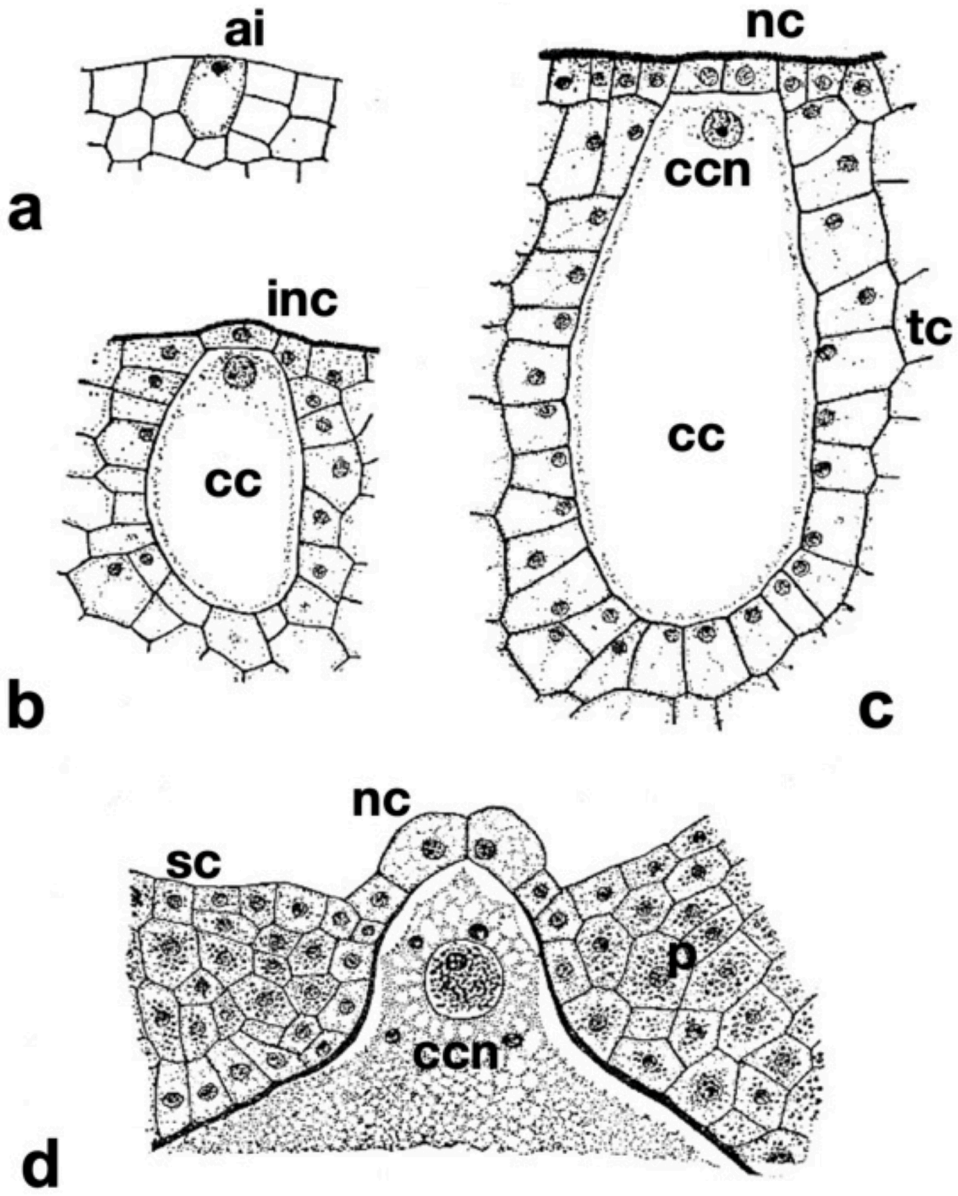

Fig. 1. Development of the archegonium in *Dioon edule*. A. archegonium primordium; B. neck cell and central cell; C. archegonium with two neck cells; D. micropylar region of the archegonium with two neck cells in the expulsion process to the archegonial chamber. Abbreviations: ai, archegonium initial; inc, neck cell initial; cc, central cell; nc, neck cell; ccn, central cell nucleus; tc, transfer cell; sc, superficial cell (Modified from Coulter and Chamberlain, 1910).

described four neck cells at the time of fertilization in *Zamia integrifolia* L.f. Other studies on *Dioon* (Chamberlain, 1919), *Bowenia* (Lawson, 1926) and *Encephalartos* (Steyn and Strydom, 1993) have reported only two neck cells emerging within the archegonial chamber. In the structure of the archegonium, a large central nucleus and a smaller nucleus that is basically the ventral canal nucleus are common to all cycads that have been studied on the ovule ontogeny. The archegonial jacket consists of a stratum of transfer cells that through cytoplasmic connections allow the passage of nutrients present in the vegetative gametophyte, mainly starch, lipids, proteins and other polysaccharides (Sánchez-Tinoco et al., 2000, 2012, 2018a, 2018b).

The neck cells protrude into the archegonial chamber (Fig. 1A) during the growth stage of the central cell, later, forming the neck canal (Chamberlain, 1935) (Fig. 1D).

In some gymnosperms, such as *Ginkgo biloba* L. (Zhang et al., 2013; Wang et al., 2014; Owens et al., 1997) and *Cycas revoluta* Thunb. (von Aderkas et al., 2022), the structure and function of the neck cells has been described. That in relation to the morphological changes that occur during fertilization and their relation to the fluids secreted to attract the antherozoid to the archegonium.

Although there are studies in some cycad species, such as those mentioned above, some of them have described the occurrence of four neck cells during the fertilization stage. In the present contribution, we focus on a pre-fertilization stage within the ontogeny of the archegonium, the central cell growth stage, and describe and compare the anatomical structure of the neck and archegonium cells in *Ceratozamia tenuis* and *Zamia furfuracea*.

2. Materials and techniques

Ovules preserved in glycerin, 96% ethyl alcohol and water (25:50:25) (GAA) (Engleman, pers com) from the Plant Anatomy Laboratory conserved ovule cycad collection of the Instituto de Investigaciones Biológicas, U.V. were used. For *C. tenuis* collections were initiated in May 1995 and for *Z. furfuracea* in September 2004. These materials were collected from “Cerro Coacoatzintla” in the municipality of Coacoatzintla, Veracruz, Mexico and “Ciénega del Sur”, municipality of Alvarado, Veracruz respectively.

The ovules were observed under the stereomicroscope to identify the stages under study, i.e., from ovule initiation to before fertilization. Subsequently, dissections were made in the longitudinal and transverse planes of the micropylar portion of the gametophyte where archegonia were visible. Following the reproductive cycle of both species (Sánchez-Tinoco et al., 2000, 2018), new ovule collections were made at the mentioned localities during May 2018 to 2022 for *C. tenuis* and in September 2019 for *Z. furfuracea*. Field-collected ovules were fixed in (FAA) composed of 37–40% formaldehyde, glacial acetic acid, 95% ethyl alcohol and water (10:5:50:35) (Sass, 1958). Both GAA-preserved and FAA-fixed ovules were washed in running water for processing by standard microtechnique of inclusion in paraffin, rotary microtome sections (12 μm) and staining with Safranin O and fast green (Sass, 1958).

Also, hand sections were taken between 10 and 50 μm thick using disposable razorblades. 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

3.1. The central cell growth stage

The growth stage of the central cell in archegonia of *C. tenuis* occurs in May, which ten months after ovule initiation. In *Z. furfuracea,* this occurs five months later in September. This stage coincides with the cellularization process in the megagametophyte.

In general, for both species, the anatomical structure of this stage is described as follows. The archegonia are located towards the micropylar end within the vegetative megagametophyte that is covered by a layer of superficial cells with the rest of the cells being the storage parenchyma in which starch grains predominate (Figs. 2B, 3C). The megagametophyte forms a depression towards the micropylar end that forms the archegonial chamber (Figs. 2A, 3A). Towards the interior of this chamber, a single cell differentiates within the archegonium that occupies the entire dimensions of the archegonium. This is the central cell that is characterized by profuse vacuolar cytoplasm (Figs. 2B, 3B). In longitudinal section, vacuoles of different diameters are seen surrounding the large central vacuole that in transverse median section can be observed at its maximum dimension. This central vacuole occupies approximately 50% of the cytoplasm of the central cell (Figs. 2G, 3C). There is also a large nucleus immersed within the vacuolated cytoplasm towards the micropylar end of the archegonium. Vacuoles can be observed in the area towards the archegonial chamber. Within the nucleus (Figs. 2C, 3C), conspicuous nucleoli stain bright red using Safranin O (Figs. 2C, 3C).

The central cell is the result of the first periclinal division of the initial cell of the archegonium (Fig. 1B), eventually will give rise to the neck cell initial at the micropyle end and the central cell internally.

In both species, as a result of an anticlinal division of the primary neck cell there are two globose neck cells that protrude into the archegonial chamber. Each globose cell has a prominent nucleus and nucleoli, as well as a reticulated cytoplasm (Figs. 2D-E, 3C-F). Here we refer to the neck cells as the complex formed by two globose cells each underlaid by a superficial basal cell with the globose cells completely separate from each other. The superficial basal cells underlying the globose cells in *C. tenuis* are anticlinally elongated and differentiated from the rest of the superficial cells of the gametophyte (Fig. 2E). In contrast, in *Z. furfuracea* these cells are isodiametric similar to the rest of the superficial cells (Figs. 2C,E, 3E-F). In repeated observations of sections from several ovules at this stage, only two neck cells were observed in both species.

There is a correlation, observable in median longitudinal sections, between the position of the neck cells, central cell nucleus and the large central vacuole (Figs. 2B-C, 3B-C). The nucleus of the central cell is located towards the micropylar region and in some sections of some ovules were observed to be very close to the proximal end of the neck cells to form a peak of vacuolated cytoplasm (Fig. 3C).

Surrounding the central cell is a layer of transfer cells each with prominent nuclei and reticulate cytoplasm. These cells form the archegonial jacket (Figs. 2B,G-H; 3B,D). Towards the interior of the jacket, the central cell wall shows pitting with cytoplasm crossing these pits. That is, there appears to be cytoplasmic connections between the jacket cells and the central cell of the archegonium.

3.2. Anatomy of C. tenuis ovules during the central cell growth stage

In May, the ovules of *C. tenuis* measure 27–33 mm in length and 15-20 mm in diameter (Fig. 2A). There are 2–6 archegonia each with two neck cells protruding into the archegonial chamber. At this time, megagametophytes can be found from precellularized to fully cellularized stages. At the latter stage, the archegonia have already differentiated. As in all other cycads studied, the archegonial chamber differentiates as a depression at the micropylar end of the megagametophyte (Fig. 2A).

In longitudinal section, the following can be distinguished at the micropylar end of the megagametophyte: the archegonial chamber, the neck cells, the central cell surrounded by the archegonial jacket of transfer cells. This archegonial apparatus is immersed within the parenchyma tissue dominated by starch grains (Fig. 2B).

In transverse section, the archegonia are observed in radial arrangement in relation to the archegonial chamber (Fig. 2F). Two neck cells protrude into the archegonial chamber with a diameter of 1.0 mm. Globose cells measure 54 μm periclinally and 64 μm anticlinally, have nuclei 23 μm in diameter and nucleoli 4 μm in diameter. The cell wall of

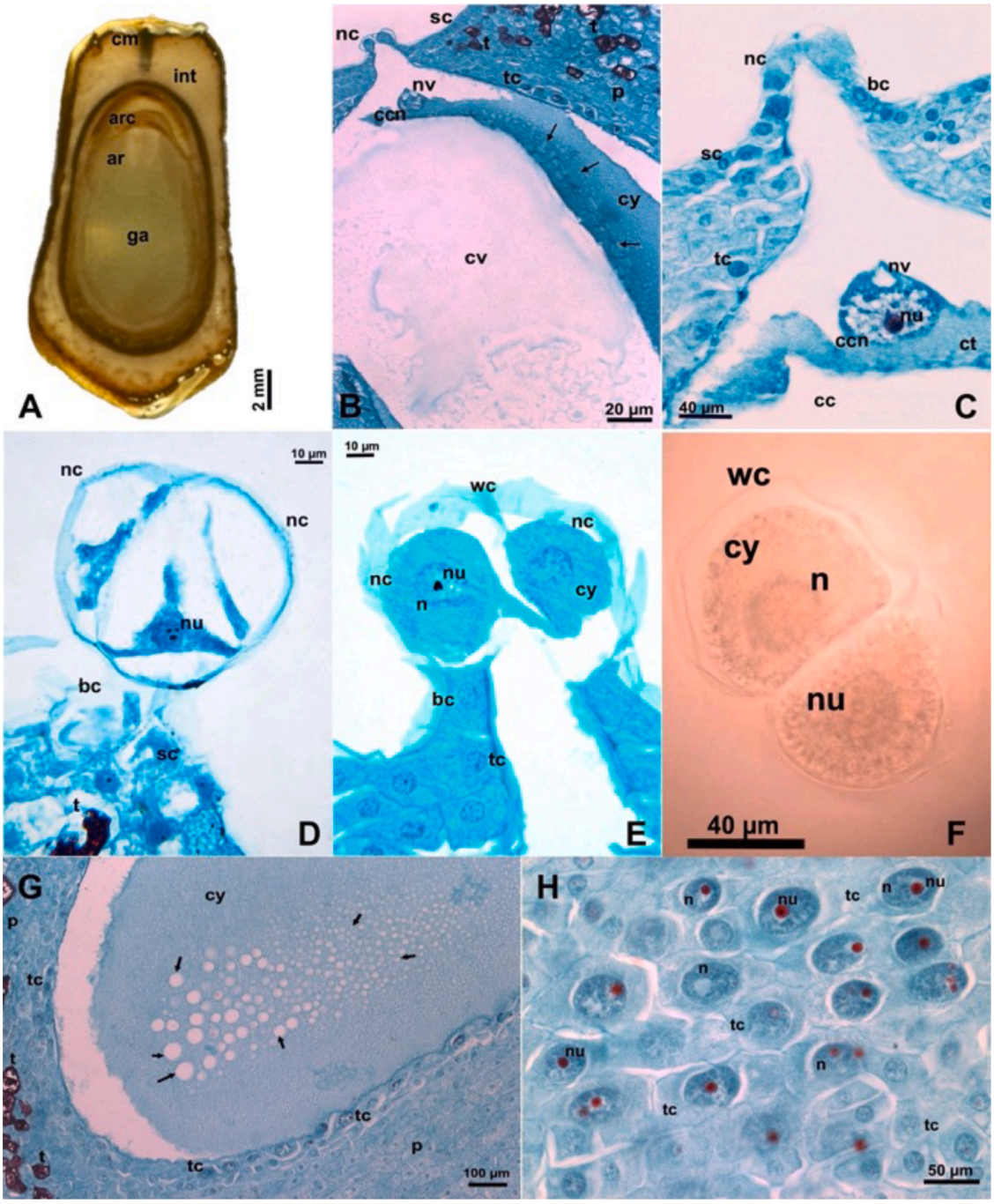

Fig. 2. A. *C. tenuis* ovule in May during the central cell growth stage. B. Longitudinal section of the archegonium. C. Longitudinal section of the micropylar region of the archegonium. D. Longitudinal view of the globose neck cells. E. Longitudinal section of the neck cells. F. transverse section of the neck cells embedded in the vegetative gametophyte. G. Longitudinal section of the central cell. H. Tangential section of the archegonial jacket. Abbreviations: ar, archegonium; arc, archegonial chamber; bc, basal superficial cell; cc, central cell; ccn, central cell nucleus; cv, large central vacuole; cy, cytoplasm; int, integument; ga, gametophyte; mc, micropillar channel; n, nucleus; nc, neck cells; nu, nucleolus; nv, nucleus vacuoles; p, parenchyma; sc, superficial cells; t, tannins; tc, transfer cell; wc, wall cell; ↑, vacuoles.

the globose cells is 0.94 µm thick. Each globose cell is underlain by an anticlinally elongated superficial basal cell measuring 36 µm anticlinally and 23 µm tangentially. These cells also differ in size from the rest of the surface cells of the vegetative megagametophyte, which are isodiametric and measure 23 µm (Fig. 2B-C,E).

In median longitudinal section, there is a layer of tangentially elongated transfer cells surrounding the central cell. This forms the archegonial jacket, which is 64 µm tangentially and 50 µm anticlinally. These transfer cells have scant cytoplasm and prominent nuclei measuring 41 µm diameter with up to five nucleoli measuring 7 µm in diameter (Fig. 2G,H). The cell walls are 2.5 µm thick.

The central cell is located interior to the jacket cells; it is large, 3 mm anticlinally and 1.20 mm periclinally. Its cytoplasm is profusely vacuolated. The vacuoles are of different sizes, the largest are 38 µm in diameter, whilst the smallest measure 2.5 µm in diameter. In median longitudinal section, the large central vacuole is at the micropyle and is 1.6 mm anticlinally and 0.80 mm periclinally. This vacuole occupies approximately 50% of the cytoplasm of the central cell and underlies a

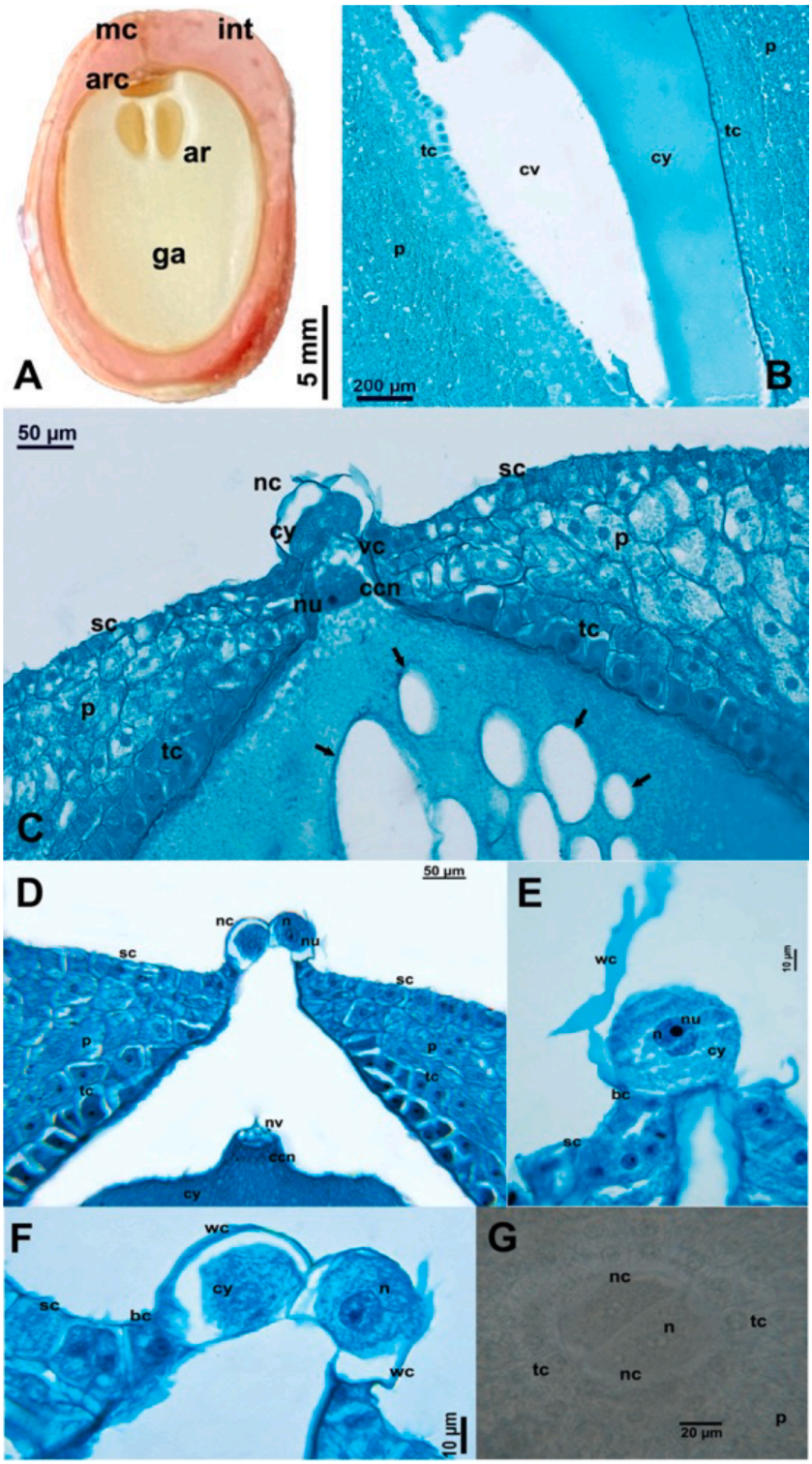

Fig. 3. A. *Z. furfuracea* ovule in September during the central cell growth stage. B. Longitudinal section of the archegonium. C. Longitudinal section of the micropylar region of the archegonium. D. Longitudinal section in the micropylar region shows the alignment of the nucleus of the central cell and the neck cells. E. Longitudinal section of the neck cells. F. Longitudinal section of the neck cells. G. Transverse section of the neck cells embedded in the vegetative gametophyte. Abbreviations: ar, archegonium; arc, archegonial chamber; bc, basal superficial cell; cc, central cell; ccn, central cell nucleus; cv, large central vacuole; cy, cytoplasm; int, integument; ga, gametophyte; mc, micropillar channel; n, nucleus; nc, neck cells; nu, nucleolus; nv, nucleus vacuoles; p, parenchyma; sc, superficial cells; t, tannins; tc, transfer cell; wc, wall cell; ↑, vacuoles.

prominent nucleus whose contents are highly reticulate (Fig. 2B). This nucleus is approximately 75 µm in diameter 2B,C). The wall of the central cell is 0.94 µm thick and has pits averaging 11 µm in diameter. Cytoplasm strands are continuous through these pits and connect the cytoplasm of the central cell with the cytoplasm of the transfer cells.

In medium longitudinal sections, an alignment between the neck cells, the nucleus of the central cell and the large central vacuole is observable (Fig. 2B,C).

3.3. Anatomy of *Z. furfuracea* ovules during the central cell growth stage

During the central cell growth stage in *Z. furfuracea* that occurs during September when the archegonia are beginning to differentiate, the ovules are 17–24 mm in length and the archegonia 3 mm in length and 1.5 mm in diameter. There are commonly four archegonia per ovule (Fig. 3A).

The neck cells complex that protrude into the archegonial chamber are formed by globose cells measuring 50 µm periclinally and 59 µm

anticlinally. Their nuclei are 15 μm and the nucleoli are 2.5 μm in diameter. The globose cell wall is 0.60 μm thick. In early September, up to two superficial basal cells are observed subtending the neck cells. At the end of the month only one superficial basal cell supports the globose neck cell. The superficial basal cell is isodiametric and 20 μm in diameter (Fig. 3E,F).

In early September, the alignment of the neck cells, the nucleus of the central cell measuring 0.6 mm anticlinally and the large central vacuole measuring 1.8 mm in length and 0.5 mm in width are observed. The nucleus also has prominent nucleoli up to 85 μm in diameter and conspicuous micropyle directed vacuoles measuring 135 μm anticlinally. The nucleus at this stage is observed forming a peak which supports the superficial basal cells that subtend the globose cells or neck cells. The location of the central vacuole is proximal to the nucleus. The central cell measures 2.5 mm anticlinally and 1.2 mm periclinally (Fig. 3B).

There is a stratum of transfer cells surrounding the central cell, the transfer cells measure 20 μm and have prominent nuclei up to 15 μm and scarce cytoplasm. Within the nuclei, up to eight nucleoli measuring 6 μm in diameter were counted. The nucleoli had a deep red staining in response to Safranin O staining (3E).

In the second week of September, in serial sections of several ovules, it was possible to record the alignment of the central cell nucleus forming a peak with the large vacuole distal to the nucleus along with the protrusion of the globose cells, subtended by superficial basal cells, into the archegonial chamber (Fig. 3C,D).

4. Discussion

In general, there are no significant differences in the anatomical structure of the neck cells at this stage in *C. tenuis* and *Z. furfuracea*. However, as expressed by their anatomy, there are features such as position within the archegonial chamber, globose shape, cell wall aspect, prominent nucleus and granular or reticular appearance of the cytoplasm, suggest an important role prior to fertilization.

Chamberlain (1910); Norstog (1972, 1990) and Norstog and Nicholls (1997) reported the importance of the neck cells in the passage of the antherozoid from the archegonial chamber to fertilize the nucleus of the egg cell. On the other hand, there are studies that refer to the participation of secreted fluids by the globose cells that have been interpreted as osmotic mechanisms that intervene in the rupture of the walls between the two globose cells. The swelling of these cells and the pressure that these cells exert allow the passage of the antherozoid and also removes flagellar apparatus (Ikeno, 1986; Lawson, 1926; Brown and Taylor, 1940; Norstog, 1972; Little et al., 2014: von Aderkas et al., 2022). Contributions on *Ginkgo biloba* by Zhang et al. (2013) and Wang et al. (2014) provide information on secretory organelles that demonstrate there are antherozoid attracting fluids, as well as antherozoid structural changes during fertilization.

Although there is information regarding the structure and participation of the neck cells during fertilization, in the present work we take up the importance that Chamberlain (1910, 1919, 1935) and Coulter and Chamberlain (1910) gave to the pre-fertilization stage, namely, the growth stage of the central cell and highlighting a profusely vacuolated cytoplasm and where a large central vacuole protrudes. We demonstrate the alignment of the nucleus of the central cell in forming a peak directed towards micropyle. Additionally, we observed prominent vacuoles within the nucleus as well as the role of the large central vacuole that their respective tonoplasts play an important role in pushing the globose cells towards the archegonial chamber and along with them a pair of subtending surface basal cells that are also pushed into the archegonial chamber. This information supplements the osmotic mechanism mentioned by Lawson (1926); Brown and Taylor (1940) and Norstog (1972), which could be the contributing factor in the rupture of the cell wall between the two globose cells. This concept is supported because we found traces of the wall that was detached from the globose cells in the archegonial chamber (Figs. 2E,3E).

A repeated series of sections led us to clarify that the cells subtending the globose cells, the superficial basal cells, are not transfer cells which are part of the archegonial jacket. This is further supported by the observations that these superficial basal cells are pushed together with the globose cells into the archegonial chamber.

Other studies related to the reproductive ontogeny of *Z. furfuracea* have highlighted the feasibility of finding important stages of reproductive ontogeny in those cycad species with annual strobilus production (Sánchez-Tinoco et al., 2000, 2012, 2018). Here, we have recorded the alignment of structures more easily in *Z. furfuracea,* This is a species that annually produces both microstrobili bearing pollen and megastrobili bearing ovules. In this case, there is annual seed production. In contrast, *C. tenuis* is a species that produces seed every two years with a longer period of ovule development (Sánchez-Tinoco et al., 2000). Thus, it was necessary to make repetitive observations with samples collected in 1995, 2018 and 2022 to compare with the annual cycle of *Z. furfuracea*.

We emphasize that during the growth stage of the central cell prior to fertilization, the traditional concept of neck cells refers to a pair of globose cells with prominent nuclei and granular or reticulate cytoplasm that are very similar to the superficial basal cells that subtend them. However, Norstog and Nicholls (1997), describe the process of globose cells dividing to form a quartet of cells just prior to fertilization.

Thus, the stage of central cell growth named by Chamberlain (1919, 1935) and Coulter and Chamberlain (1910), is possibly due to the expansion of the central cell by the profuse vacuolized cytoplasm and characterized by an anatomical arrangement that will favor fertilization involving the secretion of fluids. It should be added that it is the stage in which the globose cells are expelled after the second anticlinal division that formed them. Associated with the cell complex (globose cells and superficial basal cells), vacuoles of the nucleus of the central cell and the large central vacuole as well as the rest of the cells allow an arrangement that will favor the passage of the antherozoids leading to the consequent fertilization. That is, at this stage of growth of the central cell, structural characteristics that favor the protrusion of the globose cells into the archegonial chamber are present.

Thus, it is concluded that in the growth of the central cell in both species, there is a similar anatomical structure where within the scanty cytoplasm there are predominate vacuoles of different sizes with a large central vacuole that occupies almost 50% of the cell lumen. At this stage, there are two globose cells each subtended by a superficial basal cell for both species. This results in the alignment of the nucleus of the central cell with its corresponding vacuoles and the large central vacuole. The expansion of the large central vacuole results in the displacement of the globose cells together with their superficial basal cells into the archegonial chamber. A complex of cells, nuclei and vacuoles is well developed, and we interpret this to be part of the physiological processes that take place in the attraction of the antherozoids and consequent fertilization.

Author statement

MYS, APV and DWS planned and designed the research, collected theplant material, and wrote the final version of the manuscript. All authorsperformed the experiments, analyzed the data, and read and approvedthe final manuscript.

Declaration of Competing Interest

None of the authors have any financial or personal relationships that may be perceived as influencing their work.

Data availability

No data was used for the research described in the article.

Acknowledgments

This research was carried out thanks to the generosity and financial support of Timothy Gregory, PhD, to whom we are very grateful. We also thank Miguel Angel Perez-Farrera, PhD, for his contribution with information on phenology of the species studied. Also, we are grateful for the unconditional encouragement of Verónica Lango Reynoso, PhD, Director of the Colegio de Postgraduados campus Veracruz, during the doctoral studies of the first co-author. This work was funded in part by NSF Grants 7721495, BSR-8607049, EF-0629817 and DEB-2140319 to DWS.

References

Brenner, E.D., Stevenson, D., 2006. Using genomics to study evolutionary origins of seeds. Landscapes, Genomics and Transgenic Conifers. Springer, Dordrecht, pp. 85–106.
Brough, P., Taylor, M.H., 1940. An investigation of the life cycle of *Macrozamia spiralis* Miq. Proc. Linn. Soc. N.S.W. 65, 494–524.
Chamberlain, C.J., 1906. The ovule and female gametophyte of *Dioon*. Bot. Gaz. 27, 321–358.
Chamberlain, C.J., 1910. Fertilization and embryogeny in *Dioon edule*. Bot. Gaz. 50, 415–429.
Chamberlain, C.J., 1919. The Living Cycads. University of Chicago Press, Illinois.
Chamberlain, C.J., 1935. Gymnosperms. Structure and Evolution. University of Chicago Press, Illinois.
Coulter, J.M., Chamberlain, C.J., 1910. Morphology of Gymnosperms. University of Chicago Press, Illinois.
De Sloover, J.L., 1964. Études sur les Cycadales. III. Nucelle, gamétophyte femmelle et embryon chez *Encephalartos poggei* Asch. Cellule 64, 149–200.
Foster, A.S., Gifford, E.M., 1974. Comparative Morphology of Vascular Plants, 2nd ed. W. H. Freeman and Company, New York.
Gifford, E.M., Foster, A.S., 1989. Morphology and Evolution of Vascular Plants, 3rd ed. W. H. Freeman and Company, New York.
Ikeno, S., 1896. Das spermatozoid von cycas revoluta. Bot. Mag. (Tokyo). 10, 367–368.
Lawson, A.A., 1926. A contribution to the life history of *Bowenia*. Trans. Roy. Soc. Edinb. 54, 357–394.
Little, S.A., Prior, N., Pirone, C., von Aderkas, P., 2014. Pollen-ovule interactions in gymnosperms. In: Ramatt, K., Mérillon, J., Shivanna, K. (Eds.), Reproductive Biology of Plants. CRC Press, Boca Raton, LA, pp. 97–111.
Maheshwari, P., Singh, H., 1967. The female gametophyte of gymnosperms. Biol. Rev. 42, 88–130.
Norstog, K.J., 1972. Role of archegonial neck cells of *Zamia* and other cycads. Phytomorph 22, 125–130.
Norstog, K.J., 1990. Studies of cycad reproduction at Fairchild Tropical Garden. Mem. N. Y. Bot. Gard. 57, 63–81.
Norstog, K.J., Nicholls, T.J., 1997. Biology of the Cycads. Cornell University Press, Ithaca, NY.
Owens, J.N., Catalano, G.L., Aitken-Christie, J., 1997. The reproductive biology of kauri (*Agathis australis*). IV. Late embryogeny, histochemistry, cone and seed morphology. Int. J. Pl. Sci. 158, 395–407.
Sánchez-Tinoco, M.Y., Engleman, E.M., Vovides, A.P., 2000. Cronología reproductora de *Ceratozamia mexicana* (Cycadales). *Bol. Soc. Bot. Méx.* 66, 15–23.
Sánchez-Tinoco, M.Y., Ordóñez-Balderas, J.R., Engleman, E.M., 2012. Starch accumulation in the megagametophyte of *Ceratozamia mexicana* Brongn. and *Zamia furfuracea* L. f. Mem. N. Y. Bot. Gard. 106, 251–262.
Sánchez-Tinoco, M.Y., Engleman, E.M., Vovides, A.P., 2018a. Development cycle of the ovule and seed of *Zamia furfuracea* L. f. (Cycadales). Mem. N. Y. Bot. Gard. 117, 43–53.
Sánchez-Tinoco, M.Y., Engleman, E.M., Vovides, A.P., 2018b. Anatomical description of the cytoplasmic connections between the central cell and transfer cells in *Ceratozamia mexicana* Brongn., and *Zamia furfuracea* L.f. (Cycadales). Mem. N. Y. Bot. Gard. 117, 32–42.
Sánchez-Tinoco, M.Y., Stevenson, D.W., Vovides, A.P., López, A.A., Iglesia-Andreu, L.G., Moya, E.G., Hernández-Zárate, G., 2022. Salient ovule features of *Zamia furfuracea* L. fil.: early stages of megagametophytogenesis. Flora 296, 152168.
Sass, J.E., 1958. Botanical Microtechnique, 3rd ed. Iowa State University Press, Ames, IA.
Singh, H., 1978. Embryology of Gymnosperms. Handbuch der Pflanzenanatomie Spezieller Teil. Band X 302, 230–241. Te Borntraeger, Berlin262-287.
Singh, H., Johri, B.M., 1972. Development of gymnosperm seeds. In: Seed Biology, 1. Acad. Press, New York and London, pp. 21–75.
Steyn, E.M.A., Strydom, D.J.F., 1993. The nucellus: its position and function in the ovule of *Encephalartos*. Encephalartos 35, 14–18.
von Aderkas, P., Little, S.A, Nepi, M., Guarnieri, M., Antony, M., Takaso, T., 2022. Composition of sexual fluids in *Cycas* revoluta ovules during pollination and fertilization. Bot. Rev. 88, 453–484.
Wang, D., Lu, Y., Zhang, M., Lu, Z., Luo, K., Cheng, F., Wang, L., 2014. Structure and function of the neck cell during fertilization in *Ginkgo biloba* L. Trees 28, 995–1005.
Zhang, X., 2019. Ovule development in Cycads: observation on anatomy and nucellus morphology in *Zamia* and *Cycas*. bioRxiv 735–837.
Zhang, Z., Clayton, S.C., Cui, K., Lee, C., 2013. Developmental synchronization of male and female gametophytes in *Ginkgo biloba* and its neck mother cell division before fertilization. Physiol. Pl. 147, 541–552.