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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.