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Original article

Embryogenesis, endospermogenesis and fruit development in *Lophophytum* (Balanophoraceae): Focus on endosperm and embryo initiation

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ABSTRACT

Studies on the development of the endosperm, embryo and fruit are scarce in Balanophoraceae, since a particular pattern for the family has not been established. Moreover, discrepancies between the reported cases are being observed by various authors. The aims in this study are to describe the processes of endospermogenesis, embryogenesis and fruit development for the Argentine *Lophophytum* species. Pistillate flowers and fruits at different stages of development were analysed using conventional optical and scanning electronic microscopy. Endospermogenesis without fertilization takes place in three stages: firstly, the formation of a coenocyte of 2–12 nuclei which originate from polar nuclei. The second stage is the fusion of the endosperm nuclei, plus both nuclear and cytoplasmic material from cells of nucella. Once inside the coenocytes, fusion of all the nuclei results in a single giant nucleus reaching dimensions of 120 × 60 μm. The third stage is the endosperm formation sequence with rounds of coordinated mitosis, giving rise to nuclei of equal dimensions; the simultaneous cytokinesis gives rise to the early endosperm cells. The zygote, influenced by the endosperm, undergoes three or four rounds of mitotic division, resulting in a mass of very small cells. The mature seed is formed only of endosperm and undifferentiated embryo. The mature embryo is undifferentiated, globular, consisting of between 24 and 32 cells and it completely lacks any radicle, cotyledons or stem. The mature ovary becomes a tiny achene in both species. We proposed the existence of parthenogenesis for the Argentine species of *Lophophytum*, which is justified by the formation of the endosperm without any fertilization, which is the start of the development of the autonomous endosperm. The zygote begins its division due to the influence of the endosperm.

1. Introduction

Little has been studied in the Balanophoraceae (Kuijt, 1969), as regards endosperm and embryo development and no particular pattern has been established for the family Balanophoraceae. The greatest amount of research on the embryology refers to the genus *Balanophora* (Treub, 1898; Ernst, 1913; Chamberlain, 1914; Kuwada, 1928; Zweifel, 1939; Fagerlind, 1945a; Teryokhin and Yakolev, 1967), which is a genus in which species of great morphological reduction are found (Kuijt, 1969; Hansen, 1972; Hansen, 1980a; Eberwein and Weber, 2004). Discrepancies between the observations of different authors are found in some of the cases. Some of them have observed pollen grain germination on stigmas and a normal fertilization (Treub, 1898; Ernst, 1913; Fagerlind, 1945a), whereas Kuwada (1928) described apomictic processes (agamospermy) in *Balanophora japonica*. Su et al. (2012) use the expression "*putatively agamospermic*" for the *B. yakushimensis*, a species mentioned as agamospermic, because they doubt the observations and besides they do not discard the possibility of double fertilization in any of the species studied. Not all of the *Balanophora* species is known to be agamospermic. All the known cases with female-only individuals are restricted to particular taxa, e.g. *B. japonica, B. yakushimensis, B. elongata var. ungeriana,* and *B. fungosa* ssp. *indica* var. *globosa* (Hansen, 1972). The four taxa are all very likely agamospermic since they can produce seeds and there are no male flowers near. It is convenient to clarify that apomixis is the asexual reproduction by mitotic division without meiosis or sexual fusion, which implies the suppression of the sexual function, different from agamospermy which is reproduction by seeds although

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without fertilization (Richards, 2003). Parthenogenesis, a type of agamospermy, is when there is an embryo-sac and the embryo of the seed is formed by the unfertilized egg-cell (Rutishauser, 1982).

As regards seeds, parasitic plants generally have small and little differentiated embryos (Kuijt, 1969; Johri et al., 1992; Heide-Jørgensen, 2008). The holoparasitic species recorded, such as Hydnoraceae, Cynomoriaceae, Rafflesiaceae, have been characterized by the presence of reduced globular embryos, composed of a few cells without any differentiation of the organs and surrounded by the endosperm (Harms, 1935; Davis, 1966; Kuijt, 1969; Bhojwani and Bhatnagar, 1986; Johri et al., 1992).

In the Balanophoraceae the fruits are generally small, ovoid or cylindrical, with a decreasing diameter towards the base, most of them derived from a syncarpellate ovary (Harms 1935; Kuijt, 1969; Hansen, 1980b; Johri et al., 1992). Only *Sarcophyte* and *Chlamydophytum* species have multiple fruits formed by ovarian fusion of several flowers, making a fleshy mass (Kuijt, 1969). In *Lophophytum* species the fruit has been described as a small single-seeded achene, although its formation has not been studied (Davis, 1966; Hansen, 1980b; Johri et al., 1992).

In the Balanophoraceae even the term seed is conflictive, Holzapfel (2001), considers that it could not be applied to this family in a strict sense, as it has been proven that the structure derives completely from the embryo-sac and it is totally devoid of an integument.

Sato and Gonzalez (2016) described the megasporogenesis and megagametogenesis processes and they observed unusual behaviour in the central cell which forms a coenocyte without fertilization, but further events were not mentioned. They described an *Adoxa* type embryo-sac and this four-nucleate embryo-sac tends to take on a "J" shape during the migration of the two pairs of nuclei to the opposite poles. Each pair of nuclei undergoes a mitotic division to form an 8-nucleate embryo-sac. In the upper, chalazal pole of the embryo-sac, a typical egg-apparatus with a central egg-cell and two adjacent synergid cells is developed, while in the micropylar pole three antipodals are formed. The fusion of polar nuclei has not been observed. In this sense, a series of free nuclear divisions that result in a coenocyte structure of up to 12 nuclei has been noticed. This stage is characterized by a well-defined mature embryo-sac, the absence of antipodals and a number of nuclei in the middle-cell varying between 3–12, 6 being the most frequent number.

On the other hand, Cocucci (1991) noticed two striking features about the central cell: the huge amount of cytoplasmic stroma accompanying the organelle in comparison to the vacuole volume; and the rapid polar nuclei fusion, followed by free nuclear division prior to fertilization which leads to the formation of a coenocytic structure. He speculates that one sperm enters the cell and then fuses with one of the diploid nuclei so that a mosaic endosperm is formed where part of the cell would be 2n and part 3n.

The aim of this study is to describe the endospermogenesis and embryogenesis processes, as well as fruit development, in Argentine species of *Lophophytum,* reviewing the available literature about these processes in the Balanophoraceae in order to find if there is a general trend.

2. Materials and methods

2.1. Plant material

Two species of *Lophophytum* were used in this study:

Lophophytum leandri Echler., **ARGENTINA. Prov. Misiones:** San Ignacio y Salto Tabay; *Sato 114, 421, 422, 423* (CTES).

L. mirabile Schott & Endl. subsp. *bolivianum* (Wedd.) B. Hansen (hereinafter referred to as *L. mirabile*), **ARGENTINA. Prov. Jujuy:** Parque Nacional Calilegua, *Sato N° 430, 432, 434, 436* (CTES). **Prov. Salta:** Caraparí, *Sato 202* (CTES).

Voucher specimens were deposited in the herbarium of the Instituto de Botánica del Nordeste (CTES, Argentina).

2.2. Light microscopy (LM)

Pistillate flowers were fixed in FAA (formaldehyde, alcohol 70%, acetic acid, 5:90: 5). The material was dehydrated by histological dehydration followed by a tertiary butanol series (Gonzalez and Cristóbal, 1997); embedded in paraffin and microtomed using a Microm HM350 rotary microtome (MICROM International GmbH, Walldorf, Germany) into 12 μm transverse (TS) and longitudinal sections (LS), and then finally stained in either Safranin – Fast green (Johansen, 1940) or Safranin – Astra blue combinations (Luque et al., 1996). The presence and identification of starch were observed with polarized light and also stained with Lugol. $FeSO_4$ (Johansen, 1940) and IKI-H_2SO_4 (Ruzin, 1999) were used for the identification of tannins.

The stigmatic receptivity was tested by the bubbling of stigmata in the presence of 1% hydrogen peroxide (Galen and Plowright, 1987; Dafni and Motte Maués, 1998).

Observations were performed with a LEICA DMLB2 optical microscope equipped with a polarized light filter and a digital camera LEICA ICC50HD.

2.3. Scanning electron microscope (SEM)

The FAA-fixed material was dehydrated in an ascending acetone series, critical point dried (Denton Vacuum LLC, DCP-1, Pleasanton, EUA), and sputter coated with Gold-Palladium (Denton Vacuum, Desk II). Observati dried (Denton Vacuum LLC, DCP-1, Pleasanton, EUA), and sputter coated with Gold-Palladium (Denton Vacuum, Desk II). Observations were performed at 20 Kv with a SEM Jeol LV5800 (JEOL Ltd., Tokyo, Japan) using the Service of Electron Microscopy facility of the Universidad Nacional del Nordeste.

Measurements were made using software ImageJ (–2016). A three-dimensional reconstruction was made with the Rhinoceros 5.0 program (Versión 5, Educacional © 1993–2015, Robert McNeel & Associates). Photomicrographs of longitudinal serial sections were used to reconstruct the three-dimensional structure of endosperm and embryo.

3. Results

3.1. Developmental stages

Internal and external developmental changes in the inflorescence and flowers are shown and described. These changes were typified in three stages.

Stage I (Fig. 1A, D): The inflorescence emerges from ground level. The primary rachis is covered with tightly arranged sclerified scales completely hiding, secondary rachis with both staminate and pistillate flowers.

Stage II (Fig. 1B, E): The height of the inflorescence is completely developed and sclerified scales start to loosen and fall off. Scale abscission starts in the inflorescence medium area, exposing the secondary rachis with staminate flowers; the falling of the scales progresses towards the apex. The secondary rachis with pistillate flowers (at the base of primary rachis) is still covered by scales. At the beginning of this stage, the nuclei of the central cells of the embryo-sac have already started free division. The stigmas are wet and have pale yellowish-white colour and have positive reaction to test of H_2O_2, manifested by an intense bubbling reaction. At the same time, in the staminate flowers of the same inflorescence, the dehiscence of anthers has not started.

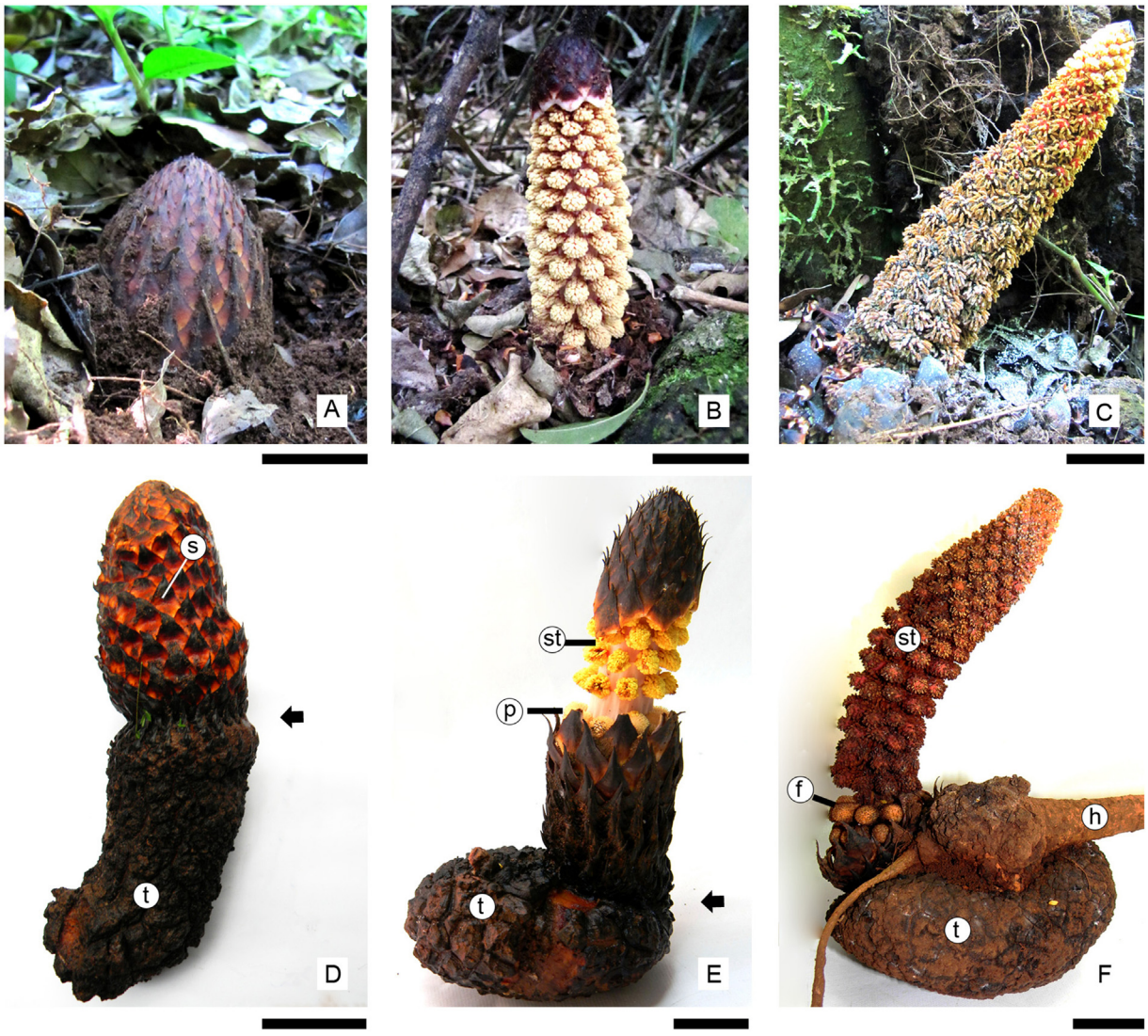

Fig. 1. Table I.: Stages of inflorescence development of Argentine species of *Lophophytum*. A-C, E-F: *L. leandri*. D: *L. mirabile*. A-C: habit of the plant in nature. D-F: complete specimens showing the underground parts. A, D: stage I. B, E: stage II. C, F: stage III. A: young inflorescence covered with scales. B: scales abscised in the middle section of the inflorescence exposing the secondary rachises with staminate flowers. C: inflorescence with senescent staminate flowers; pistillate flowers present but hidden by topsoil. D: complete plant showing the tuber (t) and the young inflorescence covered with scales (s), arrow indicates the soil level. E: complete plant showing the tuber (t), pistillate flowers (p) and staminate flowers (st), arrow indicates the soil level. F: complete plant attached to the root of host (h), showing the tuber (t), fruits adhered in secondary rachis and the post dehiscent staminate flowers (s). Scales bars: A-F: 10 cm.

Stage III (Fig. 1C, F).: The scales that cover the secondary rachis with pistillate flowers are the last ones to become detached due to abscission, but they do not fall off because this part is below the level of the ground. In *L. leandri* the inflorescence portion with pistillate flowers remains inside the mulch, which prevents the scales falling off whereas in *L. mirabile* this portion is below the soil level. The imbricate shape of the scales helps to keep them in place, thus abscission occurs at the petiole level. For this reason pistillate flowers are never exposed to the air. The scales of the peduncle of the inflorescence do not fall either, and even cover the first row of the secondary rachis with pistillate flowers. When the fruit is developing, the seeds have endosperm present. The staminate flowers have dehiscent anthers with pollen in a two-cell state.

3.2. Endospermogenesis

In a previous study (HAS & AMG) described an *Adoxa* type embryo-sac in *Lophophytum,* the embryo-sac with a typical egg-apparatus with a central egg-cell and two adjacent synergids cells, while three antipodals are formed in the micropylar pole. The fusion of polar nuclei has not been observed. In this work it was observed that the two polar nuclei migrate to the upper middle area of the central cell of the embryo sac, near to the egg apparatus. No pollen tube or fertilization was observed. The endosperm and embryo are formed in the absence of double fertilization. The nuclei of the central cell were observed undergoing free divisions, giving rise to a coenocyte (Fig. 2A–C), which increases the nuclei number to between 2 and 12, with 6 being the most frequently recorded. A great increase in the cytoplasmic content is seen inside the coenocyte which acquires a dark blue-purple colouring following safranin-fast-green staining (Fig. 2C). Small corpuscles acquire the same red colouring as nuclei are observed free in the cytoplasm. The embryo-sac increases its size, mainly in its diameter (Fig. 2G). At this embryo-sac stage, the pistillated flowers already show their receptive stigmas.

The second stage in endosperm formation involves nuclei fusion. The coenocyte has varying sizes of nuclei, from 20 μm, which are a result of fusion. Some of the large nuclei located towards the

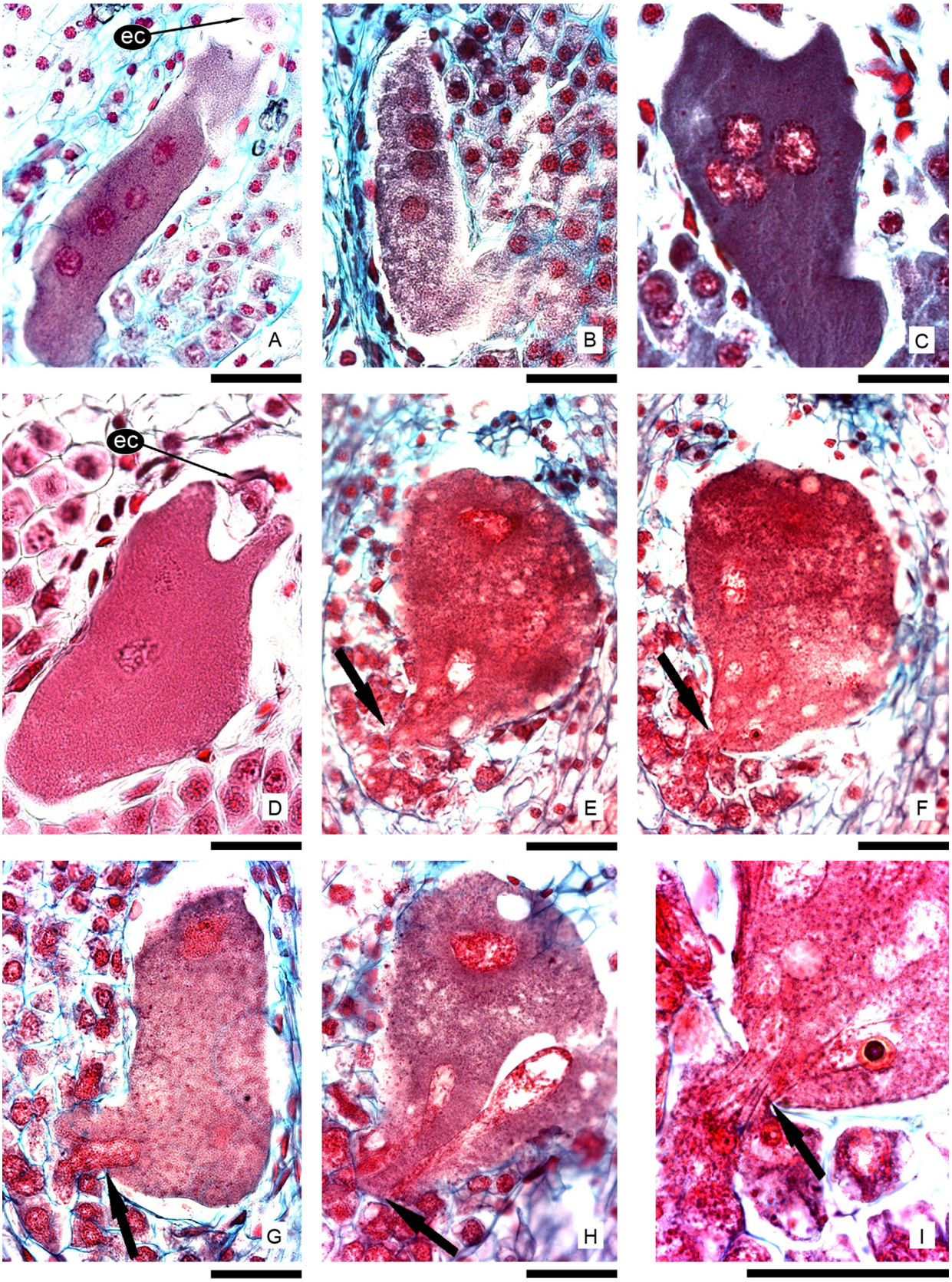

Fig. 2. Endosperm development: first stage of a coenocyte formation and later nuclear fusions, with light microscopy. A, E-I: *L. mirabile*. B-D: *L. leandri*. A-C: coenocyte with free division nuclei. D: coenocyte showing only one endospem nucleus and egg-cell (ec) in upper portion. E-F: LS successive stages showing material input from the nucellus (arrows). G-I: details of nuclei and cytoplasmic matrix input from the nucellus and fusion with coenocyte nuclei (arrows). Scales bars: A-I: 50 μm.

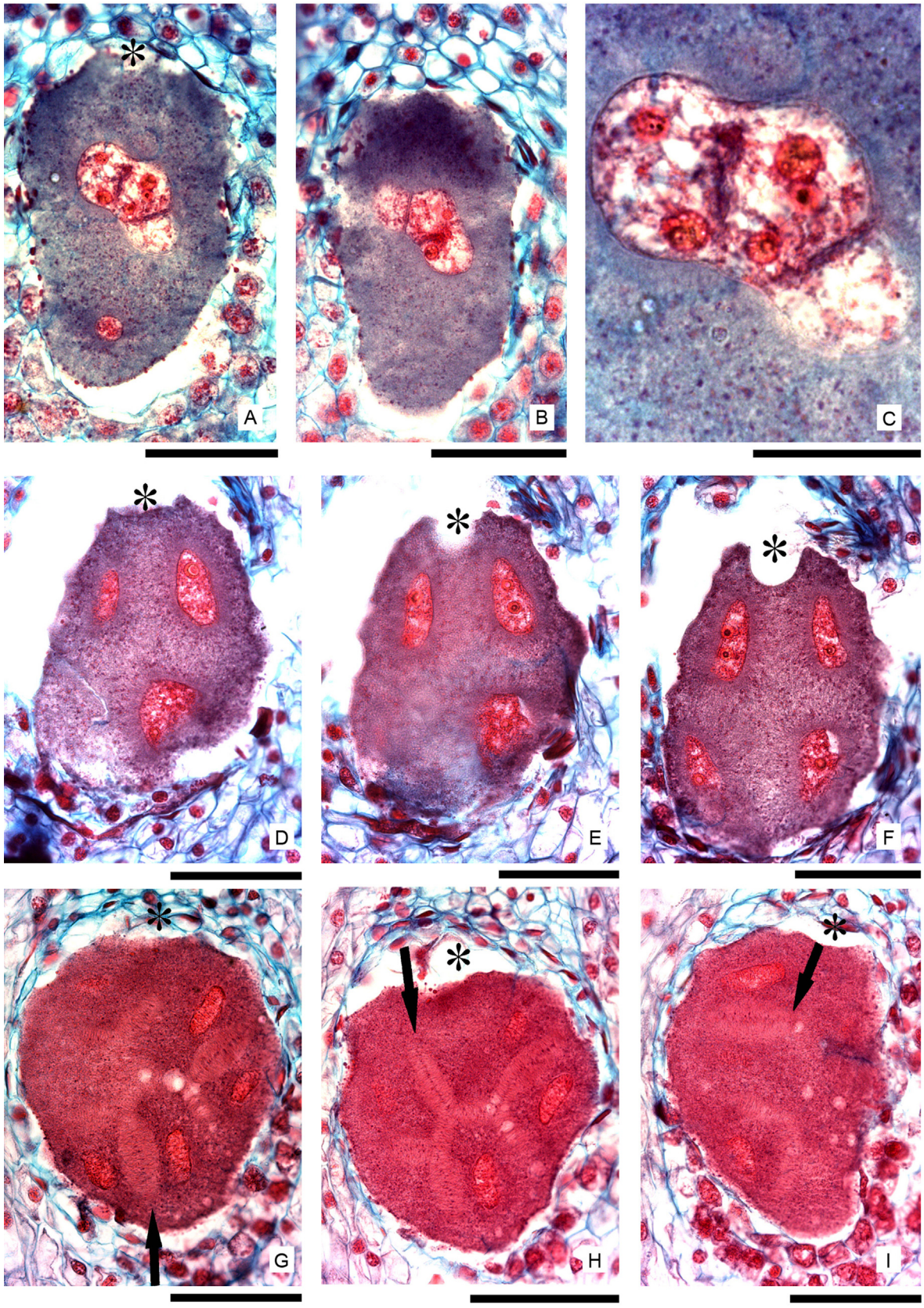

Fig. 3. Endosperm formation: subsequent karyokinesis and cytokinesis stages with light microscopy. A-C: *L. mirabile*. D-I: *L. leandri*. A-C: nuclei fusion. D-F: LS successive karyokinesis (* shows the zygote position, but not in this section). G-I: LS of successive cytokinesis of endosperm nuclei, mitotic spindles and phragmoplasts are observed (arrows), * indicates the zygote position. Scales bars: A-B, D-I: 100 μm; C: 50 μm.

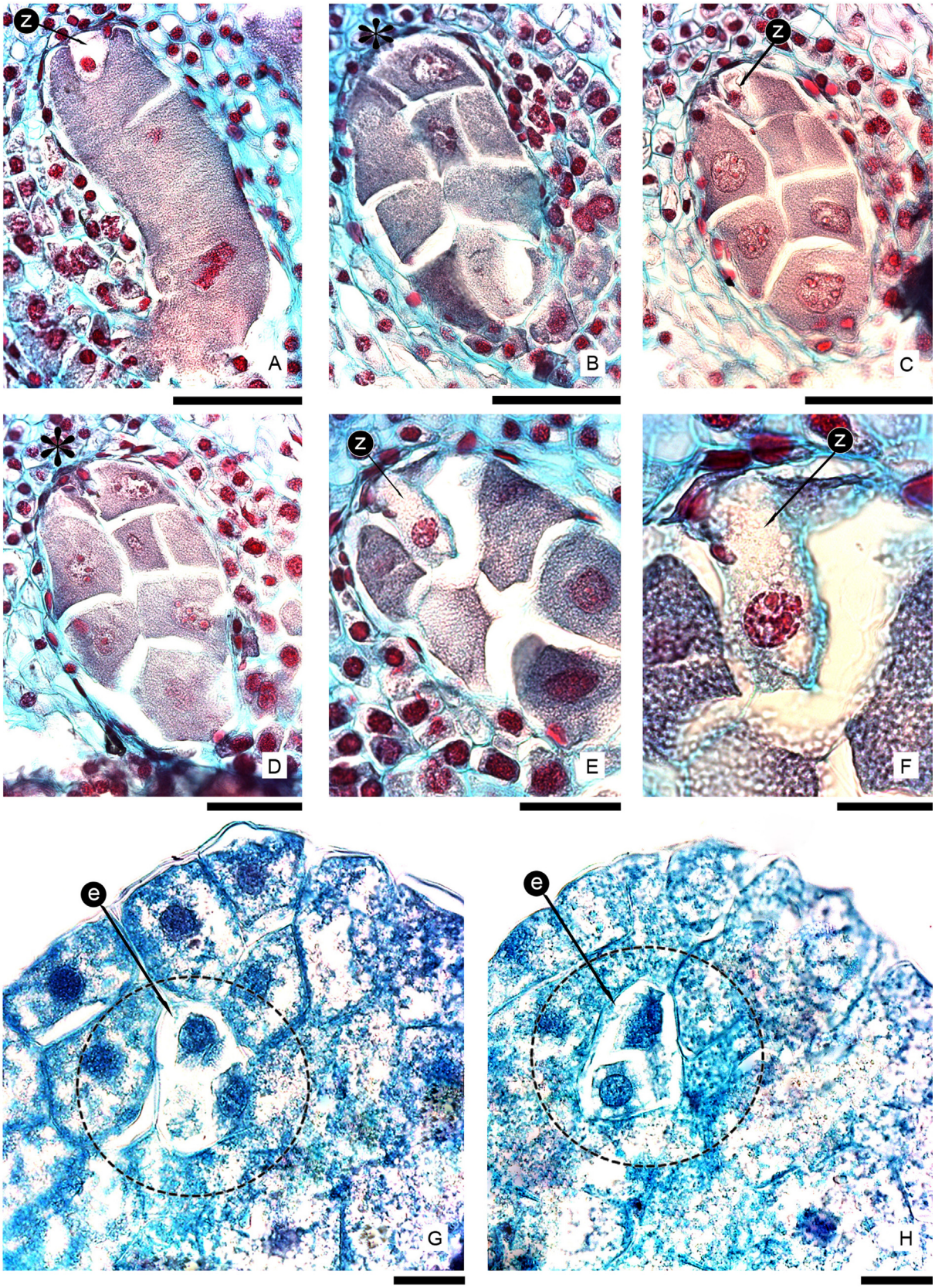

Fig. 4. Endosperm and zygote cytokinesis with light microscopy. A, G-H: *L. leandri*. B-F: *L. mirabile*. A: LS of zygote (z) and endosperm at initiation of cytokinesis. B-E: LS of successive mitoses of endosperm. E: zygote (z) with surrounding endosperm cells. F: zygote (z) detail. G-H: successive LS of seed with 4 cell embryo (e) immersed in endosperm. Scales bars: A-E: 50 μm; F-H: 25 μm.

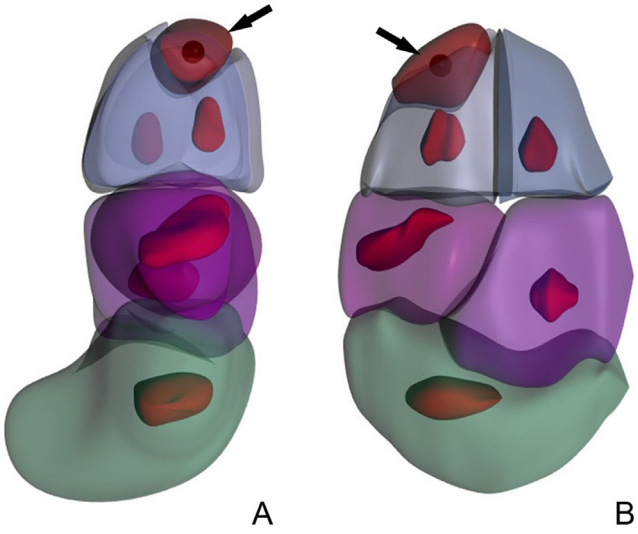

Fig. 5. Three-dimensional reconstruction of endosperm and zygote from serial sections in Fig. 4B–F. A: lateral view. B: frontal view. The arrows indicate the zygote.

micropylar pole (zone of already degenerated antipodals) acquire a teardrop shape, their sharp end projecting into the adjacent nucellar cells (Fig. 2E–I). An interruption occurs in the coenocyte wall and the nucellar cells wall, and then the nuclei of the coenocyte and the nucellar cells are fused. Several nuclei of the nucellar cells were observed chaining prior to fusion. This fusion allows the entrance of both nuclear and maternal cytoplasmic material inside the coenocyte. This event takes place in the nucellar cells located at the micropylar end of the ovule. Once inside the coenocyte, all the fused nuclei become one giant nucleus, reaching 120 × 60 μm in size. (Fig. 3A–C).

The third stage of endosperm formation is the sequence of karyokinesis coordinated inside the coenocyte, producing nuclei of equal size (50–70 μm diameter); When nuclear division reaches about 12 nuclei, simultaneously cytokinesis takes place generating the first cells of the endosperm (Figs. 3D-I; 5A-E). The mitotic spindles are very evident in this cytoplasmic division.

This process only occurs in one embryo-sac as the second embryo-sac is reabsorbed (Fig. 6A). Once coenocyte cellularization has taken place it starts dividing mitotically (Fig. 6B–C). Once developed, the endosperm gradually fills the entire ovarian cavity, where the placenta and second ovule were previously, until it is contained by the endocarp. The endosperm cells begin to accumulate abundant reserve substances as starch grains as they develop (Fig. 6D–E). Fully developed endosperm is formed from clear cytoplasm uninucleate cells ranging from 600 to 900 μm diameter.

3.3. Embryogenesis

During the endosperm development, the synergids were compressed and reabsorbed. Initially, no divisions took place in the egg cell located at the upper end of the endosperm mass (Fig. 4 D–F). The egg cell divides forming a four-cell globular embryo only when the endosperm cytokinesis is complete (Fig. 4G–H). It was not possible to detect the first division to see whether it occurred on the transverse or longitudinal plane, but no suspensor formation was verified. The globular embryo continues with three or four mitotic rounds, resulting in a mass of very small cells (Fig. 6E–F).

The mature embryo is undifferentiated, globular and it is composed of up to about 24–32 cells; it completely lacks any cotyledons or outline of a radicle (Fig. 6G). The total size of the embryo is similar to one endosperm cell. It is completely surrounded by the endosperm, located in the apical portion thereof, in the upper central area of the ovary. Its cells completely lack tannins that are omnipresent in the remaining fruit (Fig. 6 E).

Ovule nucellus tissue (it does not have any integuments) was digested during endosperm formation, therefore the seed lacks a seed coat, (Figs. 6D and E, 7I). The mature seed is only made up of the endosperm and undifferentiated embryo. It is spheroidal with a small wedge towards the upper part of the ovary (Fig. 7G-I). Its consistency is gelatinous in both species and the limits between the cells conforming it are observed on its surface (Fig. 7I).

3.4. Fruit development

The mature ovary becomes an achene, reaching an average size of 2.5 × 1.5 mm in *L. mirabile,* and 2 × 1.2 mm in *L. leandri* (Fig. 7B–E).

The pericarp is composed of the epicarp formed by the outer epidermis of the ovary, with cells completely occupied by tannins; the ones in the upper portion of the ovary are differentiated into sclereids, which even reach the mid-portion (Fig. 7B, E,F). In *L. mirabile* the sclereids only occupy the apical portion of the fruit and in *L. leandri* they spread along its side walls reaching the upper third of the fruit (Fig. 7A). Also in *L. mirabile* sclereids differentiate in the basal portion of the fruit (Fig. 7F).

The mesocarp is made up of parenchyma cells with tannins. In *L. leandri* the brachysclereid ring of the upper portion of the ovary binds to the superficial sclereids by sclerification of the intermediate parenchyma cells (Fig. 7E). The wall of the fruit is generally porous in dry state (Figs. 6D and E; 7E–G).

The endocarp develops from the internal epidermis of the carpel which differentiates into brachysclereids, which then become in the pits that protect the seed (Figs. 6D and E; 7H). In L. mirabile the ring of secreting cells is differentiated at the fruit base by forming a whitish area noticeable in dry specimens (Fig. 7D and C).

4. Discussion

4.1. Endospermogenesis-embryogenesis

Practically all Balanophoraceae embryological studies are restricted to the genus *Balanophora.* Endosperm development in the Balanophoraceae seems to be unique as regards its features. Hofmeister (1858) and Van Tieghem (1896) were the first authors to record them and they observed normal behaviour, 8-nucleate embryo sacs and even fertilization in *Balanophora globosa*. In contrast, Treub (1898) and Lotsy (1899) studied *B. elongata* and *B. globosa*, observing that one out of the eight mature embryo-sac nuclei produces endosperm and that the other seven degenerate, thus producing the embryo from one endosperm cell; in the seed the embryo is suspended by a small pedicel and the endosperm cells are filled with oily material.

Ernst and Schmid (1913) observed that the embryo sac in *Balanophora* species is made up of a diploid nucleus, from which the eight nuclei of the embryo-sac are derived and where only one of the polar nuclei progresses a large haustorial cell and another one that will generate the endosperm resulting from this first division. They also argue that the embryo derives from the unfertilized diploid egg cell, describing this phenomenon as “parthenogenesis”, though a possible fertilization is not ruled out.

Later, Chamberlain (1914) in new research on *Balanophora globosa* and *B. elongata,* observed that the embryo does not originate from an endosperm cell, rather it is the egg cell that forms it, but its subdivisions take place later than those of the endosperm, which surrounds the egg cell without it having been fertilized.

Chamberlain’s research (1914) was very significant, since similar events were observed in *Lophophytum* to those recorded in agamospermic specie *B. japonica,* such as the fact that endosperm

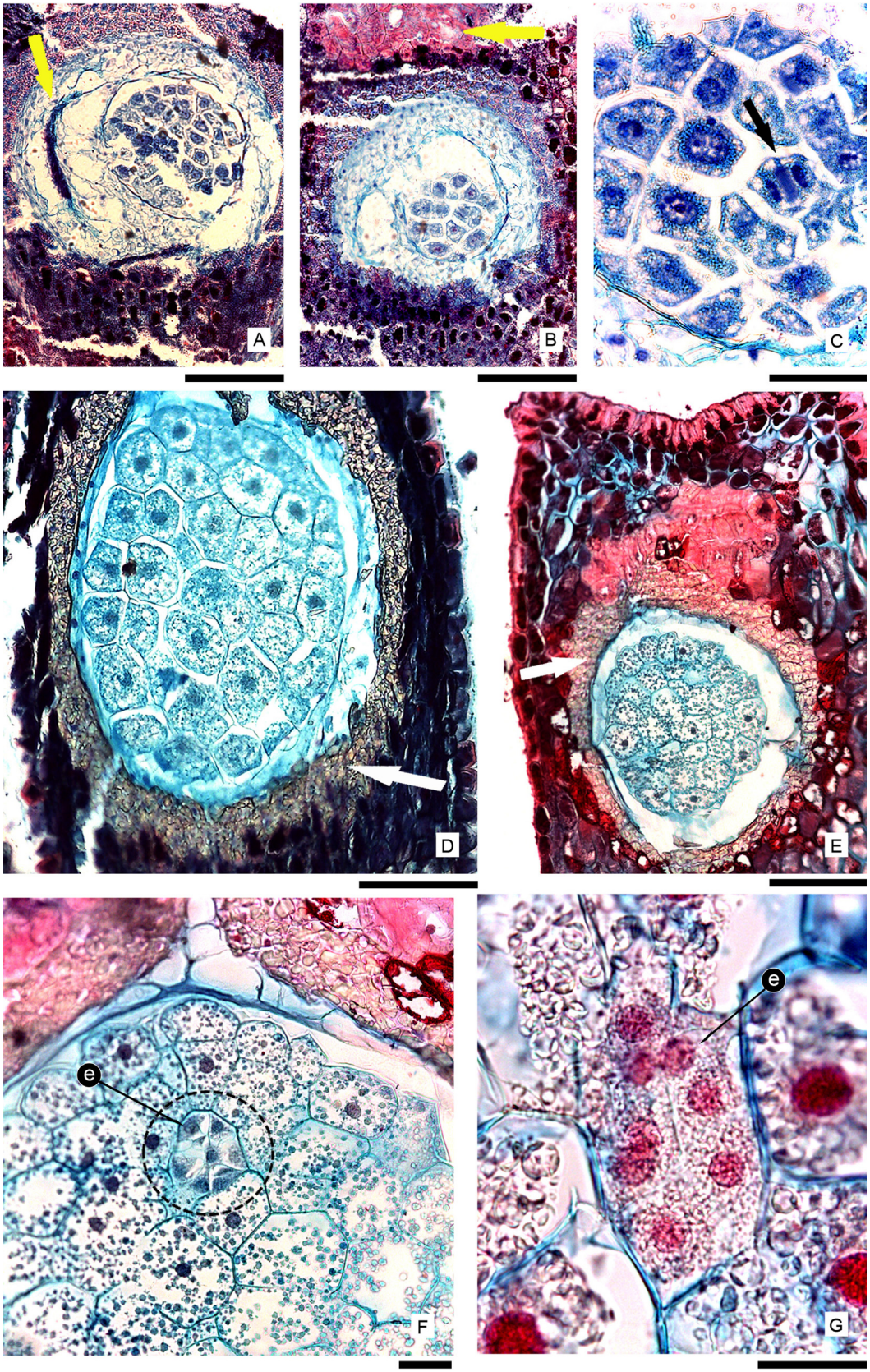

Fig. 6. Seed and fruit development with light microscopy. A-D, G: *L. leandri*. E-F: *L. mirabile*. A: endosperm cells division, one embryo-sac of the pair is indicated reabsorbing (arrow). B: LS of fruit, sclereid ring in upper portion (arrow). C: endosperm mitotic division (arrow). D-E: seed inside the fruit, the arrow shows endocarp sclereids. F: embryo (e) immersed in endosperm. G: undifferentiated embryo (e). Scales bars: A-B, D-E: 200 μm. C: 50 μm. F-G: 20 μm.

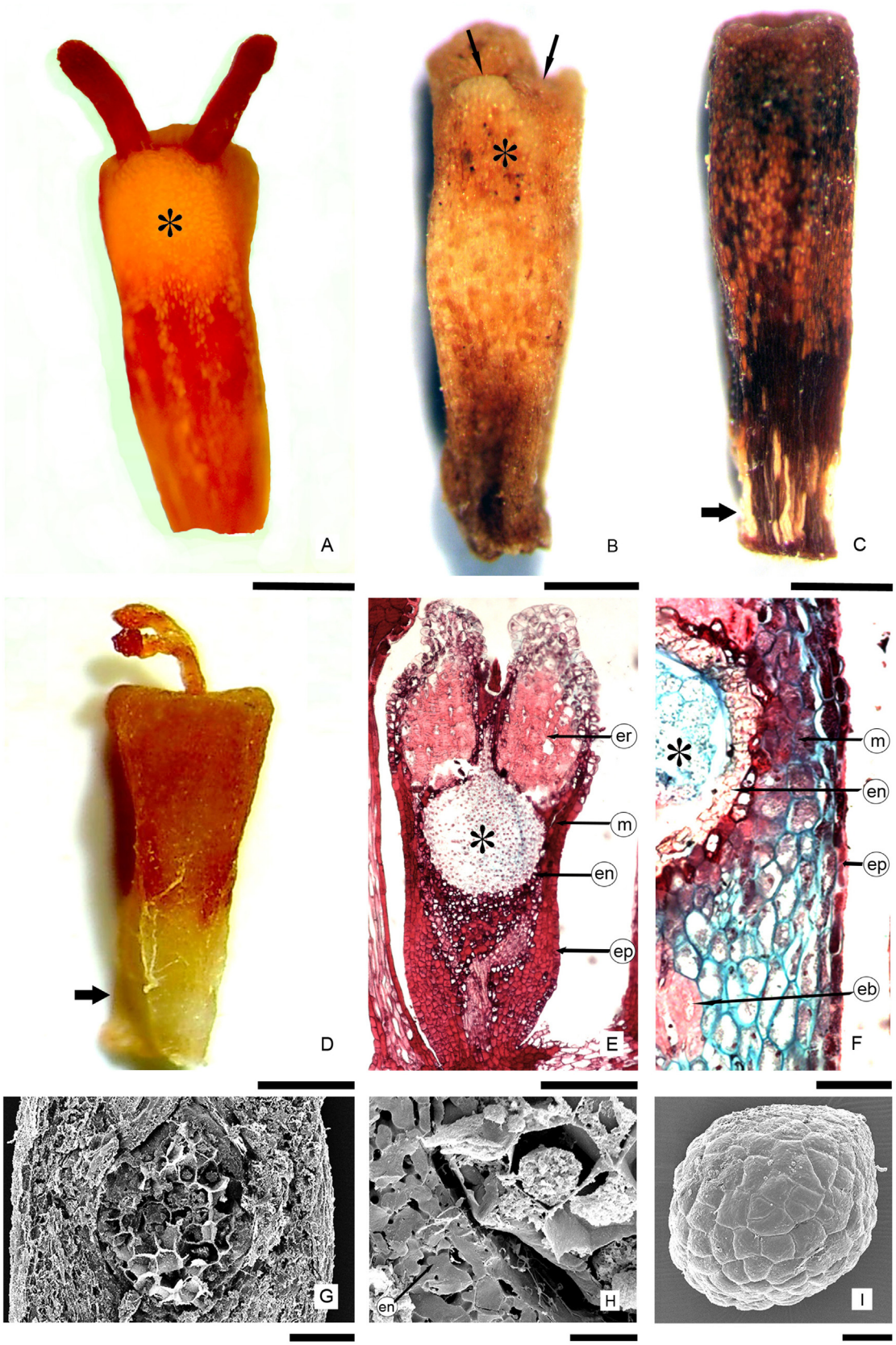

Fig. 7. Seed and fruit development. A-D: stereoscopic microscope, E-F: light microscopy, G-I: SEM. A-B, E, G-H: *L. leandri*. C, D, F, I: *L. mirabile*. A: immature fruit, asterisk indicates the zone of sclereid ring. D: immature fruit, secretory tissues at the base is observed (arrow). B: mature fruit, asterisk indicates the zone of sclereid ring; the arrows indicate the area where the styles were uncertain. C: mature fruits, the clear area with secretory tissues at the base is observed (arrow). F: wall detail showing epicarp (ep), mesocarp (m), endocarp (en) and sclereid ring (er) bordering epidermis sclereids. E: wall detail showing epicarp (ep), mesocarp (m), endocarp (en) and basal sclereids (eb). G: LS of fruit with seed. H: endocarp (en) of fruit with endosperm cells of seed. I: seed. Scales bars: A-D: 0,5 mm; B-C: 1 mm; F, G, I: 200 μm; E: 1 mm; H: 25 μm.

originates autonomously without fertilization and that it develops and surrounds the unfertilized egg cell which begins to divide long after the endosperm. The omission in the early works of Treub (1898) and Lotsy (1899) were probably the result of the difficulty imposed by the sclerotic endocarp in making the sections, coupled with the size difference between the endosperm and zygote body.

In addition, the behaviour of the central nuclei is key to the initiation of the endosperm. Even though input of nuclei from nucellar cells in *Balanophora japonica* was not recorded, the presence of a chalazal haustorium formed by an embryo sac projection is mentioned (Zweifel, 1939). Arekal and Shivamurthy (1978) noticed that the antipodal nuclei showed varying behaviour in the *B. abbreviate and B. fungosa ssp. indica var. indica*, being able to fuse between themselves and with the polar nuclei of the central cell forming the endosperm. In *Lophophytum,* a haustorium does not develop and participation of the antipodals was not observed in the endosperm formation, however, an area through which nucellus nuclei are incorporated was observed. In spite of the numerous observations, none of the three events: fertilization, fusion of a sperm nucleus with the egg cell nucleus or fusion of two polar nuclei, have been reported in *Lophophytum* by Cocucci (1991) or Sato and Gonzalez (2016). However Cocucci (1991) proposes the possibility of endosperm formation in mosaics made up of cells with different ploidy levels, arising from the fertilization of some coenocyte nuclei. This in fact does not occur because the nuclei fuse and then divide, as was previously described.

Zweifel (1939) studied *Balanophora abbreviata* and *B. indica*, mentioning that endosperm is of the cellular type and the primary division of the endosperm nucleus is followed by cytokinesis, when a wall separates a small micropylar cell from a large chalazal one. The micropylar cell gives rise to endosperm, while the chalazal develops a large uninucleate haustorium, subsequently crushed by endosperm tissue development.

In respect to the embryogenesis type in Balanophoraceae, Ekambaran and Panje (1935) observed that the third division of embryo cells in *Balanophora dioica* is periclinal in the four quadrants and that the embryo is made up of four central and four peripheral cells and all the nuclei in the cells are in the equatorial region of the embryo. They also mention that the embryo becomes embedded in the endosperm during development and has about 12 cells. Treub (1898) indicates a five cell embryo in *B. elongata* whereas Ernst (1913) reports a six cell embryo in the same species. Zweifel (1939) describes embryogenesis as both the first and second division are vertical and there is no suspensor, this type corresponds to the so-called Piperad type which is found in few families, the Balanophoraceae among them (Johri, 1992). In all the parasite species studied the embryo in a mature seed is undifferentiated, therefore, Teryokhin and Yakolev (1967) described it as a non-oriented embryogenesis.

The plane of the first divisions of the zygote could not be observed in *Lophophytum*, therefore the embryo type to which it corresponds could not be established. However, the absence of a suspensor is highlighted, which together with an undifferentiated globular embryo are the features shared by other parasitic species in the Loranthaceae, Hydnoraceae and Balanophoraceae families.

The presence of a suspensor has been associated with the function it performs when the embryo goes deeper inside the endosperm (Johri, 1984); so its absence in *Lophophytum* would be explained by the fact that the zygote goes deeper inside the endosperm before its brief further development and therefore, the presence of a suspensor is unnecessary.

It is clear that both the embryo and the endosperm in *Lophophytum* have a larger number of cells and therefore, they are larger in size than in studied species of *Balanophora*, confirming the extreme reduction in this genus within the family.

The presence of polyembryony was recorded in *Balanophora globosa* by Kuwada (1928) and in *Helosis cayennensis* by Fagerlind (1945b). *B. japonica* was described as an apomictic diplosporium species of the *Taraxacum* type, due to the formation of a restitution nucleus (Kuwada, 1928). Apomixis has been suggested for other Balanophoraceae genera, such as *Ombrophytum*, based on their underground life style, but no studies have been carried out to confirm this (Kuijt, 1969; Hansen, 1980b).

The hypothesis of parthenogenesis is suggested for the Argentine species of *Lophophytum*, which is justified by embryo and endosperm formation in the absence of fertilization, and the initiation of endosperm development is autonomous, an event also observed by Cocucci (1991) in *L. leandri*. In addition, the endosperm first surrounds the zygote, which subsequently begins to divide. Cases in which the zygote starts dividing due to the influence of the endosperm were recorded in *Lilium* species, in *Bergenia delavayi* and *Erythraea centaurium* (Rutishauser, 1982). In this case egg cell development may be prompted by penetration of a sperm nucleus or by endosperm development. At the same time, endosperm development may result from fertilization or the rare case of autonomous development, under the sole influence of pollination (Rutishauser, 1982). Ohad et al. (1996) described endosperm formation without fertilization in an *Arabidopsis* mutant.

Other observations that would justify the existence of parthenogenesis are: pollen tube development which has never been observed in the studies performed and although pistillate flowers are not exposed to the environment, there is access to them by insects which act as pollinators. When pollen exclusion experiments (unpublished observations) in pistillate flowers were performed seeds were formed although their subsequent viability is not known. There is a great similarity of these events with those reported as agamospermy by Chamberlain (1914) and Zweifel (1939) in *Balanophora* species. In most of the *Balanophora* species potential pollinators are reported, as well as for *Lophophytum* (Ferrer et al., 2011; Su et al., 2015).

However, in accordance with Su et al. (2012), we cannot guarantee that it is the only form of reproduction as there were no structural hindrances for pollination and subsequent fertilization: it would be convenient to study whether there are any physiological, biochemical or genetic obstacles.

The *Lophophytum* and *Balanophora* seeds are similar to those of other holoparasites, especially *Pilostyles* (Heide-Jørgensen, 2008), which suggests that this is a convergence phenomenon, in the same sense as posed by Kuijt (1969).

We consider that there is no inconvenience to apply the term seed in Balanophoraraceae, in contrast to the opinion of Holzapfel (2001)

4.2. Fruit

The fruit in the *Lophophytum* species has been described as a single-seeded achene (Kuijt, 1960; Davis, 1966; Hansen, 1980b). Our observations in *Lophophytum* show that the endocarp is sclerotic, the mesocarp is dry and corky and the seed is in close contact with the pericarp, so it is considered as an achene.

The *Lophophytum* fruit structure is coincidental with that observed in *Balanophora* in regard to the sclerotic endocarp formation (Zweifel, 1939; Johri et al., 1992), but with a much more developed mesocarp.

Borchsenius and Olesens (1990) mention that *L. mirabile* fruits do not float, contrary to our field observations, as when the fruit is dry it floats and it may be dragged by the water flow, which could be considered as a form of dispersal (personal observation).

5. Conclusion

The existence of parthenogenesis is suggested for the Argentine species *of Lophophytum*, justified by the formation of endosperm without any fertilization, i.e. the initiation of autonomous endosperm development, however, one cannot rule out possible fertilization. The endosperm surrounds the zygote which later begins to divide. The zygote would begin its division due to the influence of the endosperm. Further studies are needed in other species of the Balanophoraceae to be able to propose a general tendency for the family. However it is clear that parthenogenesis is a mechanism that exists in the Balanophoraceae.

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References

Arekal, G.D., Shivamurthy, G.R., 1978. Female gametophyte in two species of *Balanophora* – a reinvestigation. Phytomorphology 28, 7–13.
Bhojwani, S.S., Bhatnagar, S.P., 1986. The Embryology of Angiosperms Vani. Educational Books, New Delhi.
Borchsenius, F., Olesens, J.M., 1990. The amazonian root holoparasite *Lophophytum mirabile* (Balanophoraceae) and its pollinators and herbivores. J. Trop. Ecol. 6, 501–505.
Chamberlain, C.J., 1914. The embryogeny of *Balanophora*. Bot. Gaz. 57, 542.
Cocucci, A.E., 1991. *Lophophytum leandri* megasporogenesis and gametogenesis. In: Ransom, J.K., Musselman, L.J., Worsham, A.D., Parker, C. (Eds.), Proceedings of the 5th International Symposium of Parasitic Weeds. CIMMYT Kenya, Nairobi, pp. 31–35.
Dafni, A., Motte Maués, M., 1998. A rapid and simple procedure to determine stigma receptivity. Sex Plant Reprod. 11, 177–180.
Davis, G.L., 1966. Systematic Embryology of the Angiosperms. John Wiley & Sons, New York.
Eberwein, R., Weber, A., 2004. Exorhopala ruficeps (Balanophoraceae): morphology and transfer to Helosis. Bot. J. Linn. Soc. 146, 513–517.
Ekambaran, T., Panje, R.R., 1935. Contributions to our knowledge of Balanophora 2-Life history of B dioica. Proc. Indian Acad. Sci., 522–543.
Ernst, A., Schmid, E., 1913. Über blute und frucht von *Rafflesia*. Ann. Jard. Bot. Buitenzorg. 27, 1–58.
Fagerlind, F., 1945a. Bau der floralen organe der gattung *Langsdorffia*. Svensk Bot. Tidsskr. 39, 197–210.
Fagerlind, F., 1945b. Bildung und entwicklung des embryosacks bei sexuellen und agamospermichen *Balanophora*. Svensk Bot. Tidsskr. 39, 65–82.
Ferrer, M.S., Marvaldi, A.E., Sato, H.A., Gonzalez, A.M., 2011. Biological notes on two species of *Oxycorynus* (Coleoptera: *Belidae*) associated with parasitic plants of the genus *Lophophytum* (Balanophoraceae), and new distribution records in Argentina. Rev. Soc. Entomol. Argent. 70, 351–355.
Galen, C., Plowright, R.C., 1987. Testing the accuracy of using peroxidase activity to indicate stigma receptivity. Can. J. Bot. 65, 107–111.
Gonzalez, A.M., Cristóbal, C.L., 1997. Anatomía y ontogenia de semillas de *Helicteres lhotzkyana* (Sterculiaceae). Bonplandia 9, 287–294.
Hansen, B., 1972. The genus balanophora: J. R. & G. Foster.A taxonomic monograph. Dansk Bot. Ark. 28, 1–188.
Hansen, B., 1980a. Pollen dimorphism in lophophytum mirabile schott & endl (Balanophoraceae). Grana 19, 189–191.
Hansen, B., 1980b. Balanophoraceae. Flora Neotrop. 23, 1–80.
Harms, H., 1935. Balanophoraceae. In: Engler, A., Prantl, K. (Eds.), Die Natürlichen Planzenfamilien. Wilhelm Engelman, Leipzig, pp. 296–339.
Heide-Jørgensen, S., 2008. Parasitic Flowering Plants. Koninklijke Brill NV, Leiden, The Netherlands.
Hofmeister, W., 1858. Neuere beobachtungen über embryobildung der pahenogamen. Jahrb. Wiss. Bot. 1, 110.
Holzapfel, S., 2001. Studies of the New Zealand root-parasite *Dactylanthus taylorii* (Balanophoraceae). Englera 22, 7–176.
Johansen, D.A., 1940. Plant Microtechnique. McGraw-Hill Book Co, New York (523).
Johri, B.M., 1984. Embryology of Angiosperms. Springer-Verlag Press, Berlin.
Johri, B.M., Ambegaokar, K.B., Srivastava, P.S., 1992. Comparative Embryology of Angiosperms. Springer-Verlag Press, Berlin.
Kuijt, J., 1969. The Biology of Parasitic Flowering Plants. University of California Press, Berkeley.
Kuwada, Y., 1928. An ocurrence of restitution-nuclei in the formation of the embryo sacs in *Balanophora japonica*. Bot. Mag. (Tokyo) 42, 117–129.
Lotsy, J.P., 1899. Balanophora globosa, eine wenigstens örtlich verwitwete Pflanze. Ann. Jard. Bot. Buitenzorg 2, 174–184.
Luque, R.H., Sousa, C., Kraus, J.E., 1996. Métodos de coloraçao de Roeser (1972)-modificado- e Kropp (1972) visando a subtituicao do azul de astra por azul de alciaô 8 GS ou 8 GX. Acta Bot. Bras. 10, 199–212.
Ohad, N., Margossian, L., Hsu, D., Williams, C., Repetti, P., Fischer, R.L., 1996. A mutation th at allows endosperm development without fertilization. Proc. Natl. Acad. Sci. U.S.A. 93, 5319–5324.
Rasband, W.S., 1997. ImageJ, U. S. National Institutes of Health. Bethesda, Maryland, USA (2016) http://imagej.nih.gov/ij.
Richards, A.J., 2003. Apomixis in flowering plants: an overview. Philosophical Transactions of the Royal Society B: Biological Sciences 358 (1434), 1085.
Rutishauser, A., 1982. Introducción a la embriología y biología de la reproducción de las angiospermas. Hemisferio Sur, Buenos Aires, Argentina.
Ruzin, S.E., 1999. Plant Microtechnique and Microscopy. Oxford University Press, New York.
Sato, H.A., Gonzalez, A.M., 2016. Floral development and anatomy of pistillate flowers of Lophophytum (Balanophoraceae), with special reference to the embryo sac inversion. Flora 219, 35–47.
Su, H.J., Murata, J., Hu, J.M., 2012. Morphology and phylogenetics of two holoparasitic plants, Balanophora japonica and Balanophora yakushimensis (Balanophoraceae), and their hosts in Taiwan and Japan. J. Pl. Res. 125, 317–326.
Su, H.J., Hu, J.M., Anderson, F., Der, J., Nickrent, D., 2015. Phylogenetic relationships of Santalales with insights into the origins of holoparasitic Balanophoraceae. Taxon 64, 91–506.
Teryokhin, E.S., Yakolev, M.S., 1967. Embryology of Balanophoraceae (on the problem of homologies of the flower of Balanophora). Bot. Zhurn. 52, 745–748.
Treub, M., 1898. L'organe femelle et l'apogamie du Balanophora elongata. Ann. Jard. Bot. Buitenzorg. 15, 1–25.
Van Tieghem, P., 1896. Sur les phanérogames à ovule sans nucelle, formant le groupe des innucellées ou santalinées. Bull. Soc. Bot. France 43, 543–577.
Zweifel, R., 1939. Cytologisch-embryologische Untersuchungen an Balanophora abbreviate Blume und Balanophora indica Wall. Vierteljahrsschr. Naturf. Ges. Zurich 84, 245–306.