Chapter

Anatomy, Embryology and Life Cycle of *Lophophytum*, a Root-Holoparasitic Plant

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Abstract

The most extreme manifestation of parasitism occurs in holoparasites, plants that are totally achlorophyllous. Among them, the genus *Lophophytum* (Balanophoraceae) is characterized by an aberrant vegetative body called a tuber, devoid of stems and leaves. The genus is exclusively South American, comprising five taxa, which parasitize the roots of trees or shrubs. This review focuses on the Argentine species of the genus*: L. leandri* and *L. mirabile* subsp. *bolivianum*. Topics covered include: morphology and anatomy of the vegetative body and host–parasite connection; structure, anatomy and development of the staminate and pistillate flowers; sporogenesis and gametogenesis, embryo sac inversion; endospermogenesis, embryogenesis and fruit development. The evolutionary trend in the gynoecium and embryo sac of the Balanophoraceae is also discussed to reflect the variability. Finally, observations were made on the synchronization of the life cycles of the parasites and hosts to infer possible ways by which parasitism has evolved, until now unknown.

Keywords: embryology, embryo sac inversion, holoparasitism, host–parasite connection, legume, tuber

1. Introduction

Most vascular plants (Pteridophytes and Spermatophytes) are autotrophic, producing their food through photosynthesis. However, a significant number of plants have adopted a heterotrophic mode of life, obtaining part, or all, of their requirements from other organisms [1–4]. These can be divided into myco-heterotrophs (living in symbiosis with fungi through which they feed on decaying organic matter and the so-called parasitic plants, that grow on other plants and establish an organic union or haustorium by which they derive food directly from the host [4, 5].

There are two basic types of parasitic plants: hemiparasites and holoparasites [6]. The former possess chlorophyll and are capable of photosynthesis (at least during some phase of their cycle) and they obtain only water and mineral salts through haustoria with the host. The most extreme manifestation of parasitism occurs in holoparasites, which are totally achlorophyllous, obtaining all their nutrients from the host, on which they are totally dependent [7]. Most holoparasites are found parasitizing the roots of their hosts.

Worldwide, many of the parasitic plants represent major losses to agriculture, especially in Africa, where root holoparasites cause serious damage to cereals and legumes [8, 9]. Conversely, others are on the red lists of endangered plants, such as the Balanophoraceae [10, 11].

According to Kuijt [1] and Musselman & Press [12] there are about 3,000 species of parasitic plants, representing approximately 1% of flowering plants. Other recent studies put this number at 292 genera and ca. 4750 species [3, 4, 6, 13]. According to Heide-Jørgensen [3] the parasitic plants are distributed in 280 genera and 20 families, 90% (4,100 ssp.) are hemiparasites and only 10% (390 ssp.) are holoparasites. About 60% are root parasites and 40% are stem parasites. Holoparasites are represented in the families Orobanchaceae, Cynomoriaceae, Lennoaceae, Apodanthaceae, Cytinaceae, Raflesiaceae, Hydnoraceae and Balanophoraceae [3, 4]. Parasitism evolved independently in different groups of Angiosperms and there are thirteen lineages where at least one species is parasitic [14, 15].

It is agreed that these modifications respond to a phenomenon of evolutionary convergence [1, 3]. In this sense, Westwood (2010) emphasizes that the study of the structure of parasitic plants provides the conceptual framework for understanding the "specialization" of plants in general.

Among the more specialized holoparasites are the species of the family Balanophoraceae L. C. Richard et A. Richard, which are devoid of chlorophyll and parasitize the roots of trees and shrubs. The best summary of the known characteristics of the family Balanophoraceae can be found in Kuijt & Hansen's work [16]. These plants develop a vegetative body called a tuber, which is partially or totally underground, of variable shape and color, from whitish-yellowish to yellow, orange to reddish-orange or brownish, or even purplish. It lacks the structures of the typical cormophytic organization, as the body is not differentiated into root, stem, and leaves [1, 3, 17–21].

A peculiarity of holoparasites is the tendency to acquire foreign genes from their host plants. It has recently been demonstrated that *L. mirabile* not only harbors in its mitochondria a majority of genes from its host, but also depends on them to carry out cellular respiration. Twenty-three of the 35 protein genes were obtained from Leguminosae. But what is most interesting is that these genes have replaced the native genetic material. [22–24].

The family Balanophoraceae is distributed in tropical and subtropical areas. It has 17 genera and 42 species [3, 4, 16]. The genus *Lophophytum*, which is exclusively South American [17, 25–30], comprises five taxa:

- *L. leandri Eichler* from Misiones province (Argentina) and southeastern Brazil
- *L. mirabile* Schott & Endl. subsp. *bolivianum* (Wedd.) B. Hansen, from Jujuy and Salta provinces (Argentina), Bolivia, Brazil
- *L. mirabile* Schott & Endl. subsp. *mirabile*, that grows in Brazil
- *L. weddellii* Hook. f, from Colombia, Peru and Brazil
- *L. rizzoi* Delprete, from Goiás, Brazil

This contribution is based on the results of years of research on the genus *Lophophytum*, focusing on the Argentine species: *L. leandri* and *L. mirabile* subsp. *bolivianum* (hereafter *L. mirabile*). The bibliography used is Sato's

doctoral thesis [31] and the numerous papers derived from it [32–35]. The existing bibliography on the other species of the genus *Lophophytum* is scarce, mainly reduced to taxonomic works.

Among the topics included are: i) morphology and anatomy of the vegetative body, including the host/parasite interphase; ii) structure, anatomy and ontogeny of unisexual flowers, iii) description of embryological processes, from gamete formation, iv) morphology and anatomy of fruit and seed, v) taxonomic value of floral characteristics, vi) observations on dissemination, germination and the establishment of the parasitic relationship with the host, vii) the evolutionary trend in the gynoecium and embryo sac of the Balanophoraceae, and viii) synchronization of parasite and host life cycles.

2. Morphology and anatomy or vegetative organs

Lophophytum plants are formed of an underground vegetative body or tuber, spheroidal or slightly flattened, and 4-(9.5)-15 (38) x 3-(6.5)-12 cm in size [20, 21]. The tubers are connected to the roots of the host tree, close to the trunk. The tubers have no apex and no regions that resemble shoot or root apical meristems; there are no scales, leaves, branches, runners, or roots emerging from the tubers (**Figure 1A**–**C**). The host/parasite interface attachment point is a "woodrose", no larger than 5 cm in diameter; this region has a "coralloid" design in which the host wood is intermingled with the host tissue development (**Figure 1B**). Externally the tubers are dark brown to black and the surface is covered by polygonal or hexagonal "warts" of variable sizes between 0.4 to 1.2 cm.

Anatomically the tuber consists of an outermost black warty surface zone, and an interior body, white in *L. mirabile*, and pink in *L. leandri* (**Figure 1D** and **E**). The warty zone lacks an epidermis, stomata and trichomes. It is composed of a variable number of compact parenchyma cells without any intercellular spaces, with thin, cellulose walls and a completely tanniniferous cytoplasm. The outer cells are progressively detached, as the tuber grows. Solitary or clustered brachysclereids are dispersed between the parenchyma cells of the surface zone (**Figure 1F**).

The interior body is composed of storage parenchyma and abundant collateral bundles that are randomly distributed (**Figure 1F** and **G**). The cells of the peripheral zone showed a positive reaction for tannin by the ferrous sulfate method [36], while the parenchyma cells of the central region have abundant amyloplasts stained with IKI (confirmed by polarized light and the presence of a hilum) and other spherical wax or fat bodies (stained with Sudan IV, not rotated by polarized light, and no hilum) [36–38]. The brachysclereids occur occasionally in the outer region. Vascular bundles are dispersed in the interior body, not organized in a eustele; many of them are continuous from the interior body to the warty zone. The xylem of the vascular bundles is remarkable because the vessel elements have scalariform pitting with ingrowths (**Figure 1H**).

Inflorescences are the only aerial part of the plant and their peculiarity is their endogenous origin (in relation to their own tissues), a characteristic unique to Angiosperms [3, 20, 21, 31–33]. Each tuber usually has one inflorescence, however up to six inflorescences may be produced per plant (**Figure 1A, B** and **D**). The inflorescences are monoecious, consisting of one main axis or primary rachis of 2-(21)-40 cm tall, which rises above the soil surface. Short secondary rachises carrying unisexual flowers are inserted in the axil of each bract of the primary rachis; the proximal ones with pistillate flowers and the distal ones with staminate flowers.

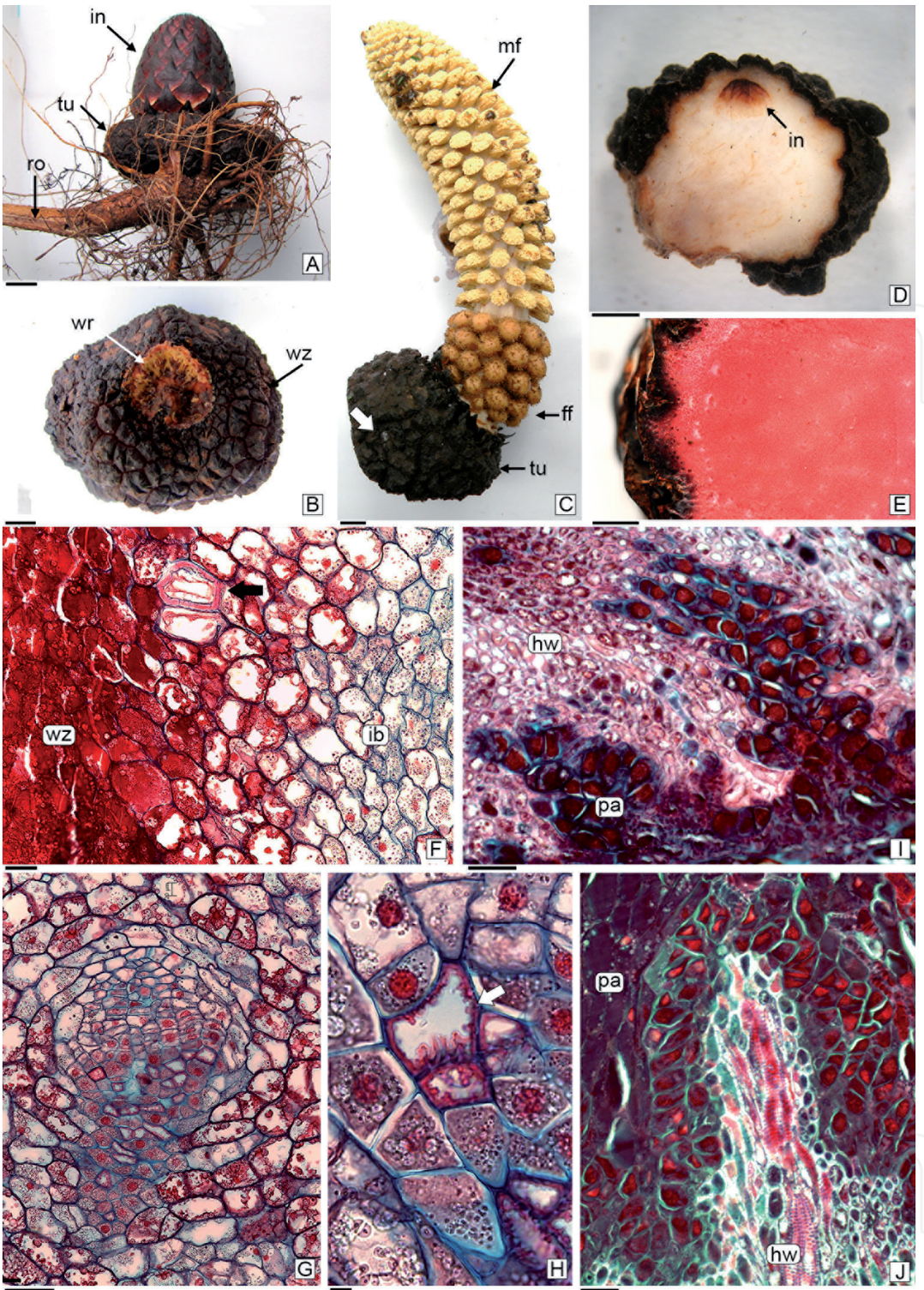

Figure 1.
A, B, E–H: L. leandri; C, D, I, J: L. mirabile. A: Hypertrophied root (ro) of P. rigida with tuber (tu) and immature inflorescence (in) fully covered by scales. B: Tuber showing the warty surface (ws) and the woodrose host/parasite interface (wr). C: Tuber with fully developed inflorescence showing the pistillate (pf) and staminate flowers (sf), the arrow indicates the site where the tuber broke away from the host root. D and E: Longitudinal section through unfixed small tubers showing the warty surface zone, natural color of internal body and the primordium of inflorescence. F: Warty surface zone (wz) showing a group of brachysclereids (arrow) and parenchyma cells of the internal body (ib). G: Transection of vascular bundle. H: Detail of vessels with wall ingrowths (arrow). I and J: Host wood (hw) intermixed with parasite cells (pa). Scales, A,L: 2 cm; B,D: 1 cm; E: 0.5 cm; F-G,I-J: 50 μm; H: 10 μm.

Immature inflorescences are completely covered by black scales (**Figure 1A**). The scales are shed at flower maturity, starting in the medium region where the staminate flowers are first exposed (**Figure 1C**).

2.1 Tubers / host Interface

The root where the parasite is installed stops its growth and elongation after infection, forming a woody gall (**Figure 1A**) [20, 21]. Tuber development is always observed in woody roots, larger than 1 cm in diameter. Infections are focused on the cambium, where the parasite cells divide intensely producing a strong undulation of the cambial zone (**Figure 1I**). One of the main consequences of the infection is the alteration of the axial and radial systems typical of secondary wood (**Figure 1J**).

In the affected xylem, the vessels are narrow and abundant, oriented in concentric rings. The fibers between the vessels are replaced by lignified parenchyma cells, with the same circular distribution of vessels. In the phloem, the tangential bands of the normal wood are almost completely replaced by parenchyma cells, very few fibers, and cells with crystals can be observed, disorganized and dispersed, but no sieve tubes elements are detected. This interaction of the host tissues (both xylem and phloem) with those of the parasite was the origin of the choraloid design of the interphase.

3. Structures of flowers

3.1 Staminate flower

Each staminate flower is composed of two stamens and 1–2 perianth pieces. *L. mirabile* has a single yellowish-white piece, and there are two deep red pieces in *L. leandri* (**Figure 2A–C**) [31, 32]. In both species, these pieces are fleshy cushion-like organs, formed of tanniniferous parenchyma with vascular supply, and covered with a unistratified epidermis with scattered sclereids. The epidermis lacks stomata and is covered by a thin cuticle (**Figure 2D**).

The stamens are composed of a short filament, and bitechae anthers. In the anther of *L. leandri* the anterior and posterior pollen sacs of each theca are about the same length, but the thecae are inserted at different heights in the connective tissue (**Figure 2E**). This displacement is the result of the adjustment that the thecae must undergo due to the presence of the upper piece of the perianth. In *L. mirabile* the anterior pollen sacs of each theca are 3/4 of the length of the posterior sacs (**Figure 2F**).

Each anther is tetrasporangiate (**Figure 2G**). The mature anther wall consists of the following layers (**Figure 2G–J**): unistratified epidermis with tanniniferous cytoplasm, without any stomata; bi-stratified endothecium with U-shaped fibrous thickening and tanniniferous cytoplasm; one middle layer, which in *L. mirabile* also has fibrous thickenings, forming a tri-stratified endothecium (**Figure 2G**). The tapetum is unistratified, and of secretory type (**Figure 2H**). No orbiculae are observed on the tapetal membrane and/or anther locule. The connective has a single vascular bundle; the surrounding parenchyma cells show the characteristic fibrous thickening of the endothecium, both on the dorsal and ventral sides of the connective. The development of the anther wall can be considered as being within the basic type proposed by Davis [39], although there are variations in the behavior of the middle layers, which develop thickening, constituting a pluristratified endothecium at maturity.

Primary sporogenous cells undergo several mitotic divisions, giving rise to uninucleate microspore mother cells. In meiosis I tetrahedral tetrads are formed by simultaneous cytokinesis, which remain surrounded by a callose wall. Mature pollen grains are released in a bicellular state, they are spheroidal, tricolpate-sincolpate, with a thin exine, and no protruding sculptures (**Figure 2K**).

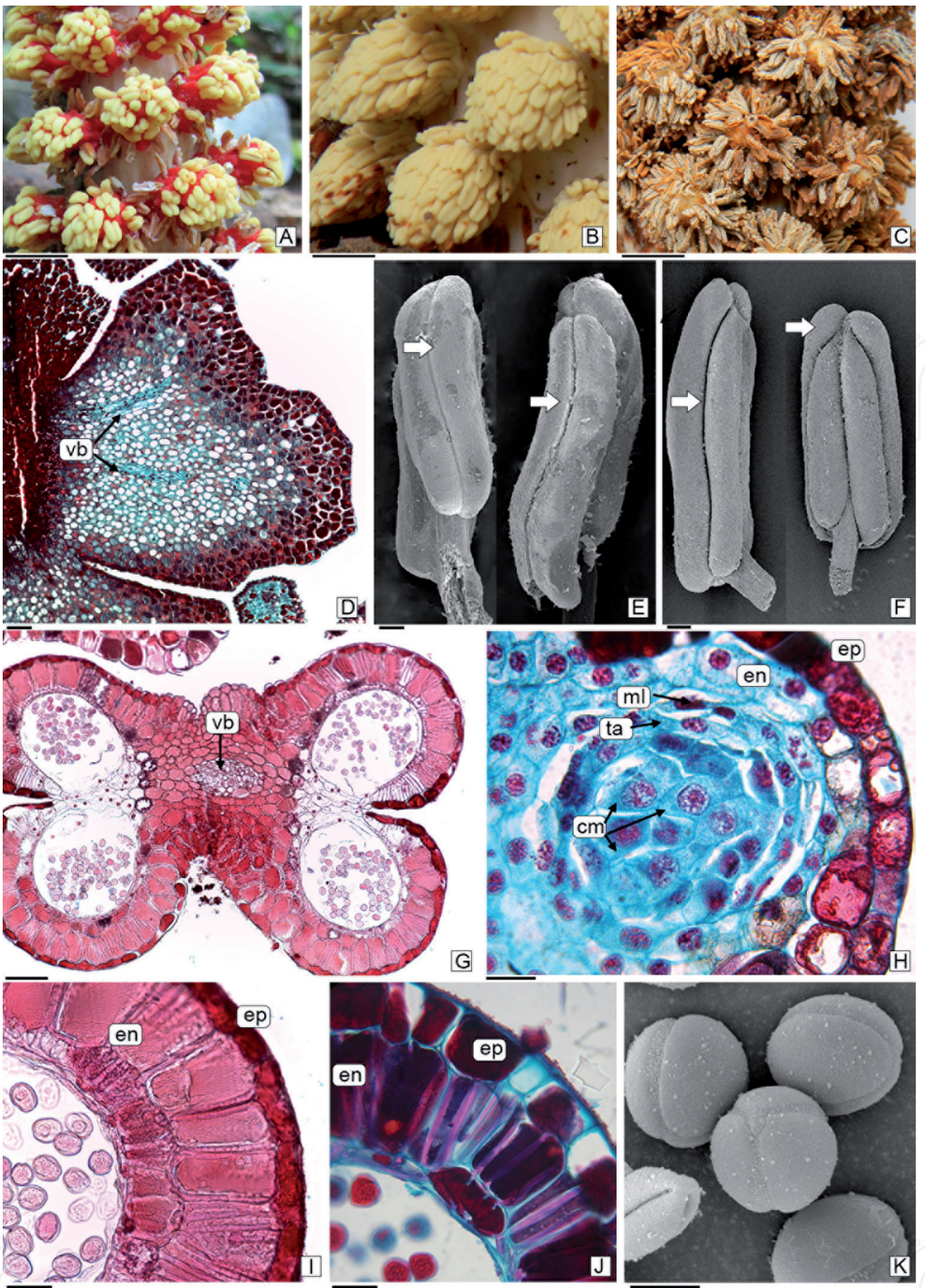

Figure 2.
Staminate flowers. L. leandri: A, D, F, H, J. L. mirabile: B, C, E, G, I, K. A and B: Mature inflorescence without scales, detail of secondary branches showing red perianth pieces and staminate flowers. C: Staminate flowers with dehiscent anthers. D: Stamens, anterior (left) and abaxial (right) views, showing offset in the insertion of thecae. E: Adaxial (left) and lateral (right) view showing different lengths of the pollen sacs. D and E: Arrows mark the lines of dehiscence. F: Longitudinal section of perianth piece with vascular bundles (vb). G: Transection of anther. H: Young pollen sac. I and J: Mature anther walls. K: Pollen grains. Abbreviations: cm: Microspore mother cells; ep: Epidermis; ml: Middle layers; en: endothecium; ta: Tapete. Scales: A–C: 1 cm; D, E: 0,2 mm; F: 0.1 mm; G, H: 0.2 mm; I, J: 20 μm; K: 10 μm.

3.2 Pistillate flower

In both species of *Lophophytum* the pistillate flowers lack a perianth and are reduced to one pistil formed of a superior ovary, two styles and capitated stigmas (**Figure 3A–C**) [31–33]. The ovaries of *L. leandri* are arranged compactly in the axil of clavate bracts. These bracts have a thin basal portion and a capitated distal portion, which covers the top of the ovary (**Figure 3A**). *Lophophytum leandri* has

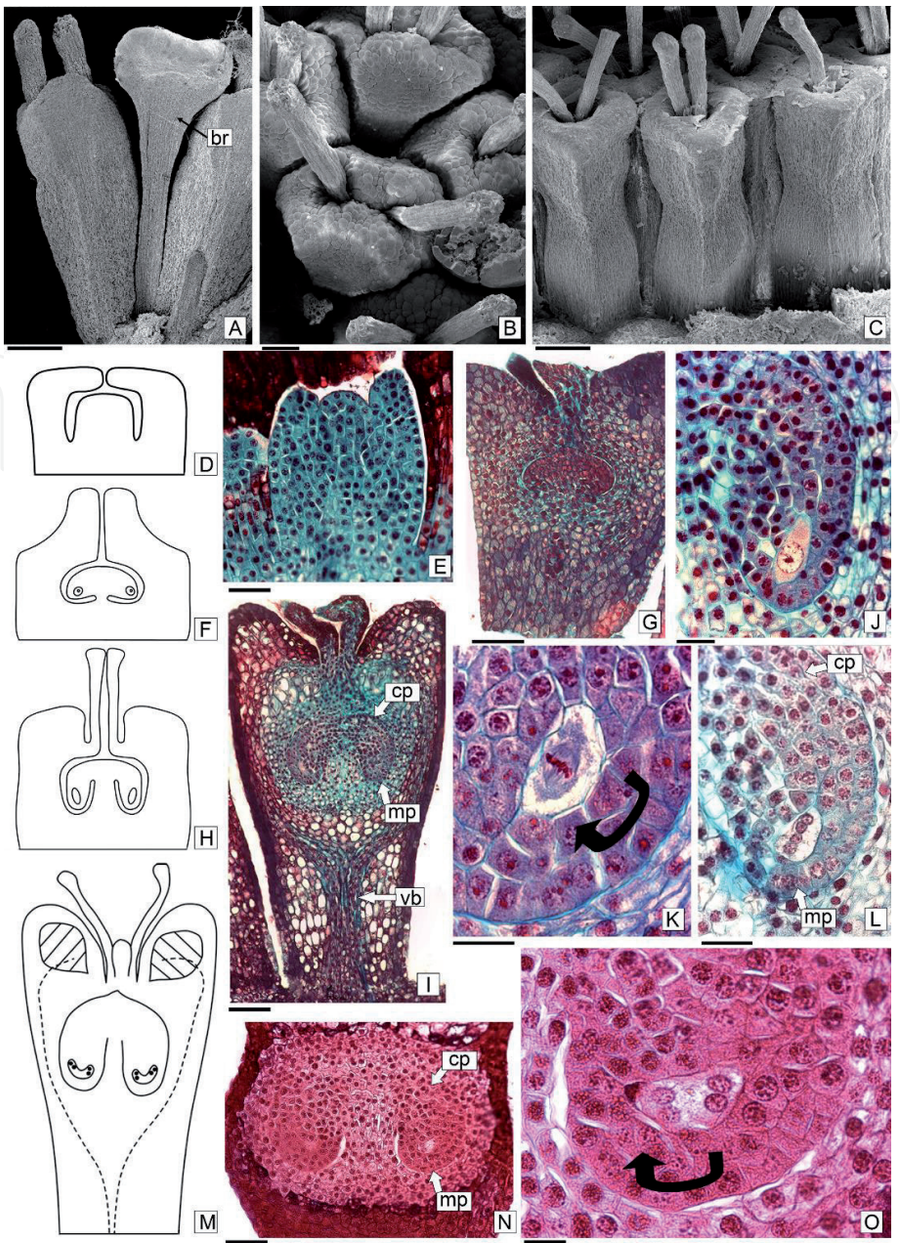

Figure 3.
Pistillate flowers. L. leandri: A, B, E, G, I, K. L. mirabile: C, J, L, N, O. A–C: Scanning electron microscope of pistillate flowers, showing bract (br) and stigma positions. D, F, H, M: Successive stages of ovary and ovule development. D and E: Longitudinal section of ovary at the early stage, cup-shaped with a central placenta. F and G: Ovary with bilobed placenta. H and I: Ovary with two ategmic ovules in stages of megaspore mother cell. J: Megaspore mother cell. K: Metaphase I. L: Linear tetrad of megaspores. M–O: Tetranucleate embryo-sac in horizontal position. O: Details of four megasporic nuclei separated by a central vacuole. Note: All photographs were taken with the ovules in the same position (photo A); arrows indicate the direction of rotation of the megaspore mother cell/embryo-sac. Abbreviations: cp: Chalazal pole; mp: Micropylar pole; vb: Vascular bundles. Scales: A, C: 0,5 mm; 0,2 mm: B; 100 μm: E, G, I, N; 50 μm: J, K, L, O.

two cavities in the apex of the ovary, on which two separate styles are inserted respectively (**Figure 3B**). *Lophophytum mirabile* lacks bracts, the ovary is cylindrical and is also compactly arranged, acquiring a well-defined hexagonal or square shape. The apex of the ovary has a single cavity where both styles are inserted (**Figure 3C**).

During the development of pistillate flowers, the pistil primordium is initiated from hemispheric meristems on the surface of secondary rachis of a young inflorescence (**Figure 3D** and **E**). In *L. leandri*, the bracts develop first, and then ovarian primordia are formed in their axils. In *L. mirabile* only ovarian primordia are formed. In both species the meristem acquires a cup-shaped form. In the center

of the pistil a sub-spherical protrusion corresponding to the placenta is developed. Until the closure of the ovary, the ovarian cavity is unique and almost undetectable; the placenta is located at the base of the ovary and is perfectly distinctive from the tissue of the carpels.

The placenta grows occupying the whole cavity of the single locule, the upper end acquires a sharpened shape and it is united postgenitally to the top of the carpels; therefore the mature ovarian cavity is divided into two locules (**Figure 3F** and **G**). The placenta is laterally enlarged giving rise to two-lobed projections in each locule, which are 90° curved towards the base of the ovary, resulting in two ovule primordia. This primordium of ovules occupies the entire cavity of the locules.

Two ategmic ovules are inserted on the upper portion of a central column placenta. The two locules are almost completely obstructed by the ovules (**Figures 3I** and **4A, B**). As the ovules develop, they are reduced to the nucellus and lack of integuments, but the female gametophyte is developed inside (**Figure 2J–O**). The term micropyle is not applicable in its usual sense, therefore it has been designated as a "micropylar pole" at the apex of the nucellus, which is where the megaspore mother cell develops. Vascular supply is absent in the placenta and the ovule, so the chalaza and the funiculus cannot be defined. Therefore, the basal portion is called the "chalazal pole" where the nucellus is attached to the placenta (**Figure 3D, E, J, L, N, O**).

The mature ovary wall consists of several layers of parenchyma and two zones are recognized: the outer layers are tanniniferous and the internal layers have starch grains (**Figures 3I, N** and **4B**). Different types of sclereids have been differentiated between the two zones: both species present a brachysclereid ring in the apical portion of the ovary (**Figure 4C**). In *L. mirabile* four clusters of macrosclereids are also formed at the base of the ovary, which alternate with the vascular bundles. Vascular bundles show scarce development; elements of the xylem vessels have ingrowths.

The styles are solid and are formed of elongated parenchymal cells with dense tanniniferous cytoplasmic contents. Cells of the center of style are smaller but are not differentiated into transmission tissue. The stigmas are capitate and have depressions on their surfaces, where adhered pollen grains can be observed.

Megasporogenesis [31–33]: In both ovules, a conspicuous cell develops below the nucellar epidermis and acquires archesporial features, this cell gives rise to the megaspores mother cell (MMC) (**Figure 3J**). The MMC meiosis I and II happen normally, resulting in four identical nuclei, which are arranged a linear tetrad or "T" shaped (**Figure 3K, L**). These four nuclei migrate in pairs to opposite poles of the cell, all of which participate in the formation of a tetrasporic type of embryo-sac (**Figure 3M–O**). The polarity of the embryo-sac is determined by the displacement of the nuclei and the presence of a central large vacuole. At this stage of the tetranucleated coenocyte the embryo-sac is in a horizontal position relative to the main axis of the gynoecium, this apparent shift is due to the growth of the ovule by increased cell division on the dorsal side of the nucellus.

Megagametogenesis: During the migration of the two pairs of nuclei to opposite poles, the four-nucleate megagametophyte extends and becomes "J" shaped (**Figure 4A, D**). Each pair of nuclei undergoes a mitotic division creating an eight-nucleate embryo-sac (ES). Four nuclei remain near to the chalazal pole and the other four move towards the micropylar pole, separated by vacuoles. Three nuclei remain at each end of the ES, and the fourth nucleus moves towards the center of the central cell, where the cytoplasm is gradually increased in density (**Figure 4E**). The three nuclei at the micropylar end of the embryo-sac are compacted and reduced; cytokinesis occurs at this pole and creates three antipodes (**Figure 4F**). At a later stage, when the triad of nuclei at the chalazal pole is

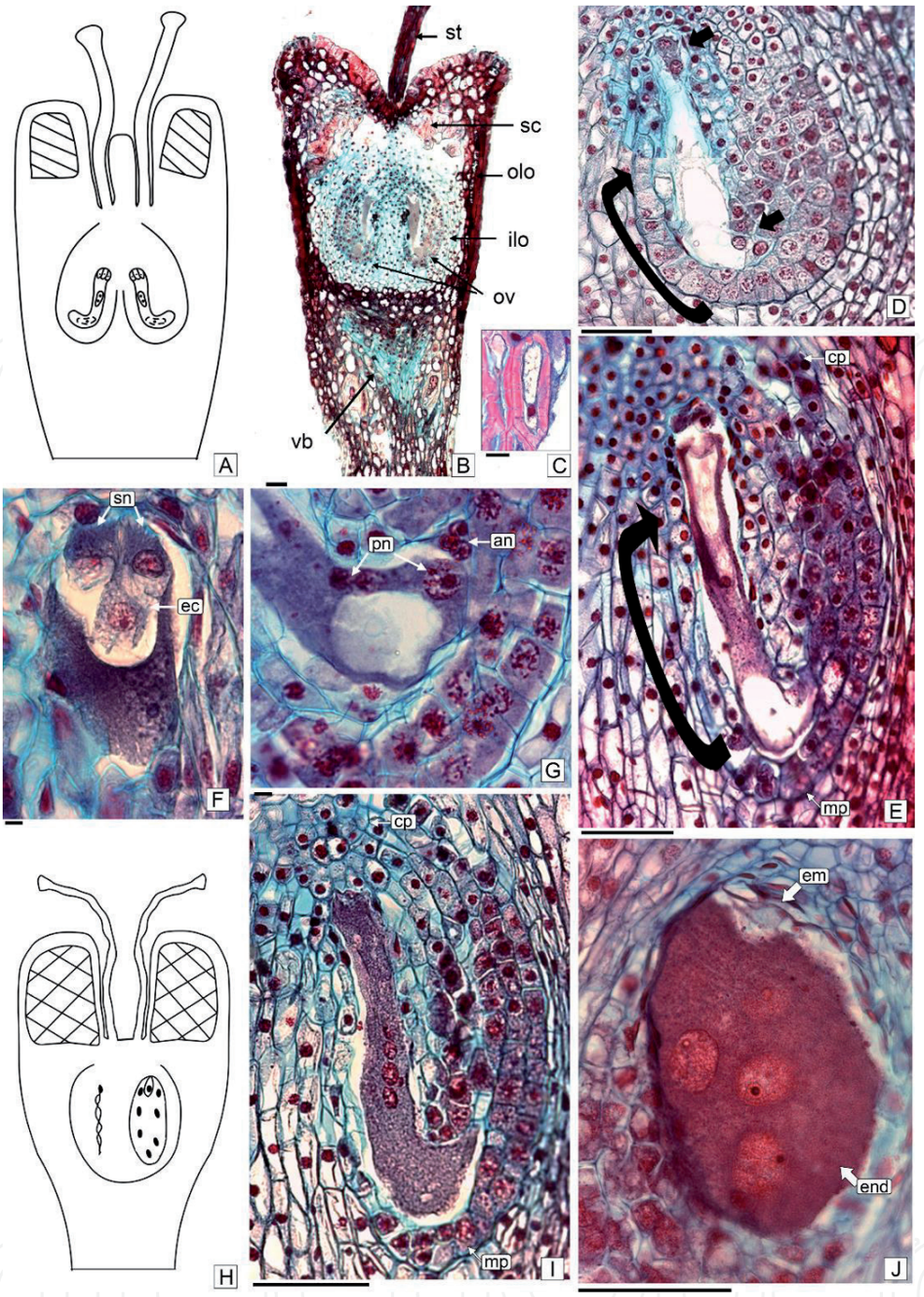

Figure 4.
Embryo sac (ES) development in L. leandri; A and B: Scheme and light microscopy photo of ovary, showing position of ovules and embryo sacs. C: Detail of sclereids. D: Tetranucleate ES. E: Tetrasporic, 8-nuclei ES (arrow indicates the direction of rotation of ES). F: Antipodes (an) and part of the central cell (pn). G: Egg-cell (ec) and the pair of synergids with filiform apparatus (sn). Abbreviations: cp: Chalazal pole; Ilo: Inner layer of ovary; mp: Micropylar pole; olo: Outer layer of ovary; ov: Ovule; sc: Sclereids; st: Stigma. Scales: B, D, E: 100 μm; C, F, G: 10 μm.

organized as the egg-apparatus, the antipodal cells disappear. At the chalazal pole of the ovule, the typical egg-apparatus is developed, composed of an egg-cell in a central position and two adjacent synergids-cells, all of which determine an inverted embryo-sac (**Figure 4E** and **G**). The synergids-cells are smaller than the egg-cell; their vacuole being oriented towards the center of the megagametophyte, and a prominent filiform apparatus is developed. This tetrasporic, 8-nuclei embryo-sac follows an Adoxa type organization (**Figure 4F**).

4. Embryology

Despite the fact that a large number of pollen grains are produced, no pollen tubes or fertilization can be observed [31, 33, 34]. The endosperm and embryo are formed in the absence of double fertilization. The formation of the endosperm occurs in three stages. The fusion of the polar nuclei has not been seen, in contrast, endosperm formation starts with a series of free nuclear divisions that result in a coenocytic structure of up to 12 nuclei, with six being the most frequently recorded (**Figure 5A** and **B**). The second stage in endosperm formation involves nuclei fusion (**Figure 5C**). 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. This fusion allows the entrance of both nuclear and maternal cytoplasmic material inside the coenocyte. Once inside the coenocyte, all the fused nuclei become one giant nucleus, reaching 120 x 60 μm in size (**Figure 5C**). The third stage of endosperm formation is the sequence of karyokinesis, producing about 12 nuclei of equal size (50 to 70 μm diameter) (**Figure 5D**). Cytokinesis then takes place, which generates the endosperm cells (**Figure 5E** and **F**). This process only occurs in one embryo-sac as the second embryo-sac is reabsorbed.

The egg cell divides forming a four-cell globular embryo only when the endosperm cytokinesis is complete (**Figure 5E** and **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 (**Figure 5G–I**). No suspensor formation has been verified.

The hypothesis of the existence of parthenogenesis is proposed for the Argentine species of *Lophophytum*, justified by the formation of embryo and endosperm in the absence of fertilization and the beginning of endosperm development is autonomous.

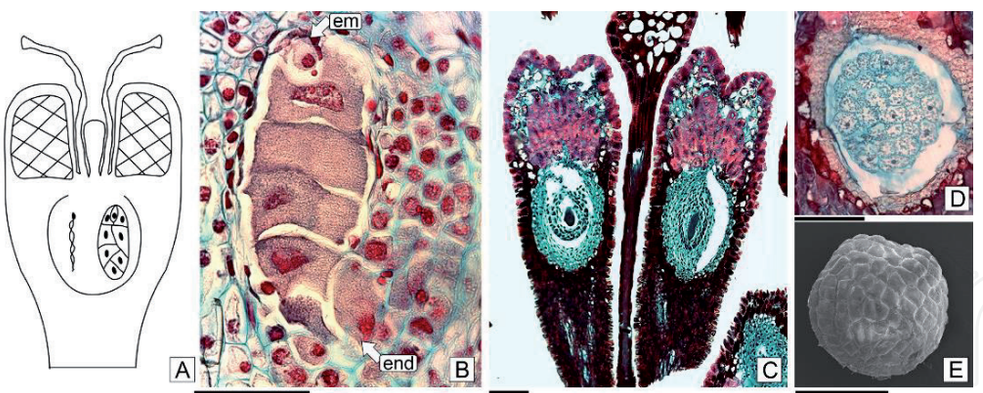

Figure 5.
A, E: scheme of endosperm development. A B: cygote (em) and endosperm cytokinesis. C: fruits. D-E: seeds (light and scanning microscope). Scales: B: 100μm; C: 200μm; D-E: 500 μm.

5. Fruit and seed

In *Lophophytum* the fruits are achenes, reaching an average size of 2.5 x 1.5 mm in *L. mirabile*, and 2 x 1.2 mm in *L. leandri* [31, 34]. As they are indehiscent, they constitute the unit of dissemination or diaspore. The epicarp is derived from the outer epidermis of the ovary, with cells completely occupied by tannins; those in the upper portion of the ovary are differentiated into sclereids (**Figure 5G**). In *L. mirabile* the sclereids occupy the apical and basal portion of the fruit, and in *L. leandri* they spread along its side walls reaching the upper third of the fruit. The

mesocarp is made up of parenchyma cells, also with tannins. The endocarp develops from the internal epidermis of the carpel which differentiates into brachysclereids (**Figure 5H**).

The ovule nucellus tissue is digested during endosperm formation, therefore the seed lacks a seed coat. The mature seed is only made up of the endosperm and undifferentiated embryo; its cells completely lack tannins that are omnipresent in the remaining fruit (**Figure 5H** and **I**). The seed is spheroidal with a small wedge towards the upper part of the ovary (**Figure 5I**).

The inflorescence of *Lophophytum* has between 50 and 65 secondary rachises with pistillate flowers, each bearing between 350 and 700 fruits, with each plant producing an average of 25,000 fruits. These may remain aggregated since they are on the secondary rachis and they do not fall until the inflorescence axis becomes decayed. However, in both species of *Lophophytum* the larvae of Oxycorynus consume the parenchymatous axis of the secondary rachis and thus the fruits are separated from the rachis, facilitating their dispersal [35].

It has been recorded that the rodent *Dacyprocta aguti* L. (Rodentia, Agoutidae) digs up the plants of *L. mirabile* to consume the tubers and inflorescence axes, especially the staminate flowers. The female portion, with fruits, remains disintegrated in the ground. In the NW of Argentina, inhabitants have mentioned that the rodent *Agouti paca* L. (Rodentia, Agoutidae) consumes the plants of *L. leandri* very assiduously. However plants gnawed by animals have never been observed in the populations of *L. leandri* under observation in Misiones [31].

6. Taxonomic value of floral characteristics

A vegetative body lacking stems and leaves makes it necessary to look for other characters of taxonomic value, such as those related to the floral parts [31]. Several morphological characteristics of the staminate and pistillate flowers allow easy distinction of material from the two Argentine species of *Lophophytum* (**Table 1**).

In flowers of *L. leandri* the perianth pieces have been described by Burkart [25] as reduced ovaries. In the present study it is confirmed that the fleshy excrescences accompanying the stamens do not show any female reproductive structures that could be considered as reduced ovaries, nor any remnants of them. Hansen

	L. leandri	***L. mirabile***
PF: clavate bracts	present, the flowers are in the axils of the bracts	absent
PF: insertion of styles in top of the ovary	two cavities	one cavity
PF: sclereids in ovary	one ring of sclereids at the top of the ovary	four clusters of sclereids in the basal portion of the ovary
SF: number and color of the perianth pieces	two deep red pieces	a single yellowish-white piece
SF: length of the pollen sacs	the anterior and posterior pollen sacs of each theca are about the same length, but the thecae are inserted at different heights in the connective	the anterior pollen sacs of each theca are 3/4 of the length of the posterior sacs

Table 1.
Differential morpho-anatomical characteristics of pistillate flowers (PF) and staminate flowers (SF).

[17, 26] described them as parts of the perianth and used them in the taxonomic delimitation of the species.

In the core Eudicot, the absence of perianth parts is not common, except in wind-pollinated plants and the Balanophoraceae [16, 40]. In the *Lophophytum* species studied, the protective function of flowers is carried out by woody bracts covering the inflorescence. *L. leandri* shows an additional second protective line, represented by the clavate bracts, which accompany each pistillate flower.

7. The evolutionary trend in the gynoecium and embryo sac of the Balanophoraceae

The analysis of the anatomy and development of pistillate flowers and the study of the functional architecture of the ovules, carpels and embryo-sac provide embryological data of great importance to complement the phylogenetic studies of the family Balanophoraceae, and even of the order Santalales.

The presence of four vascular bundles in the ovary, two ovules, and two styles and stigmas, suggests the occurrence of two carpels in *Lophophytum*. The bi-carpellated ovary is a widespread condition in the Balanophoraceae s.l., except in *Balanophora* [41], and *Dactylanthus* [42] that have a single carpel and one style.

The reduction of ovules, fusion of the ovules with the carpels, and the number of loculi are variable characteristics in the family (**Table 2**). The complete fusion between the ovules and the carpels determines the absence of a locular cavity in *Balanophora* [41] and Helosis [46–49]. In *Corynaea* [45] and *Rhopalocnemis* [44] they have a single locule due to the absence of postgenital fusion. In *Lophophytum*, the two ovarian cavities are determined by the postgenital fusion of the tip of the placenta with the apex of the ovary.

The ovules of *Lophophytum* are the only ones in the whole family that are still distinguishable from the placenta, although they are ategmic. In *Corynaea*, *Dactylanthus* and *Rhopalocnemis* [42, 44, 49] the term placental-nucellar complex (PNC) has been used instead of ovules, as the boundary between the nucellus and the placenta is blurred. The most extreme reduction occurs in *Helosis* and *Balanophora*, where there are no recognizable ovules; the ovary, placenta and nucellus are completely fused into a compact mass where the embryo sacs develop, there is no ovarian cavity [41, 44, 50]. All genera in the family, except *Balanophora* (with 1 MMC), have two MMCs, but only one ES completes its development and forms an embryo.

Genera	Locules	MMC	Embryo-sac			References
			origin	type	shaped	
Lophophytum	2	2	4-sporic	*Adoxa/Polygonum*	J	[18, 31, 32, 43]
Rhopalocnemis	1	2	1?	—	Straight	[44]
Corynaea	1	2	2-sporic	*Allium*	Straight	[45]
Helosis	absent	2	2-sporic	*Helosis* (four-celled ES)	Straight	[46–49]
Balanophora	absent	1	1, 4-sporic	*Polygonum*	U/J	[50, 51]

Table 2.
morpho-embryological features known for pistillate flowers in species of Balanophoraceae s.l.

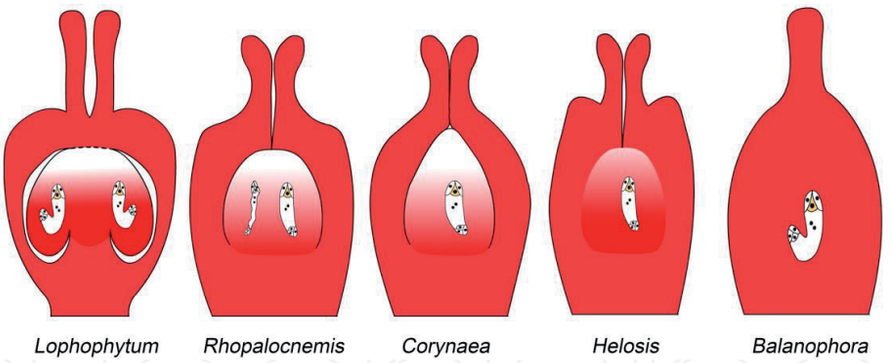

Figure 6.
Hypothetical line of sequences of congenital fusion and reductions within the gynoecium of the Balanophoraceae at the embryo-sac development stage. Without scales bars.

Among the genera of the family, monosporic, bisporic and tetrasporic ES have been described, with a bisporic ES with Allium-like development being the predominant type (**Table 2**). A new type of embryo sac (bisporic four-celled embryo sac, provided with a typical egg apparatus and a uni-nucleated central cell) has been described for Helosis [49].

In the **Table 2**, a hypothetical line of successive steps that includes several major modifications, such as: a gradual reduction in the integuments; gradual loss of identity of the ovule and placenta, both structures that are still recognized in *Lophophytum*, while in the other genera of the Balanophoraceae it is not possible to discern discrete ovules, presenting a PNC, a consequence of this reduction is the loss of the chalaza, funicle and absence of vascularization; and progressive fusing of the placenta/ovules/carpels, with the consequent reduction of the ovarian cavity, until its complete disappearance, which is found in *Helosis*, *Balanophora* and in some *Loranthaceae* (**Figure 6**).

Lophophytum [31] and *Balanophora* [50] studies show the rotation of the ES growing within the nucellus, with the egg- apparatus oriented towards the apex of the ovary, in the region that is more favorable to pollen tube access.

8. Synchronization of parasite and host life cycles

8.1 Host, environment, and distribution

8.1.1 Lophophytum leandri

- Host: *Parapiptadenia rigida* (Benth.) Brenan. A leguminous tree, of 10 to 15 m in height, generally associated with the banks of watercourses. Deciduous in the studied areas. Common name "angico colorado".

- Vegetation: humid forest, composed of a compact mass of 20 to 30 m in height, formed of at least four strata: large trees, shrubs, lianas, and epiphytes.

- Distribution: In Argentina *L. leandri* coexists with *P. rigida* in Misiones and to a lesser extent in Corrientes (**Figure 7**). Much of the environment of the species *L. leandri* has been modified by anthropogenic action and the existing and more accessible populations are being decimated by the local people, who commercialize them as medicinal and ornamental plants.

Figure 7.
Map of location of species of Lophophytum and their host in Argentina.

8.1.2 Lophophytum mirabile

- Host: *Anadenathera colubrina* (Vell.) Brenan. A leguminous tree, of 10 to 25 m in height, that grows on riverbanks, generally near watercourses where it is considered a slope fixer due to its rooting type. Characteristic mameloned bark. Deciduous in this area, in August–September it is found defoliated, with dry pods still on the branches. Common name "cebil colorado".

- Vegetation: pedemontane forest up to 600 m, riverbanks in forests with a predominance of A. colubrina.

- Distribution: *L. mirabile* subsp. *bolivianum* and its host were found growing in the provinces of Salta and Jujuy (Argentina), **Figure 7**). In the Calilegua National Park (Jujuy) there are large populations of the parasite and host, representing an important area for their conservation; this species is little known in the northwest and its commercialization has not been recorded as in the case of *L. leandri*. For both species, the areas recorded in the literature and collected in the past are currently being used for agriculture.

8.2 Host/parasite relationship

In the study area, *L. leandri* was found parasitizing exclusively on specimens of *Parapiptadenia rigida* and it was found that it carries out its entire cycle connected to it. Likewise, *L. mirabile* was found parasitizing roots of *Anadenanthera colubrina* var. *cebil*, on which it fulfills its entire life cycle [31]. Both are obligate parasites.

Based on the above descriptions, a schematic representation of the reproductive cycle of the Argentine species of *Lophophytum* has been established. The morphological changes of the structures present in both the pistillate and the staminate flowers are correlated with data on their embryology at progressive stages of development. In **Figure 8** it can be seen that the upper part shows anther and microgametophyte formation. In the central region the ontogeny of the pistillate flower and development of the megagametophyte are represented up to fruit formation. The lower region shows the tuber and inflorescence stages. The vertical lines link the developmental stages between the staminate and pistillate flowers on the same inflorescence. The reproductive cycles of both species are completed in 90 days, developing between the months indicated on the timelines for each species.

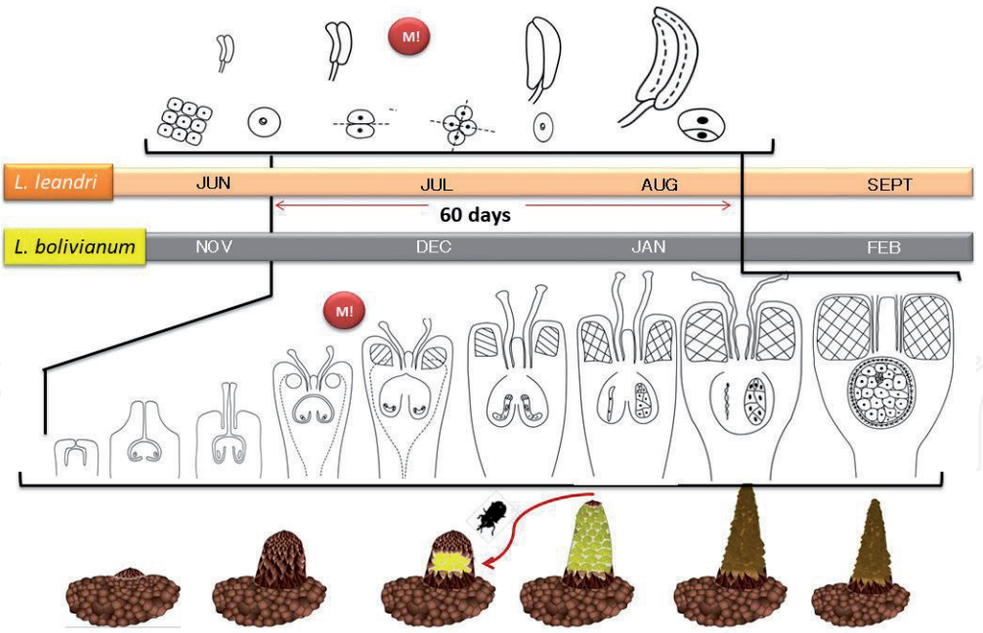

Figure 8.
Schematic representation of the reproductive cycle of species of Lophophytum over time.

The flowering of *L. leandri* is concentrated between July and August (**Figure 8**). During this period, the host is at the end of its winter dormancy, with practically no foliage and with pods still attached to the branches. These pods open and drop seeds simultaneously with the flowering of *L. leandri*. Seeds of *P. rigida* were found germinating among mature inflorescences already fruiting and with seeds of *L. leandri*, which are generally distributed at the foot of *P. rigida* trees. The seeds of *P. rigida* germinate immediately upon dispersal; they have no dormancy period. In mid-November *P. rigida* resumes vegetative growth, by which time the inflorescences of *L. leandri* are completely disintegrated. The weather throughout this period is humid and conducive to the development of the host seedlings.

In September, specimens of *L. mirabile* have been found with a tuber and the scaly peduncle of the inflorescence still underground, without any developed reproductive structures (**Figure 8**). In November A. colubrina is in full bloom and with regrowth. *L. mirabile* starts flowering at the end of November which may continue until the end of February. Although the seeds of A. colubrina var. cebil are disseminated before the flowering of *L. mirabile*, they have to become scarified in order to germinate, so the appearance of *A. colubrina* var. *cebil* seedlings coincides with the rainy season and with the flowering of *L. mirabile*. In this case, too, the pods and seeds of the host are usually found very close to the plants of *L. mirabile*, and seedlings are even found around the inflorescences of the parasite.

It has been recorded that the seeds of both the host trees (*P. rigida* and *A. colubrina*) fall underneath the canopy of the tree, directly onto the mature infructescences of the parasites. The legume embryo germinates rapidly and it is common to see the young legume seedlings growing directly on the decayed *Lophophytum* infructescences, maximizing the possibility of contact between the roots and the *Lophophytum* embryo. In both pairs of species (*Parapiptadenia*/*L. leandri* and *Anadenanthera*/*L. mirabile*) the time of germination of the legume seeds coincides with the time of the parasite fruit production which would facilitate close contact between the taxa for the establishment of parasitism. It also shows a process of co-evolution of each pair of species in relation to the environment in which they live. Unfortunately, it has not been possible to collect material that shows the morphogenesis of this process, and more collections are needed.

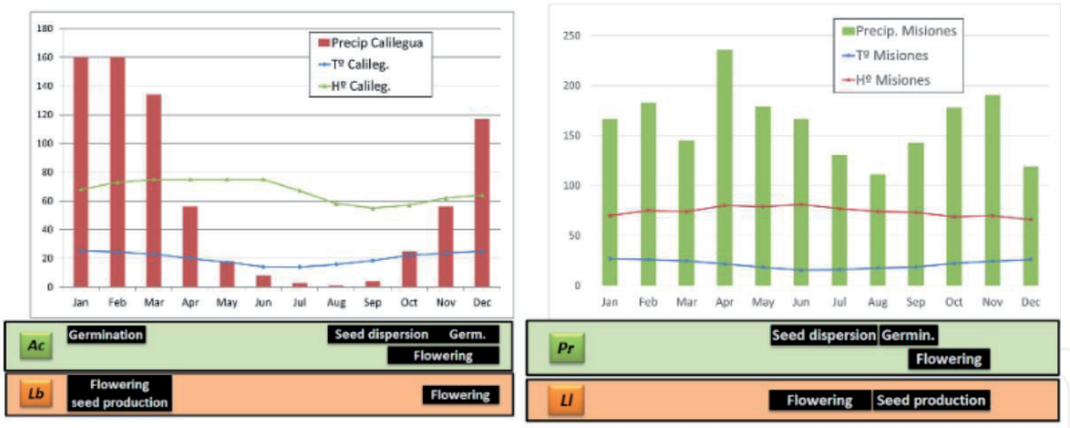

Figure 9.
Phenological stages of the parasites, their hosts and the climatic data of their respective environments over the year (Ll: L. leandri, Lb: L. mirabile, Ac: A. colubrina, Pr: P. rigida; Precip: Precipitation, T°: Temperature, H°: Humidity).

During the six years of observations [31], phenological data were collected on both the parasitic species and their hosts. Climatic data were also collected for the two regions of Argentina where this parasitic association occurs. Graphs were made comparing the temperature, humidity, and precipitation data with the main phenological stages of each parasite and its host (**Figure 9**). Thus, it was possible to interpret the phenological events of the plants (both parasite and host) better in relation to the climate. In all the years of observations, we have never detected a host tree showing any symptoms of damage caused by the holoparasites. We have collected specimens that were on trees many years old (more than 30 years) and that were vigorous, fulfilling their reproductive cycle with apparently normal flowering and fruiting.

9. Conclusions

In the vegetative bodies of *Lophophytum*, like other members of the Balanophoraceae, organs such as leaves, stems or roots are completely absent [1, 3, 20, 21, 31, 52, 53]. The reductions are also extreme when considering the anatomical structures, such as typical meristems or epidermis, and even stomata are absent. The vegetative body is covered by several layers of tanniniferous parenchyma and sclereids, which are progressively detached, similar to a peridermis. The interface between the parasite and the host has a choraloid design, which facilitates the exchange of both water and photosynthates from the vascular tissues of the host legume towards the *Lophophytum*.

In contrast to the strong reductions in the vegetative body, the staminate flowers show reductions only in their sterile floral parts. The development processes of the anthers, microsporogenesis and microgametogenesis occur normally and in correspondence with the antecedents of the majority of the angiosperms studied, and there are no substantial differences between the two species analyzed [32]. The secretory and uninucleate tapetum characteristics are shared with other genera of the family, such as *Helosis* [47–49], and *Corynaea* crassa [45].

The pistillate flowers are another example of the absence of a perianth, but here the reduction of parts also extends to the reproductive structures [31, 33, 34]. The absence of integuments determines the presence of ategmic ovules. The lack of differentiation of the chalaza, funiculus and vascularization makes it very difficult to establish a concrete boundary between the placenta and nucella. The terms

anastroph/orthotroph are not applicable in *Lophophytum*. Differentiation of the megaspore mother cell is the feature used to establish the micropylar and chalazal poles. The megagametophyte is of the tetrasporic type, with 8-nucleate nuclei at the maturity of an *Adoxa*-like organization, with the typical egg apparatus. *Lophophytum* is a clear case of megagametophyte inversion, confirmed by ontogenetic studies of numerous flowers and not as isolated cases [34]. The embryo sac develops aggressively within the nucellus and rotates during megasporogenesis and megagametogenesis, finally acquiring a "J" shape with the egg apparatus oriented towards the chalazal pole and the antipodes towards the micropylar pole. This would favor the proximity of the egg cell and the pollen tubes that will eventually enter through the styles. However, double fertilization has not been recorded, suggesting the existence of parthenogenesis.

The development of the endosperm is nuclear in nature, but has particularities, as it is possible to divide it into several stages culminating in a fully cellular endosperm [34]. In *Lophophytum*, similar events to those observed in the apomictic species of *Balanophora* have been recorded, such as the fact that the endosperm develops autonomously without fertilization, that it develops enveloping the zygote, which starts dividing much later than the endosperm. The mature embryo is globular and undifferentiated as in other holoparasites, such as *Pilostyles* and *Orobanche* [54–57]. They lack a seed coat, due to the absence of integuments in the ovule. The term seed in the strict sense could not be applied to the Balanophoraceae, as it has been shown that the structure is derived entirely from the embryo sac and is completely devoid of teguments, as already mentioned by Holzapfel [42].

The dispersal unit of *Lophophytum* is a uniseminated achene [16]. The diaspores of *Lophophytum* are dispersed by rodents that feed on them, separating the achenes from the inflorescence axis. Dispersal is favored by the previous action of Oxycorynus larvae on the axes of the secondary rachis [35].

From the comparative analysis of reductions and fusions in the gynoecium of the Balanophoraceae with the results observed in *Lophophytum*, a line of possible successive steps is proposed which includes several profound modifications: i) gradual loss of identity of the ovule and placenta: both structures are still recognizable in *Lophophytum*, whereas in the other Balanophoraceae they are not distinguishable. ii) gradual reduction of integuments, loss of the chalaza and funiculus and absence of vascular supply in the ovules. iii) progressive fusion of the placenta/ova/carpels, with a consequent reduction of the ovarian cavity until its complete disappearance in *Balanophora*. *Lophophytum* is the only genus of Balanophoraceae in which the ovules are still clearly identifiable from the placenta. In the other genera of the family the boundary between the ovules and placentas is blurred, so the term placental-nucellar complex is still used for these cases.

The family Balanophoraceae is an excellent example of how knowledge of embryological data expands the possibility of establishing their phylogenetic relationships [3, 4, 16, 56, 57]. Given the lack of vegetative characteristics due to the particular structure of these plants, the importance of floral characteristics for taxonomic identification is emphasized. All data acquired from the flower structure and anatomy make species identification possible.

In the family Balanophoraceae, knowledge of the germination process and the initiation of the host relationship (establishment of the initial haustorium) was only achieved in *Balanophora abbreviata* [50]. One of the unfinished points of this review is the germination process and the connection of the haustorium with the host root. Host and host specificity are revalidated, at least for the Argentine species of *Lophophytum*, where *L. leandri* spends its entire life cycle on *Parapiptadenia rigida*, whereas *L. mirabile* spends its entire life cycle on *Anadenanthera colubrina* [31]. There is a direct relationship between the life cycle of the hosts and that of the

parasites with respect to the coincidence of their reproductive phases. The seeds of the parasites only mature when the host seeds are ready for germination. The flowering period of the Argentine species is concentrated in contrasting periods, with *L. mirabile* flowering in midsummer, and *L. leandri* in winter.

The studies carried out here are a clear example of the process of co-evolution between a holoparasite and its host. Each species of *Lophophytum* develops its reproductive stages at the time of year when its seeds can come into contact with the seedlings of its host, so the chances of establishing a parasitic relationship are optimized.

Acknowledgements

This research was funded by the NATIONAL UNIVERSITY OF THE NORTHEAST (PICTO 199-2011 and PI-SGCyT 12P001, both to AMG).

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