

## FLORAL STRUCTURE, DEVELOPMENT OF THE GYNOCIDIUM, AND EMBRYOLOGY IN *SCHINOPSIS BALANSAE* ENGLER (ANACARDIACEAE) WITH PARTICULAR REFERENCE TO APOROGAMY

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**Premise of research.** Most studies of Anacardiaceae embryology have focused on seed and fruit development in different genera. None of them, however, included species of *Schinopsis*. Considering the absence of embryological data and the precedents of chalazogamy in the Anacardiaceae family, the aims of this study were to complete the embryological studies in *Schinopsis balansae*, providing information about micro- and megasporogenesis and gametogenesis, fertilization, and embryogenesis, and to investigate the development of the gynoecium and the pathway of the pollen tube.

**Methodology.** Flowers and fruits of *S. balansae*, a dioecious tree from the family Anacardiaceae, were collected and fixed in the field. The embryology was examined using fluorescence microscopy, LM, and SEM. Microtome section series were used to reconstruct the structure and pollen tube pathway.

**Pivotal results.** Staminate flowers have only rudimentary gynoecia, and the anthers are bithecal and tetrasporangiate. The tapetum is secretory and uninucleate, and the pollen grains are bicellular. The morphologically bisexual flowers are functionally pistillate, and staminodia without sporogenous tissue or pollen grains are present. The pistillate flowers have a superior tricarpellate ovary with three styles, each ending in a capitate stigma. The gynoecium is pseudomonomerous, possessing one fertile carpel (with one locule and a single anatropous, crassinucellar, and unitegmatic ovule) and two aborted lateral carpels that neither produce an ovule nor form a locule. Embryo sac development conformed to the *Polygonum* type. Bicellular pollen grains germinate on the stigma and penetrate the transmitting tract inside the styles. At the apical portion of the ovary, pollen tubes grow through the carpel wall and reach the dorsal portion of the bent funicle, which is in close contact and forms a functional ponticulus. Inside the funicle, the pollen tubes continue through the vascular bundle, where they are branched. One branch continues inside the vascular bundle to the chalaza. Fertilization was aporogamous: the pollen tubes encircled the embryo sac, reaching one synergid. Embryos follow the Onagrad type. The endosperm development is of the coenocytic/multicellular type.

**Conclusions.** The structural floral features described here are shared by other species of Anacardiaceae. The results of the embryological studies in *S. balansae* provide information about micro- and megasporogenesis and gametogenesis, fertilization, and embryogenesis and describe for the first time the developments of the gynoecium and the unique pollen tube pathway and fertilization; the term funiculogamy was proposed to define this type of pollen tube penetration.

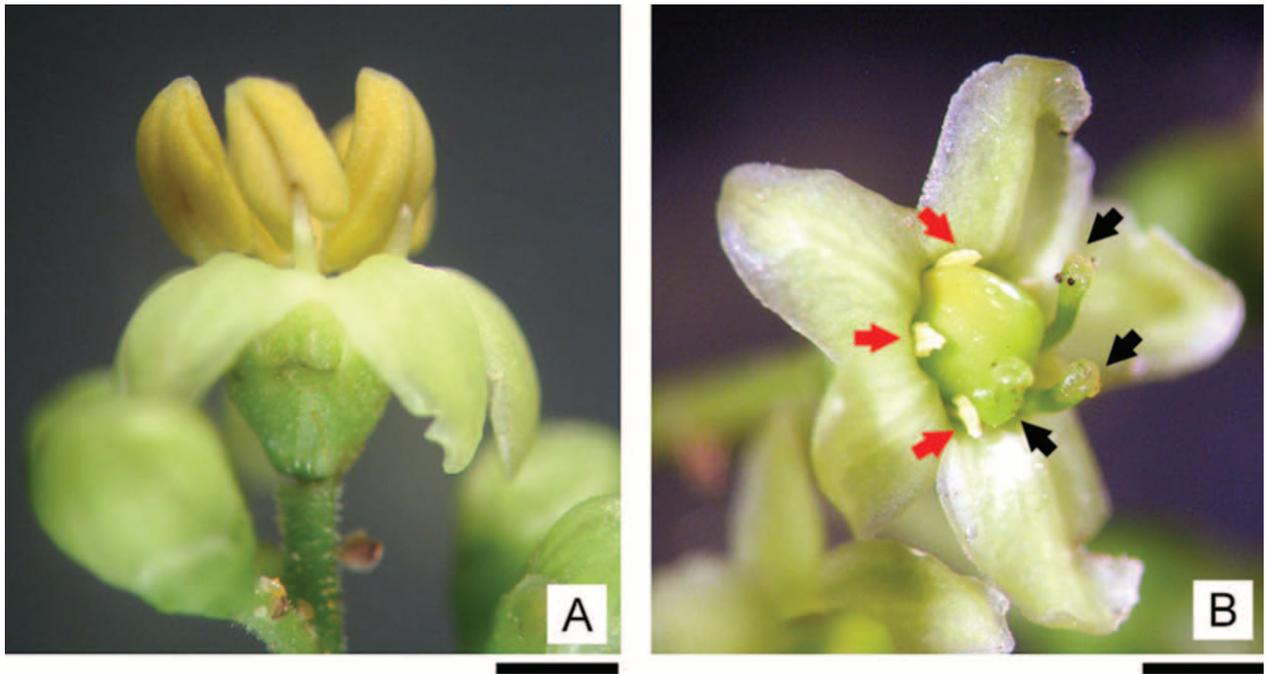
**Keywords:** Anacardiaceae, aporogamy, dioecious, embryo, endosperm, gametogenesis, pollen tube growth, sporogenesis, *Schinopsis balansae*.

### Introduction

*Schinopsis balansae* is a hardwood tree that forms forests called “quebrachales” in Argentina, Bolivia, Paraguay, and Brazil. The tree is a plant of great commercial and cultural significance; therefore, it was declared Argentina’s “National Forest

Tree.” This tree was described as polygamous-dioecious (i.e., the staminate and morphologically bisexual flowers are borne separately on different plants; fig. 1A, 1B; Muñoz 1990, 2000). A comprehensive study showed that *S. balansae* is functionally dioecious, the staminate flowers have well-developed stamens and rudimentary gynoecia, and the morphologically bisexual flowers are functionally pistillate, with sterile stamens and fertile and superior gynoecium (Gonzalez and Vesprini 2010; Gonzalez 2013). Detailed anatomic studies showed that the gynoecium is pseudomonomerous (i.e., characterized by one functional carpel with a single locule and a dorsal style and stigma and two

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**Fig. 1** *Schinopsis balansae* flowers. A, Staminate flowers. B, Pistillate flowers with three styles and capitulate stigmas (black arrows) and rudimentary stamens (red arrows). Scale bars = 1 mm.

vestigial carpels that lack a locule and are reduced to two lateral styles/stigmas). The transmitting tissue (TT) differentiated in the three styles, but only the dorsal one connects the stigma and locule; those of the sterile carpels end in the parenchyma of the carpel wall without reaching the locule. These characteristics showed the absence of internal compitum (Gonzalez 2013). The ovary of the functional carpel has a single subapical, anatropous, unitegmic, and pendent ovule. The micropyle is opposed to presumably functional dorsal style and covered by a massive integument. The position of the micropyle and the pollen tube (PT) pathway and fertilization of *Schinopsis* have never been studied before. Previous studies showed that parthenocarpic fruits can develop without pollination (Gonzalez and Vespri 2010), but the development of fertile fruits and seeds is also still unknown.

In Anacardiaceae, the ovules have an unusual dorsal bend on the convex side of their curvature, forming a contact zone with the ovary wall. In *Mangifera* and *Pistacia*, a ponticulus or small bridge between the ovary wall and the dorsal side of the ovule is present, which functions to facilitate the PT pathway (Copeland 1955; Grundwag 1975, 1976; Joel and Eisenstein 1980; de Wet et al. 1986; Martínez-Pallé and Herrero 1995; Shuraki and Sedgley 1997). Bachelier and Endress (2009) studied more than 20 genera and described a functional ponticulus; the contact is often enhanced by a projection of the dorsal side of the bent funicle into the base of the stylar canal rather than a projection of the ovary itself. Chalazogamy (i.e., PTs penetrating the ovule through the chalaza) has been found in both subfamilies (Anacardiaceae and Spondiadiaceae); a functional ponticulus has been reported in only a few closely related genera in the Anacardiaceae with *Schinopsis* (Bachelier and Endress 2009).

Most studies in Anacardiaceae embryology have focused on seed and fruit development in different genera and were mostly of economic importance; none of them, however, included species of *Schinopsis*. Considering the absence of embryological data and the precedents of chalazogamy in the Anacardiaceae, the aims of this study were to (1) investigate the development of the gynoecium to establish the relationship between carpels and ovules; (2) determine the functionality of each stigma/style and pathway of the pollen tube transmitting tract (PTTT); (3) complete the embryological studies in *S. balansae*, providing information about micro- and megasporogenesis and gametogenesis, fertilization, and embryogenesis; and (4) analyze the potential functional link between the presence of a ponticulus and chalazogamy in Anacardiaceae.

## Material and Methods

### Plants

Flowers and fruits of *Schinopsis balansae* were collected at various developmental stages in Corrientes, Chaco, and Santa Fé provinces (Argentina) and were fixed in FAA (70% ethanol:glacial acetic acid:formalin [18:1:1, v/v/v]). Voucher specimens are deposited in the herbarium of the Botanical Institute of the Northeast (Corrientes, Argentina; AM Gonzalez 200 and 306).

### LM

Staminate flowers, dissected fertile gynoecia from functionally pistillate flowers, and fruits were dehydrated according to

Johansen (1940; modified by Gonzalez and Cristóbal 1997) and then embedded in paraffin. Transversal sections (TS) and longitudinal sections (LS), 8–10  $\mu\text{m}$  thick, were prepared with a rotary microtome. In functionally pistillate flowers, LS were made in either a medium plane (the ovary was divided into a right and left portion of the fertile carpel) or a frontal one (the ovary was divided into an anterior plane, where the two lateral styles are attached, and a posterior plane, where the median style of the fertile carpel connects). Sections were stained with safranin–fast green (Ruzin 1999) or safranin–astra blue (Luque et al. 1996). Digital photomicrographs were taken using a Leica DM LB2 microscope fitted with a Leica ICC50HD digital camera. To test the stigma activity in pistillate flowers at the anthesis stages, a 3% hydrogen peroxide solution was dropped on the fresh stigma; the bubble production is taken as an indicator of stigma receptivity (Dafni 1992; Valdiani et al. 2012).

PT growth was monitored with 0.1% aniline blue in 0.1 N  $\text{K}_3\text{PO}_4$  under a fluorescence microscope (Martin 1959). Two procedures were used: (a) preparations of whole fertile gynoecia and dissected styles and ovules that were previously cleared and softened in a strong (8 N) sodium hydroxide solution and (b) preparations of dissected ovules and fertile gynoecia that were dehydrated, embedded into paraffin, and sliced with a rotary microtome into 10- $\mu\text{m}$ -thick LS. The observations and digital microphotography were carried out using a Leica DM 1000 fluorescence microscope and a Canon EOS Rebel TDi digital camera. Additional drawings were made based on photomicrographs of the sections.

### SEM

Male and functionally pistillate flowers at different stages of development were analyzed with an SEM. The flowers fixed in FAA were dehydrated with acetone and critical-point dried. Dried flowers were further coated with gold palladium and examined using a Jeol LV 5800 microscope at 20 kV. SEM analyses were performed at the Electron Microscopy Service of the Universidad Nacional del Nordeste (Corrientes, Argentina).

## Results

### *Male Flower Structure: Anther Development, Microsporogenesis, and Microgametogenesis*

Staminate flowers produce five fertile stamens (fig. 1A). The anthers are bithecal, tetrasporangiate, and longitudinally dehiscent (fig. 2G). In transection, the anther primordium appears as a fourlobed structure composed of an L1-derived epidermis, one layer of L2-derived subepidermis, and an L3-derived core (fig. 2A). The L1 layer gives rise to the epidermis and the stomium. Periclinal divisions in L2 cells further give rise to two cell layers, the inner primary sporogenous cells and the outer primary parietal cells (fig. 2B). The former then differentiate to generate the sporogenous cells, while the latter first divide to form endothelial cells and secondary parietal cells and then divide again to produce the middle cell layer and the tapetum (fig. 2C, 2D); this anther wall development corresponds to the Basic type (type I of Davis 1966). The L3 layer contributes to the connective tissue and vascular bundle.

The anther wall comprises five cell layers prior to maturation: an epidermis, an endothecium, two middle layers, and a tapetum. The tapetum forms a glandular single layer. The cells are uninucleate, and the cytoplasm accumulates tannin-like substances (fig. 2D). During maturation, the endothecium cells develop prominent U-shaped fibrous wall thickenings, and the epidermal cells are enlarged (fig. 2E). In the stomium, the epidermal and subdermal cells are smaller than the locule anther wall and lack wall thickenings in the endothecium (fig. 2F).

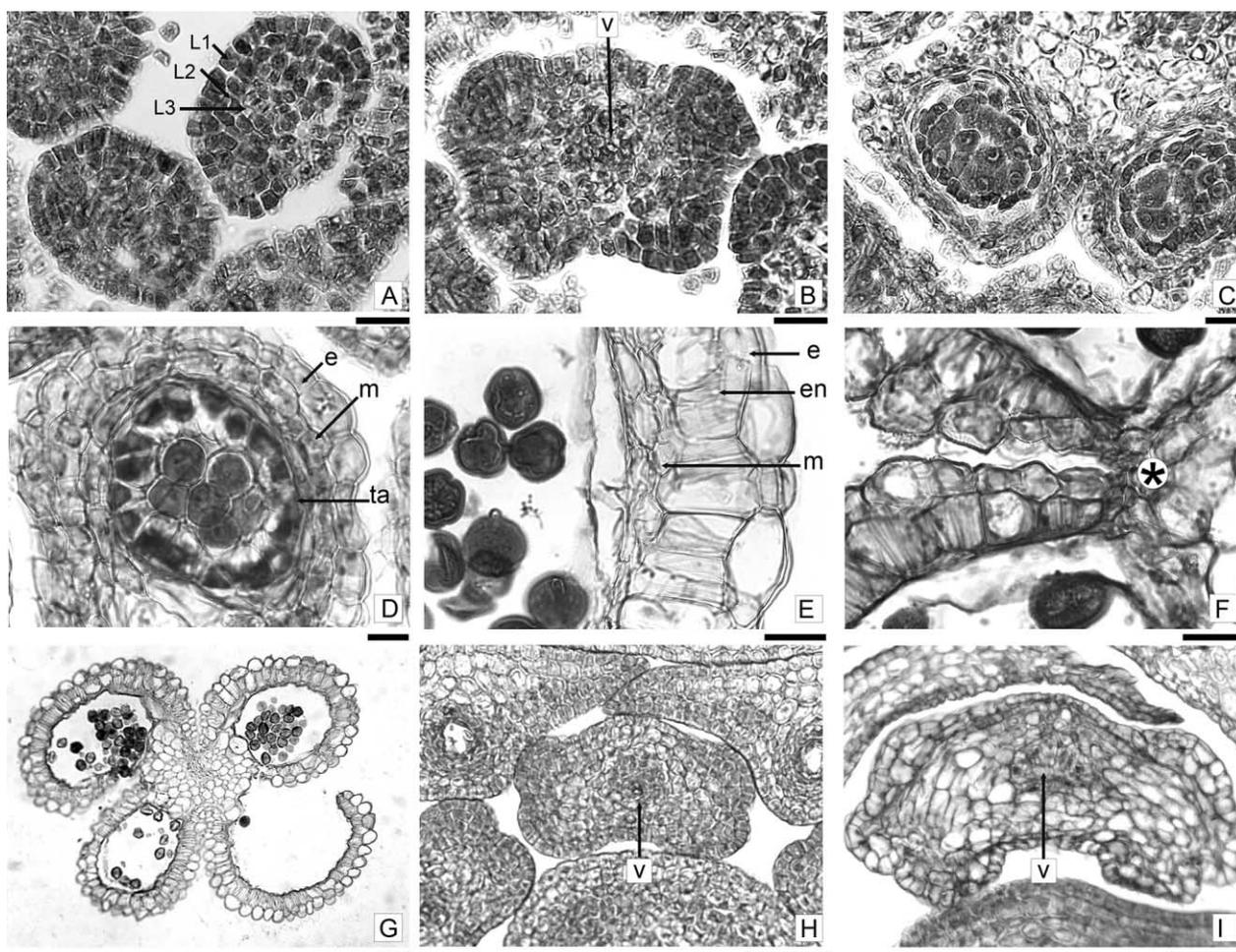
The sporogenous cells developed into microspore mother cells, with a prominent nucleus and dense cytoplasm (fig. 2C). Meiosis in the microspore mother cell is accompanied by successive cytokinesis, resulting in mostly tetrahedral tetrads covered by a refractive callose wall (fig. 2D). Mature pollen grains are two-celled (fig. 2E). In staminate flowers, the gynoecium is absent, and the tip of the floral meristem is covered by nectariferous tissue (fig. 3Q).

### *Functionally Pistillate Flower Structure: Gynoecium Development*

In the pistillate flowers, the androecium is reduced to five staminodes that lack sporogenous tissue (figs. 1B, 2H, 2I, 3K). The gynoecium is trimerous, and the ovary has one locule and a single ovule (fig. 3I). The two lateral carpels are reduced and do not produce an ovule or form a locule. In some cases, the two lateral carpels are so reduced that the gynoecium possesses only one or two styles (fig. 3L, 3M).

The gynoecium development is initiated by the formation of a hemispherical protuberance at the center of the floral primordium (fig. 3A). The gynoecium primordium rapidly adopts a triangular cup-like shape with a central depression; each angle of the triangular shape corresponds to the dorsal side of a carpel, and the central depression is the locule of the fertile carpel (figs. 3B, 4A). The subsequent appearance of two other lateral holes corresponds with the incipient locule of the two other carpels, which are reduced and form only a style and a stigma. This structure demonstrates the existence of three congenitally fused carpels (fig. 3C). Only the fertile locule enlarges further. The margins of the three holes grow to form three styles (fig. 3D–3F). The ventral side of the fertile carpel grows faster than the dorsal and lateral ones and forms the topologically upper part of the ovary (figs. 3D–3H, arrows; 4B, 4C). This portion keeps growing and forms the narrow end of the mature, ovoid, and laterally flattened ovary (figs. 3I, 3J, 4C–4I). Due to the synascidiate base of the carpels and the continued growth of the floral apex, the insertion of the three styles was displaced down to the midlength of the ovary; the two lateral styles are inserted at the middle zone of the ovary, and the dorsal style is inserted at a slightly lower level (fig. 3K). The stigmas are capitate and covered with glandular papillae that release a dense secretion insoluble in alcohol or acetone (figs. 3N–3P, 4K).

Postgenital fusion of the carpels can be achieved by either dentonection or capillinection, two terms coined by Sigmond (1930). In the apical portion of the ovary, the fusion of the ventral and dorsal sides of the ovary is produced by dentonection (i.e., fusion of the epidermal cells of both margins in contact). This attached zone remains well defined and recognizable even in the mature ovary (fig. 4C–4J, asterisks). Throughout the entire length of the mature style, the suture line on the ventral side



**Fig. 2** Development of the anther wall, microsporogenesis, and microgametogenesis in transverse sections of stamens in staminate flowers. *A*, Anther wall formation with L1-derived epidermis, L2-derived subepidermis, and L3-derived core. *B*, Four-layered anther wall. *C*, *D*, Five-layered anther wall with differentiated tapetum and microspore mother cells before meiosis. *E*, Detail of mature anther wall. *F*, Stomium area (asterisk) before anthesis. *G*, Anther at the time of dehiscence. *H*, *I*, Transverse sections of the anther of staminoids in pistillate flowers without sporogenous tissue. *H*, Four-lobed stage. *I*, Mature stage. e = epidermis, en = endothecium, m = middle layers, ta = tapetum, v = vascular bundle. Scale bars: *A–F* = 20  $\mu\text{m}$ ; *G–I* = 50  $\mu\text{m}$ .

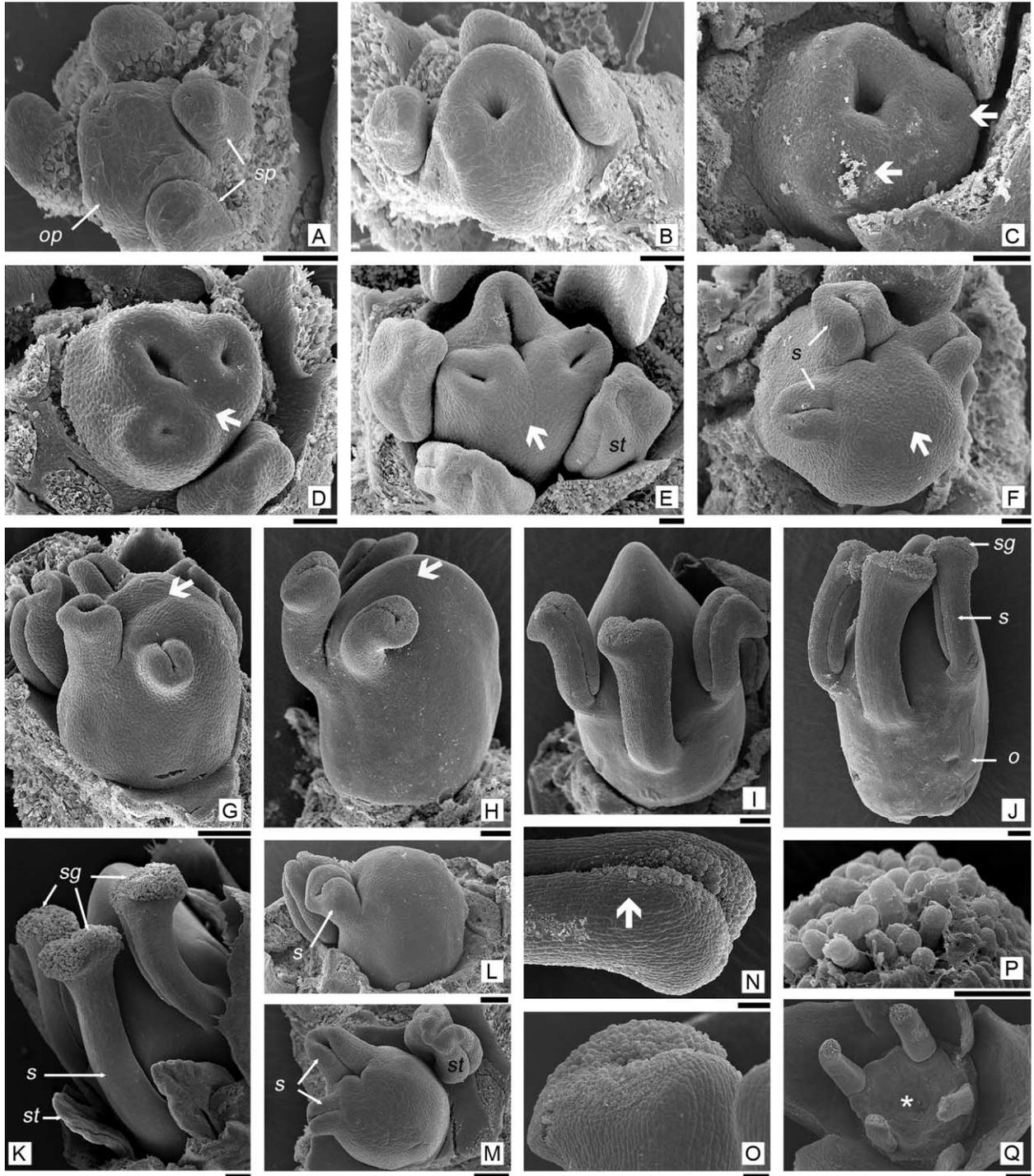
is well delimited, indicating postgenital fusion by dentonection (figs. 3*N*, 7*C*). In the basal region of stigmas, both carpel sides are fused by capillinection or close intertwining of unicellular papillae of the margins (fig. 4*K*). With time, both types of suture are transformed into a TT.

#### Ovule Development

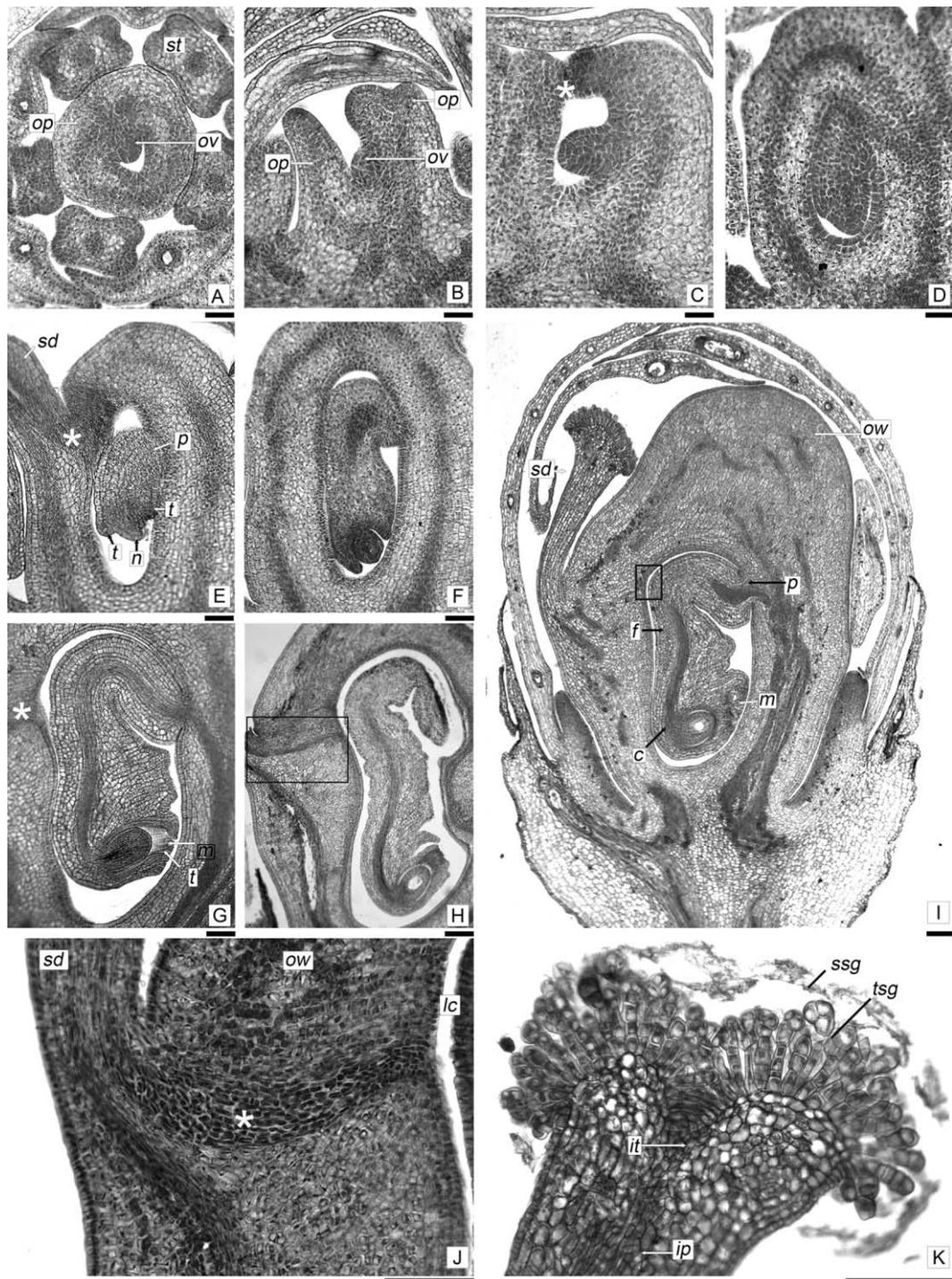
The ovule primordium develops from divisions of cells of inner epidermal and subepidermal layers of the basal-lateral region of the gynoecium before the closure of the ovary (fig. 4*A*, 4*B*). As the ovary grows and closes, the ovule primordium consists of an emergence or a bulge of the locule at midlength (fig. 4*C–E*). However, with the asymmetric development of the ventral side of the carpel, the insertion of the ovule is uplifted (or shifted upward) and is apical when the gynoecium is mature (fig. 4*F–I*).

During ovule primordium growth, the nucellar apex is oriented toward the base of the locule (figs. 4*D*, 5*A*). The single integument is initiated from dermal and subdermal layers, forming a ring below the tip of the primordium (figs. 4*E*, 5*A*). The development of the single integument delimits a very elongated funicle and a short nucellus (fig. 5*B*). The integument becomes remarkably thick by anticlinal and periclinal cell divisions (fig. 4*F*). The distal portion of the integument continues to grow, and the margin is split. Consequently, the micropyle has two or three massive integumentary ridges, each with four to six layers of cells (figs. 4*G*, 5*D*).

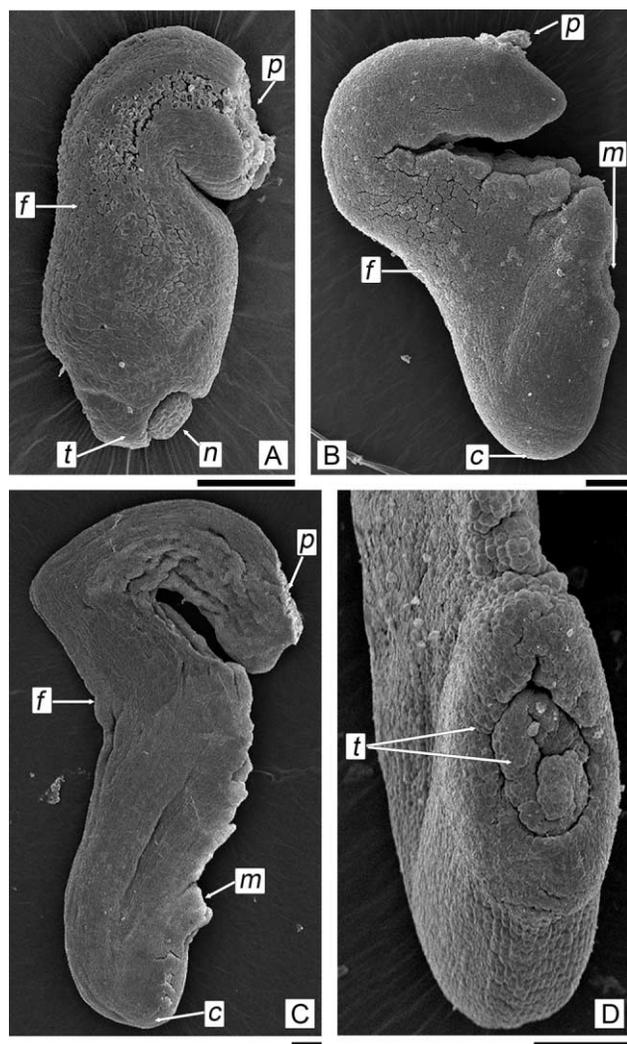
At anthesis, the ovule fills the entire locule (fig. 4*I*). The extensive growth in length and curvature of the bent funicle leads the micropyle to be close to the insertion of the ovule, face the placenta, and be opposite to the dorsal style (figs. 4*H*, 4*I*, 5*B*, 5*C*). The dorsal portion of the bent funicle is in close contact with the base of the dorsal style, where the PTTT ends and forms a



**Fig. 3** Gynoecium development of pistillate flowers of *Schinopsis balansae* (SEM). *A*, Gynoecium primordium. *B*, Gynoecium primordium with the locule of the fertile carpel. *C*, Appearance of two lateral holes (arrows) corresponding to the sterile carpels. *D–F*, Top view of the ovary closure; arrows indicate the ventral side of the fertile carpel. *G–J*, Lateral views of the ovary showing successive stages of style development. *K*, Lateral view of the pistillate flower with staminodia. *L*, Gynoecium with only one style. *M*, Gynoecium with two styles. *N*, Detail of the style and stigma; note the ventral median longitudinal furrow (arrow). *O*, Capitulate stigma. *P*, Detail of stigmatic papillae. *Q*, Staminate flowers with nectariferous tissue (asterisk). o = ovary, op = ovary primordium, s = style, sg = stigma, sp = stamen primordium, st = stamen. Scale bars: *A–F*, *N–P* = 50  $\mu\text{m}$ ; *G–M*, *Q* = 100  $\mu\text{m}$ .



**Fig. 4** Gynoecium development of pistillate flowers of *Schimopsis balansae*. *A, B*, Ovary and ovule primordia. *A*, Transection. *B–K*, Longitudinal median sections. *C–H*, Successive stages of ovule development. *I*, Mature ovary showing the placentation; the box indicates the zone of functional ponticulus. *J*, Detail of ovary wall in the area of fusion of the ventral and dorsal sides of the ovary, corresponding to the box in *H*. *K*, Detail of stigma. *c* = chalaza, *f* = funicle, *ip* = interlocking of papilliform epidermal cells, *it* = intertwining of papillae, *lc* = locule, *m* = micropyle, *n* = nucellus, *op* = ovary primordium, *ov* = ovule, *ow* = ovary wall, *p* = placenta, *sd* = dorsal stigma, *ssg* = secretion of stigma, *st* = stamen, *t* = tegument, *tsg* = papillae of stigma. Scale bars = 100  $\mu$ m.



**Fig. 5** Development of the ovule of *Schinopsis balansae*. A, B, Young ovule during anatropous curvature. C, Mature ovule. D, Detail of micropyle showing the single and splitting integument. c = chalaza, f = funicle, m = micropyle, n = nucella, p = point of attachment to the placenta, t = integument. Scale bars = 100  $\mu$ m.

functional ponticulus (box in fig. 4I). One unbranched vascular bundle extends throughout the entire length of the funicle of the ovule and connects the placenta to the chalaza (fig. 4C). The vascular bundle has few central tracheary elements surrounded by phloem, with abundant parenchymatic cells characterized by a dense cytoplasm.

#### *Megasporogenesis and Megagametophyte Development*

The archesporial cell develops in the nucellus and is separated from the nucellar epidermis by two parietal layers; ovules are crassinucellar. The archesporial cell proper grows and differentiates directly into a megaspore mother cell (MMC), a conspicuous cell with a nucleus and a distinct nucleolus (fig. 6A). This differentiation occurs before the closure of the integument to form the micropyle.

The MMC undergoes meiosis, resulting in a linear tetrad of megaspores (fig. 6B, 6C). The three micropylar megaspores degenerate and only the chalazal one remains and develops further into a megagametophyte. The embryo sac development is monosporic and follows the *Polygonum* type of octonucleate bipolar megagametophytic ontogeny (fig. 6D–6H). The mature embryo sac consists of seven cells: the egg cell, two synergids, the central cell, and three small antipodal cells. Synergids are hooked and have a conspicuous filiform apparatus (fig. 6E, 6F). The egg cell is larger than the synergids, and the egg cell's nucleus is located toward the chalazal end, displaced by a large vacuole; the synergids have the opposite polarity. The paired polar nuclei of the central cell fuse before fertilization (fig. 6F–6H). The three antipodal cells show dense cytoplasm without vacuoles (fig. 6G).

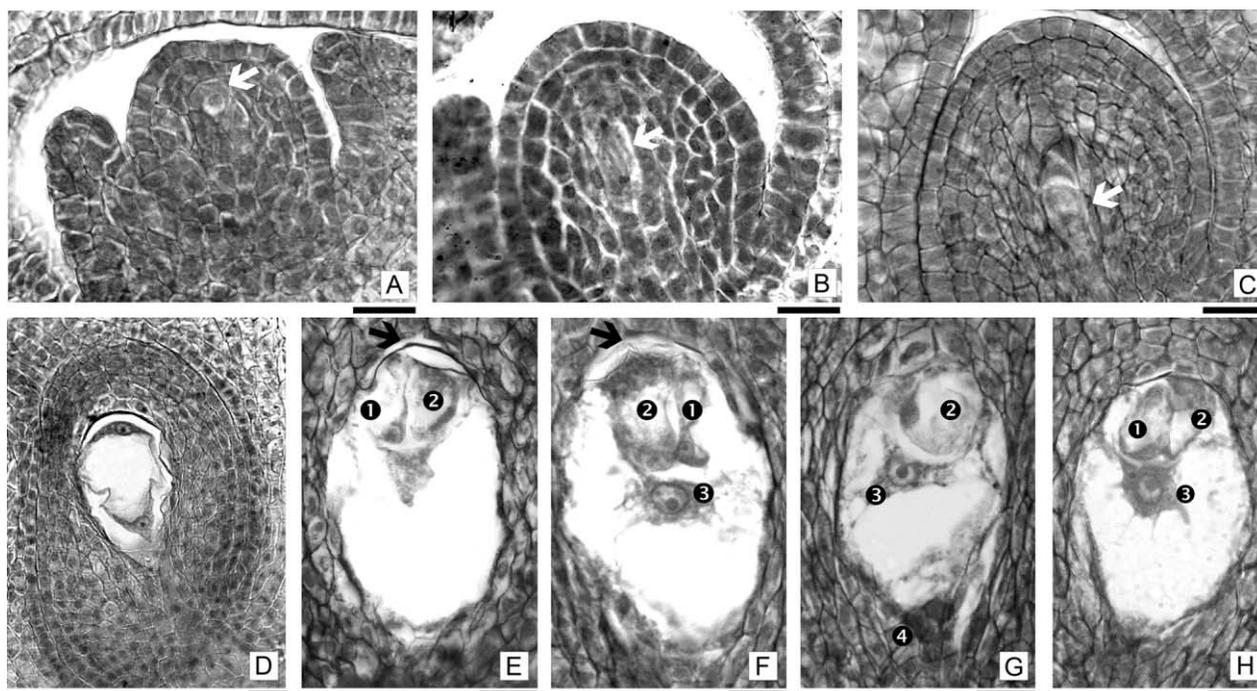
#### *PTTT(s)*

The stigma surfaces are covered by densely packed glandular papillae, which form the beginning of the PTTT (figs. 4K, 7B). The papillae are formed by a uniseriate foot and multiseriate head, coated by a thin and smooth cuticle. The cuticle is not detached from the cells of the head, so it could be assumed that the stigmatic secretion is transcuticular. As described above, the stigmatic papillae intermingle in the apex of the style slits, where they gradually shorten and lose heads, becoming shorter interspersed papillae (fig. 4K).

The styles are formed by a ground parenchyma supplied with three vascular bundles (fig. 7C). The TT lines the slit that runs along the inner angle of each style, extending from the stigma to the union of styles and ovary (fig. 7A, 7L). The PTTT is made up of elongated and glandular cells, with a conspicuous nucleus, tiny vacuoles, and dense cytoplasm (fig. 7D). The inner morphological surface in the plicate portion of each carpel (i.e., the inner epidermis of both sterile and fertile carpels) differentiates into a PTTT, and because of the architecture of the gynoecium (and the lack of a symplicate region), there is no external or internal compitum.

The three exposed stigmas of anthetic pistillate flowers were receptive, observed by the presence of bubbling on testing with hydrogen peroxide. Open-pollinated flowers of *Schinopsis* always present pollen grains on the stigma. However, only three to four germinated pollen grains were observed on each stigma. The pollen grains germinate onto the stigmatic papillae, and the PT penetrates between the multicellular papillae to reach the intermixed papillae and ground transmitting tube and down into the styler canal (fig. 7B). The PTs growing through the dorsal style of the ovary reach the locule, following the PTTT (fig. 7B–7D). Otherwise, the PTs entering through the lateral styles run along the PTTT surfaces that line the channel inside the ovary wall (fig. 7L–7N). In contrast with the PTTT of the fertile carpel, which connects the stigma directly to the dorsal side of the funicle, the PTTT of the reduced carpel ends in the ovary lateral walls. However, PTs can still reach the locule, going through the ground parenchymatous tissue, the hypodermis, and the inner epidermis of the carpel, tissues that lack glandular features of TT.

In both cases (through dorsal and lateral styles), several PTs reach the apical portion of the locule. In this region, the dorsal side in the region of the bend of the funiculus is almost in contact with the inner epidermis of the carpel (fig. 4I). The PTs pen-



**Fig. 6** Sporogenesis and gametogenesis of *Schinopsis balansae*. A–H, Megasporogenesis and megagametogenesis in longitudinal sections of pistillate flowers. A, Megaspore mother cell (arrow). B, Meiosis (arrow). C, Linear tetrad with three degenerating micropylar megaspores and a functional chalazal megaspore (arrow). D, Two-nucleate megagametophyte. E–H, Longitudinal sections of a different ovule's photomicrograph at the mature embryo sac stages, showing the filiform apparatus (arrows), egg cell (1), synergid cell (2), secondary nucleus (3), and antipodal cells (4). Scale bars = 20  $\mu$ m.

trate the epidermis of the funiculus and cross the parenchyma tissue up to the vascular bundle (fig. 7E, 7F). The PTs have thin cellulosic walls and clear cytoplasm (fig. 7D–7K, 7N). Inside the bundle, the PT branches off (fig. 7G–7J). A first division grows toward the placenta but degenerates quickly and never reaches the insertion of the funiculus. A second branch continues across the vascular bundle between the xylem and phloem, extends to the chalaza, penetrates the nucellus, encircles the embryo sac, and reaches the egg apparatus (fig. 7A, 7K). In front of the filiform apparatus, the PT is widened and finally penetrates one synergid. Fertilization is aporogamous. In about a dozen ovules, the presence of two to four PTs was observed, which even reaches egg apparatus in pairs, but always only one PT was observed to enter the synergid. In more than 150 gynoecia studied, no PT was observed to reach the female gametophyte by going through the micropyle.

#### *Endosperm and Embryo*

The development of the endosperm is precocious and nuclear ab initio, and fertilization is followed by intensive division of the primary endosperm cell nucleus, but not by cytokinesis. This leads to the production of a coenocyte or multinucleated cell that corresponds to the endosperm (fig. 8A–8D). Each endosperm nucleus possesses two to four nucleoli and is surrounded by a mass of cytoplasm, delimited by a dense cortical array of microtubules and forming a nucleocytoplasmic domain (fig. 8B). Beyond fertilization, the antipodal cells are still recognizable and persist at the chalazal side of the embryo sac; their

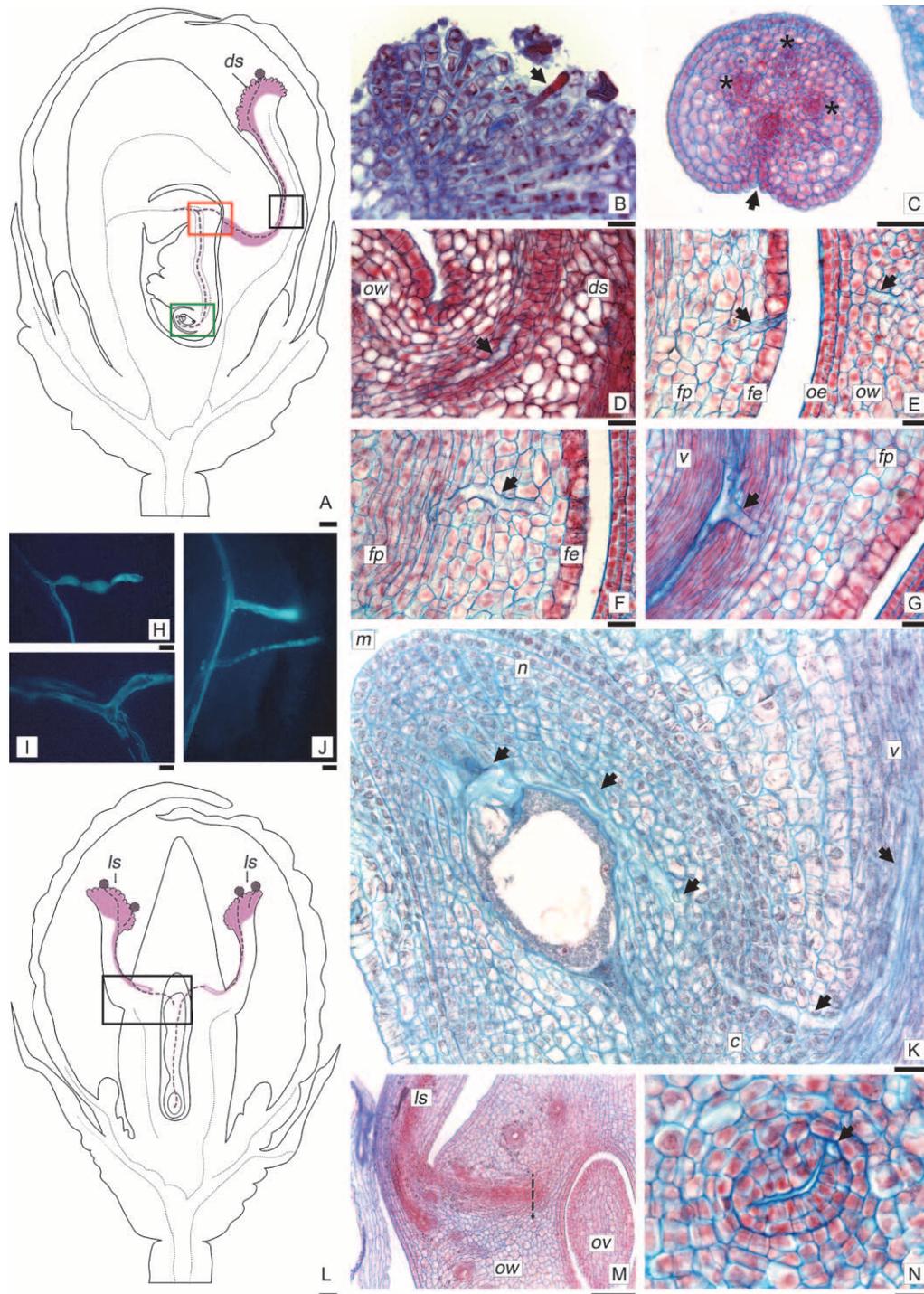
nuclei are clearly detectable and possess nucleoli and a well-defined nuclear membrane (fig. 8C). Several quasi-synchronous rounds of mitosis lead to approximately 40 nuclei surrounding a large central vacuole (fig. 8D, 8E). The cellularization process starts in the micropylar region when the embryo reaches the globular stage of development (fig. 8F).

The morphological development of *Schinopsis* embryos follows the Onagrad type of embryogenesis. The first division of the zygote is transverse, producing a two-celled embryo (fig. 8E). The basal cell divides transversely to give a suspensor of two cells, and the upper cell develops into the hypophysis. The cells of the hypophysis have divided longitudinally several times. The terminal cell, oriented to the chalaza, develops into the embryo per se, which divides twice vertically and then transversely to form an eight-celled proembryo (the octant stage). Subsequent cell divisions in the octant embryo result in the formation of the globular and heart-shaped embryo (fig. 8F–8I). The heart-shaped globular embryo elongates, and the cotyledons, hypocotyl, and radicle become distinct (fig. 8J, 8K). The embryo progressively fills the embryo sac, while the endosperm is almost entirely digested.

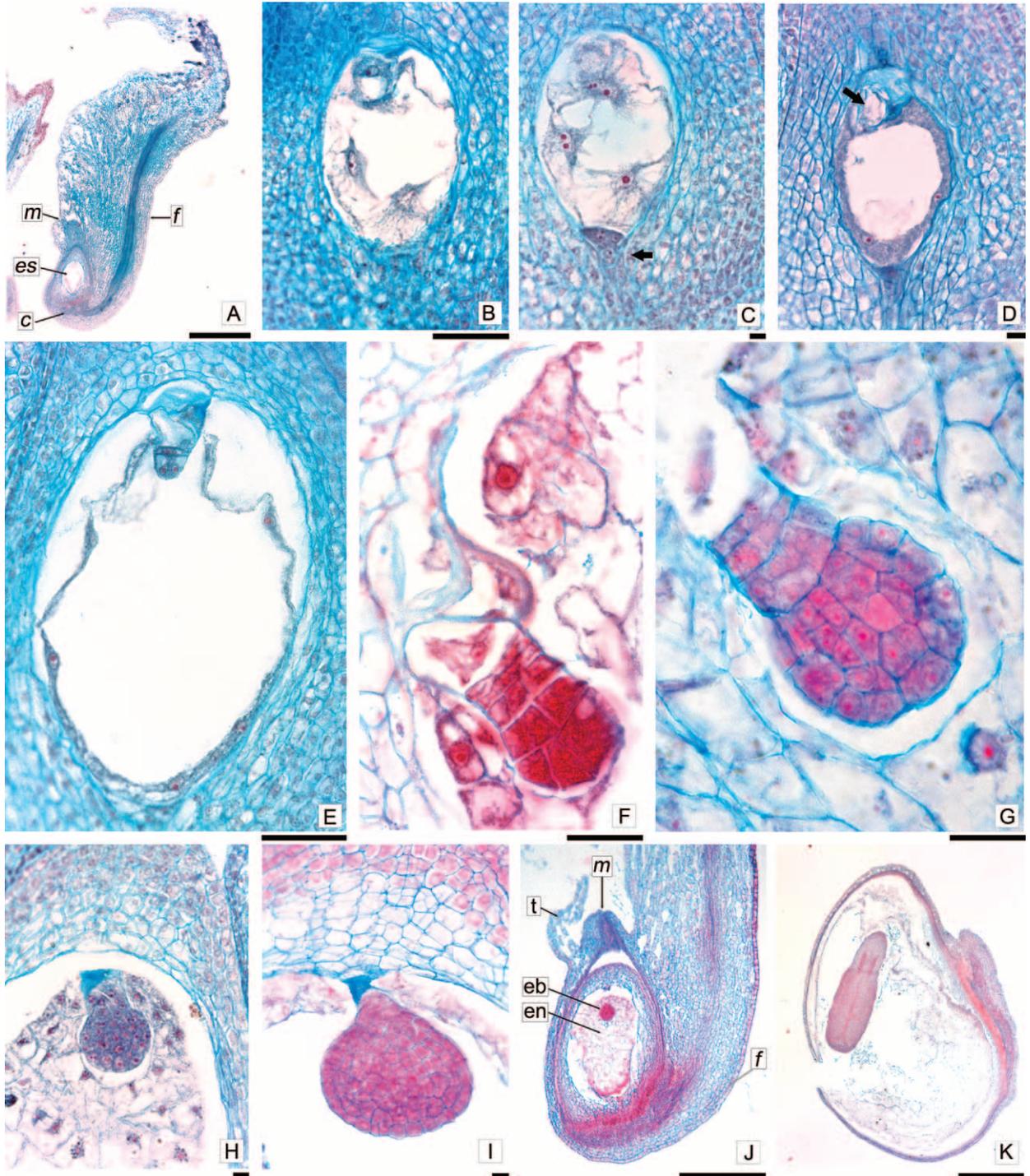
#### Discussion

##### *Anther Wall Development, Microsporogenesis, and Microgametogenesis*

The results observed in microsporangium development of *Schinopsis balansae* are consistent with the common pattern



**Fig. 7** Pollen tube transmitting tracts of *Schinopsis balansae*. A, Diagram of the pistillate flower in the longitudinal medium plane. Pink shading shows the transmitting tissue, the dashed line shows the pollen tube, and the dotted lines show the vascular bundles. B, Longitudinal section stigma with multicellular papillae and germinated pollen grain (arrow). C, Transversal section style with three vascular bundles (asterisks) and transmitting tissue lining the slit (arrow). D, Longitudinal section of the area of the dorsal style and ovary (black box in A); the arrow indicates a pollen tube. E–J, Longitudinal section photomicrographs corresponding to the area marked with a red box in A. E, Pollen tubes (arrows) growing through the carpel wall and epidermis and the parenchyma tissue of the funiculus. F, Pollen tube (arrow) reaches the vascular bundle of the funicle. G–J, Branched pollen tube growing over the vascular bundle by LM (G) or fluorescence microscopy (H–J). K, Pollen tubes (arrows) reach the chalaza, enter by the nucellus, encircle the embryo sac, and penetrate a synergid (green box in A). L, Diagram of the pistillate flower in the longitudinal frontal plane, showing lateral styles (shading and lines defined as in A). M, Detail of the area of the lateral style, ovary, and ovule (black box in L), showing the blind channel inside the ovary wall. N, Transversal section of the channel with a pollen tube (arrow; section made by the plane indicated with a dashed line in M). c = chalaza, ds = dorsal style, fe = funicle epidermis, fp = funicle parenchyma, ls = lateral style, m = micropyle, n = nucellus, oe = ovary epidermis, ov = ovule, ow = ovary wall, v = vascular bundle. Scale bars: A, L, M = 100  $\mu\text{m}$ ; B, D–K, N = 20  $\mu\text{m}$ ; C = 50  $\mu\text{m}$ .



**Fig. 8** Embryo and endosperm development of *Schinopsis balansae*. *A*, Ovule containing mature embryo sac. *B*, *C*, Embryo sac after double fertilization. *B*, Zygote and two endosperm nuclei delimited by a cortical array of microtubules. *C*, Antipodals and three endosperm nuclei. *D*, Embryo sac containing syncytial endosperm with peripheral nuclei and a central vacuole. *E*, Proembryo (four-celled). *F*, Globular embryo and cellularization of endosperm process in the micropylar domain. *G*–*I*, Advanced globular embryos and cellular endosperm. *J*, Ovule with globular embryos and cellular endosperm. *K*, Advanced stage of seed formation, with the embryo showing formation of cotyledons. c = chalaza, eb = embryo, en = endosperm, es = embryo sac, f = funiculus, m = micropyle, t = tegument. Scale bars: *A*, *J*, *K* = 500  $\mu\text{m}$ ; *B* = 50  $\mu\text{m}$ ; *F*–*I* = 10  $\mu\text{m}$ .

observed in angiosperms in general (Lersten 2004; Scott et al. 2004) and in the Anacardiaceae family in particular (Aleksandrovski and Naumova 1985; Kubitzki 2011). The family was described as having tetrasporangiate anthers with longitudinal dehiscence and a secretory and binucleate tapetum. The mature anther wall is characterized by the epidermis and the endothecium with fibrous bands. Simultaneous cytokinesis follows meiotic divisions in the microspore mother cells. The mature pollen grains are bicellular. The only feature that differs in *S. balansae* is the uninucleate tapetum. Observations made with LM in this study did not allow settling the presence of orbicules; it would be interesting to see a deeper analysis with TEM.

#### *Gynoecium and Ovule Development*

The Anacardiaceae is currently split into two subfamilies: Anacardioidae and Spondioidae (Pell 2004; APG III 2009; Pell et al. 2011). Anacardioidae includes four of the five tribes of Engler (1892): Anacardiaceae, Dobineae, Rhoaceae, and Semeocarpeae. According to the intrafamilial classifications of Engler (1892), *Schinopsis* belongs to the tribe Rhoaceae. In several genera of this tribe, the gynoecium is tricarpellate, monosymmetric, and pseudomonomerous (Wannan and Quinn 1990, 1991), indicating a basic and widespread feature within the tribe. This ontogenetic study verifies the presence of three clearly distinguishable carpels at early stages of development of the gynoecium of *S. balansae*, confirming the morphological condition of pseudomonomerous, as defined by Weberling (1989; Endress 1994; Ronse de Craene and Smets 1998), in which there is only one fertile locule in the mature ovary but there is direct evidence that more than one carpel is initiated.

The gynoecium ontogeny of *S. balansae* is similar to other pseudomonomerous gynoecia of Anacardioidae: *Pistacia vera* (Hormaza and Polito 1996), *Pistacia lentiscus* (Bachelier and Endress 2007), *Schinus terebinthifolius*, and *Schinus polygamus* (Rodrigues et al. 2004; Bachelier and Endress 2009). All these species share the presence of two abortive carpels without locules or ovules. The morphological differences correspond to the final insertion of the ovule, which is lateral-basal in *Pistacia* and apical-lateral in *Schinopsis* and *Schinus*, and the three united styles and stigmas of *Pistacia*. According to the mode of carpel closure, Endress and Igersheim (2000) described four types of gynoecium structure in basal angiosperms; the gynoecium of *S. balansae* is a transitional form between angiospermy types 3 and 4, because they combine an almost complete postgenital fusion, except for a small area of incomplete secretory canal. This area represents a relic of two reduced carpels.

Anacardiaceae are distinguished by having one apotropous ovule (syntropous; Bachelier and Endress 2009), in contrast to the two epitropous ovules in the Burseraceae. The arrangement and insertion of the ovule are topics extensively studied within the family. The most widespread condition in the family is an apically attached funicle bearing an ovule, which has a superior micropyle (Bachelier and Endress 2009). In this study of the development of *Schinopsis*, it was possible to observe that the insertion of the ovule primordium is basal. During growth of the ovary, the ovule insertion was displaced to the lateral-apical portion of the ovary. In *Schinopsis*, it may be possible to derive the final apical-lateral placentation from a basal initial one. In the ontogeny of the ovule primordium, both protodermal and

subdermal layers are involved, as also observed by Rodrigues et al. (2004) in *S. terebinthifolius* and *S. polygamus*. Bachelier and Endress (2009) and other previous authors (Bouman 1984; Johri et al. 1992) consider the unitegmic condition as derived in Anacardiaceae because it has been reported in only a few Anacardioidae, including *Amphipterygium*, *Pistacia*, and *Lithraea*. *Schinopsis* should be included among them.

The gynoecium of *Schinopsis* has undergone two evolutionary modifications, both toward reduction. In the staminate flowers, this reduction may lead to a complete disappearance of the gynoecium; in the pistillate ones, this may lead to reduction in carpel expansion and ovule number and absence of sporogenous tissue in the anthers of the pistillate flowers (Gonzalez 2013). These two trends are also found in any Anacardiaceae/Anacardioidae with (cryptic) functionally dioecious flowers and was also observed by Wannan and Quinn (1990, 1991) and Bachelier and Endress (2009). The complete absence of the gynoecium in functionally male flowers is also found in many unrelated taxa with functionally unisexual flowers.

#### *PT Pathways and Fertilization Mode*

The gynoecium of *S. balansae* lacks a compositum. Three independent PTTTs move down through the styles, but only one reaches the locule (Gonzalez 2013). This ontogenetic study shows that this PTTT corresponds to the fertile carpel. The remaining two PTTTs—belonging to sterile carpels—do not reach the ovary and stop their course inside the carpel wall. This incomplete tissue path is a consequence of the reduction in the development of the lateral carpels. Regardless of the style that the PT descends, all PTs reach the locule by crossing the tiny space between carpelar and funicular epidermis and penetrating the ovule. Any PT that reaches the vascular tissue within the ovule's funiculus divides into two branches. The PT branch that follows the vascular tissue through the chalaza encircles the embryo sac and reaches the egg complex. It seems that the PTs of *S. balansae* do not need a complete pathway of transmission tissue to reach the egg apparatus.

In species of *Mangifera indica* (Joel and Eisenstein 1980), *Spondias dulcis*, and *Anacardium occidentale* (Bachelier and Endress 2009), the funicle and the styler canal are postgenitally connected via the ponticulus (i.e., a bridge of tissue between the ovary wall and the surface of the ovule). Bachelier and Endress (2009) also described a ponticulus in species of both subfamilies of Anacardiaceae, defining it as a zone of close contact (lacking a bridge of tissue) between the dorsal side of the funicle and the PTTT, considered, however, as a functional ponticulus. The same type of ponticulus is described here for *S. balansae*. The ponticulus appears as a prominent feature in the Anacardiaceae family.

In Anacardioidae and Spondioidae, the occurrence of chalazogamy has been observed (Copeland 1961; Grundwag and Fahn 1969; Kubitzki 2011), but the ponticulus has been shown to be a path for PTs only in the former subfamily (Shuraki and Sedgley 1994; Martinez-Palle and Herrero 1998). To be strict, the first entry of PTs is by the funiculus, not by the chalaza, so I considered the term chalazogamy not precise or descriptive enough. Since in *S. balansae* the tube enters by the funicle, the PT course should be called funiculogamy. This pathway may be a potential unique feature of Anacardiaceae.

Many studies suggest that PT guidance is controlled by sporophytic and gametophytic tissues (Herrero and Hormaza 1996; Lord and Russell 2002; Erbar 2003; Dresselhaus and Franklin-Tong 2013; Bleckmann et al. 2014). Johnson and Preuss (2002) divided the process into five steps: phases I and II involve the recognition and growth of PTs into the stigma. Phase III describes the PTs' navigation into the transmitting tract or "superhighway." In phase IV, the PTs' growth on the septum surface is described: they traverse the path to the micropyle of the ovule exposed to an air-filled chamber. In *Arabidopsis*, several studies showed that growth of the PT from the surface of the ovary to the micropyle can be divided into two phases (IV and V) controlled by distinct mechanisms (Shimizu and Okada 2000). In *S. balansae*, the last step to the micropyle was skipped: when the PTs that navigate through lateral styles reach the end of the channel covered by TT, they continue in a straight line to the locule, passing through the parenchyma tissue. Obviously, a stronger signal attracts the PTs away from the signal provided by the TT.

Herrero (2003) recognized three unusual features of growing PTs, frequently without any clear explanation: (a) wandering PTs that change their direction of growth, (b) branching PTs, and (c) the occurrence of chalazogamy. PT pathways in *Schinopsis* have two of these three characteristics: branching and chalazogamy (funiculogamy). PTs branching inside the fertile gynoecia were already described to occur occasionally in *Oenothera* and *Prunus* (Sniezko 1996, 1997; Đorđević et al. 2010). In *Prunus*, Đorđević et al. (2010) speculate that the strange behavior of PTs could affect the number of fertilized ovules and the set of seeds inside the fruits. In *Schinopsis*, parthenocarpy was already described (Gonzalez and Vesprini 2010), and this could be the result of the unusual behavior of PTs.

#### *Female Gametophyte, Endosperm, and Embryo*

Angiosperms have more than 15 different patterns of female gametophyte development (Yadegaria and Drews 2004). The developmental pattern shown by more than 70% of flowering plants is the *Polygonum* type, and this pattern is found in *S. balansae* and other Anacardiaceae, including economically important *Pistacia* and *Mangifera* (Copeland 1955; Maheshwari et al. 1955; Grundwag and Fahn 1969; Aleksandrovski and Naumova 1985).

After fertilization, the initial endosperm cell divides repeatedly without cell wall formation, resulting in a nuclear endosperm. Cellularization occurs via the formation of radial microtubule systems and alveolation. This two-step pattern was

named the coenocytic/multicellular type by Lersten (2004). This endosperm is by far the most common type, present in 161 angiosperm families including commercially important crop plants (Davis 1966; Lersten 2004). During seed maturation, the endosperm is almost fully consumed by the embryo, the seed is exalbuminous, and the embryo is of *Onagrad* type, as in other Anacardiaceae (Johansen 1950; Kubitzki 2011).

#### Conclusions and Perspectives

In this study, the structure and development of the gynoecium in functionally pistillate flowers of *Schinopsis balansae* has been described. This is the first detailed morphological study of the floral anatomy and embryology in both unisexual flowers. According to Bachelier and Endress (2009), all Anacardiaceae have highly similar ovule structure, with a bent funicle that connects to the base of the style via the ponticulus, as is the case in *S. balansae*. The term funiculogamy is proposed instead of chalazogamy to emphasize the unique PT pathway and fertilization via the funicle (instead of the chalaza). This term should apply to all those species of subfamily Anacardiaceae where chalazogamia was mentioned (see above), because in all species previously analyzed, the PT actually enters through the funicle and not the chalaza.

Another interesting feature in *S. balansae* development is that the PTs run inside the ovule, following the path of the vascular bundle. This same path was described in *Pistacia* (Grundwag and Fahn 1969; Martínez-Pallé and Herrero 1998) and *Schinus terebinthifolius* (Pereira Fagundes et al. 2007). It would be worth exploring the detailed ultrastructure of the pathway and the branching of the PT in its journey inside the ovule toward the egg cell. Furthermore, *S. balansae* represents a new opportunity to analyze the source of PT attraction signals. Further investigation of subjects like presence of pairs of PTs reaching the egg apparatus, as well as the possibility of heterofertilization, is needed in order to evaluate the occurrence of parthenocarpy in this species.

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