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Embryo and endosperm development in *Pitcairnia encholirioides* (Pitcairnioideae – Bromeliaceae): An endangered species of the Atlantic Forest

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ABSTRACT

Pitcairnia encholirioides is a threatened species endemic to the inselbergs of the Atlantic Forest. The study of the embryogenesis and endospermogenesis of *Pitcairnia encholirioides* aims to the establish guidelines for its *in vitro* propagation. Using anatomical analysis, samples of flowers in anthesis and fruits at different developmental stages were analysed. In the results was observed the first division of the sporophytic zygote occurs in the transverse plane and originates the apical and basal cells, forming a uninodal embryo through Asterad-type embryogenesis. Endosperm formation begins with the formation of free nuclei, which undergo centripetal cellularization, beginning in the chalazal domain, characterizing this endosperm as the coenocytic/multicellular type. The differentiation of the aleurone layer occurs at the end of this process. The relationship between fruit maturation and embryo/endosperm developmental stages was established, thus setting forth guidelines for the development of *in vitro* culture protocols aimed at the conservation of *P. encholirioides*, an endangered Bromeliad species of the Atlantic Forest.

1. Introduction

Pitcairnia encholirioides has a single population endemic to an inselberg located in the municipality of Santa Maria Madalena in the state of Rio de Janeiro (RJ), Brazil. Within this population, sexual reproduction seems to be compromised owing to high rates of pollen sterility (Mendes et al., 2016) and low seed production (Pereira et al., 2008). The formation of sterile pollen grains in *P. encholirioides* results from cytological changes during anther development, which are related to the premature degeneration of the tapetum, reduced callose deposition, and the absence of gametogenesis (Mendes et al., 2016). The high incidence of pollen sterility (Mendes et al., 2016), together with other limitations reported for the species, such as reduced flowering and low seed production (Pereira et al., 2008), indicates that sexual reproduction occurs with lower frequency than clonal growth. Clonal growth is considered a common reproductive strategy among the Bromeliaceae (Benzing et al., 2000).

In fact, clonal growth is very common in plants (Olejniczak, 2003); it evolved as an alternative to sexual reproduction in many Bromeliaceae species. This is especially true of saxicolous bromeliads, probably as an adaption to extreme environments (Benzing et al., 2000). In inselbergs, for example, seedling establishment and survival may be limited by time and space for short periods under favourable conditions (Coelho et al., 2008) as a result of superficial soils, low soil cover, and high insolation and evaporation (Meirelles et al., 1999; Scarano et al., 2005). In such heterogeneous environments, clonal growth may enable the persistence of populations and the selection, exploration, and colonization of new microenvironments (Sampaio et al., 2004; Coelho et al., 2008).

In addition to the reproductive limitations described for *P. encholirioides*, this species is endemic and threatened with extinction (MMA, Ministério do Meio Ambiente, 2008), and its already small population suffers from constant habitat fragmentation. Together, these factors have encouraged some initiatives for its conservation, such as cultivation in the arboretum at the Research Institute of the Botanical Garden of Rio de Janeiro with the aim of seedling production based on studies of its seed physiology (Pereira et al., 2008), as well as *in vitro* production of explants (leaves) for large-scale propagation (Resende et al., 2016). Anthers, endosperm, and embryos have been increasingly used as explants for the *in vitro* propagation of plantlets for reintroduction to their natural environment. The aim of the present study was to determine the relationship between the maturation of the fruits

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and the respective embryo and endosperm developmental stages to help establish guidelines for *in vitro* culture protocols with the aim of conserving *P. encholirioides*.

2. Material and methods

2.1. Plant material

Flowers in anthesis and fruits at different developmental stages were collected from 30 *Pitcairnia encholirioides* individuals that occurred in the inselberg of Santa Maria Madalena, Rio de Janeiro, Brazil. Each inflorescence/infructescence has a cluster of approximately 255–340 flowers. To make sure that we would have samples at different developmental stages, we collected approximately 60–90 flowers and fruits of each inflorescence/infructescence.

2.2. Preparation of material for light microscopy

The collected plant material was fixed at ambient temperature in 2.5% glutaraldehyde in a 0.1 mol L^{-1} sodium phosphate buffer at pH 7.2, washed in the same buffer (Gabriel, 1982), and dehydrated in an ethanol series. The material was then embedded in hydroxyethyl methacrylate, according to the protocol by Gerrits and Smid (1983), and mounted in blocks. Sections of 3 µm each were cut on a Leica RM2245 microtome equipped with glass knives, mounted on glass slides, stained with 0.05% toluidine blue O (O'Brien et al., 1965), and examined using bright-field light microscopy. The following histochemical tests were performed: Periodic Acid-Schiff Reagent (PAS) for the detection of the total polysaccharides (O'Brien and McCully, 1981), IKI (Lugol's iodine) for starch (Johansen, 1940), Coomassie Brilliant Blue R-250 at 0.25% in 7% acetic acid for total protein (Southworth, 1973), and Sudan Black B at 95% in ethanol for lipids (Jensen, 1962). The observations and image acquisitions were performed using an Olympus BX-50 light microscope equipped with an Olympus DP73 digital camera.

2.3. Classification systems adopted for embryo and endosperm characterization

Embryo development was characterized using the classification systems by Johansen (1950) and Cocucci (2005), and endosperm development was tracked using the system designed by Lersten (2004).

3. Results

3.1. Fecundation

In *Pitcairnia encholirioides*, the pollen tube enters the ovule through the micropylar canal (Fig. 1a), which, in this species, consists of only the inner integument. Following the attachment of the pollen tube to the synergid filiform apparatus (Fig. 1b), the synergid receives the cytoplasmic content of the pollen tube, which includes the vegetative cell nucleus and the two male gametes derived from the generative cell of the pollen grain. This step is followed by the plasmogamy of one male gamete and the egg cell, the other male gamete, and the secondary nucleus. This secondary nucleus was formed following the fusion of the polar nuclei (Fig. 1c). Following karyogamy, two zygotes are formed. One follows endospermogenesis and originates the endosperm, while the other, the sporophyte, follows embryogenesis and originates the embryo.

3.2. Embryogenesis

Immediately after fecundation, the zygote is established (Fig. 2a), and it presents a large vacuole close to the micropyle and a prominent nucleus at the chalazal pole. The zygote undergoes a transverse mitotic division that is asymmetric owing to the position of the vacuole, which confers polarity to the cell. This division results in a bicellular proembryo, which is formed by a basal cell (*bc*) close to the micropyle and an apical cell (*ac*) turned towards the chalaza (Fig. 2b). Because of this asymmetric division, *bc*, which possesses a large vacuole, is larger than *ac* (Fig. 2b).

The basal cell (*bc)* then undergoes a transverse division, originating the inferior *(ic)* and superior *(m)* cells and forming the tricellular proembryo (Fig. 2c). The apical cell (*ac*) divides longitudinally, originating cells *q* and *q'* and forming a T-shaped four-celled proembryo (Fig. 2d). The *ic* then undergoes a transverse division, originating cells *n* and *n'* (Fig. 2e) and forming the five-celled proembryo, followed by the first longitudinal division of *m*, forming the six-celled proembryo (Fig. 2f).

The proembryo then undergoes two transverse divisions, *q* and *q'*, to form the octant proembryo, which is made up of eight cells (Fig. 2g). The cell division of the quadrant constitutes an external cell layer (Fig. 2h, black asterisks), which will form the protoderm, and the differentiation of these cells leads to the formation of the post-octant embryo, which will establish two zones, (*l)* and (*l')* (Figs. 2i & 3 a). At this stage, an increase in the number of cells in layers *m* and *n* and a transverse division of *n'*, originating cells *o* and *p* (Fig. 2h), are also observed, followed by a transverse division of *o*, originating cell layer *o'*. A longitudinal division of *p* originates cells *p* and *p'* (Fig. 2i). In addition, the most internal cells of the newly differentiated peripheral layer will act as precursors of the fundamental meristem (Fig. 2i).

In the stage that follows, the establishment of the globular embryo is observed (Fig. 3a). The more external cells characterize the protoderm and, together with the more internal cells (derived from *l* and *l'*), divide to give rise to the embryo body (Fig. 3a & b). In parallel with these divisions, the number of cells derived from *m*, *n*, and *o* increases, whereas *o'* only undergoes one transversal division, and *p* and *p'* do not divide (Fig. 3c). Subsequently, the embryo is formed by successive divisions of cells derived from *l* and *l'* in the region of the future fundamental meristem, whereas the protodermal cells divide only anticlinally, accompanying the growth of the embryo (Fig. 3d).

As development continues, a difference in the cell division rate is observed between *l* and *l'*, which results in the bending of the apical region of the embryo, indicating the beginning of cotyledon formation

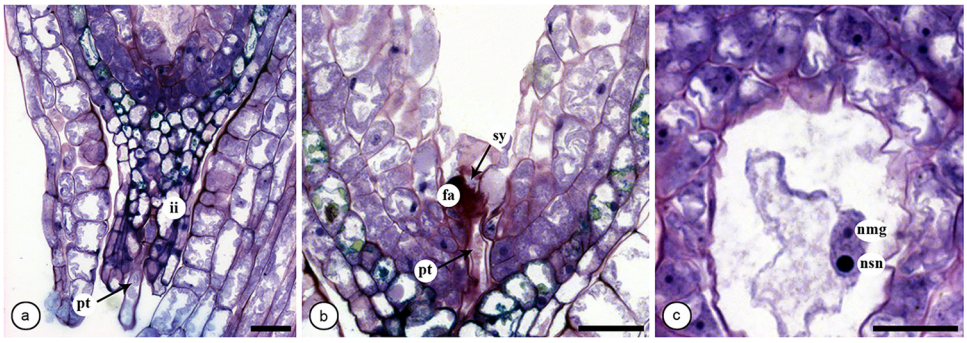

Fig. 1. Processes related to the fecundation of *Pitcairnia encholirioides*. (a) Pollen tube (pt) penetration into the ovule through the micropylar canal, which is made up of the inner integument (ii). (b) Detail of the basal part of the ovule, showing (pt) penetration into the synergid (sy) through the filiform apparatus (fa). (c) Detail of the nucleolus of the male gamete (nmg) and of the secondary nucleus (nsn) resulting from the fusion of the two polar nuclei. Bars = 100 µm (a and b); 50 µm (c).