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Anatomical development of the pericarp and seed of *Oncidium flexuosum* Sims (ORCHIDACEAE)

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ABSTRACT

Interpretation of the anatomical structure of the ovary and fruit of the Orchidaceae family is still controversial, which makes it difficult to understand the development and dehiscence of the fruit. The genus *Oncidium* is polyphyletic and is currently the subject of taxonomic studies. In this study, we have investigated the anatomical development of the pericarp and seed of *Oncidium flexuosum* Sims to determine important diagnostic characters that, along with molecular data, can assist in defining this group. We have found a new anatomical characteristic of the family: the presence of precursor cells for fruit dehiscence, which were visible from the beginning of development and located on the outer walls of the sterile valves. In contrast with what has been observed by different authors with other species, in the mature fruit of *O. flexuosum*, only the endocarp of the fertile valves and a few cells near the exocarp and the vascular bundle in the sterile valves show parietal thickening, while the rest remains parenchymatous. During the development of the ovule and embryo, we have shown that the embryonic sac of this species has eight nuclei and that the embryo has a long and elaborate suspensor.

Introduction

Structural and developmental studies of Orchidaceae fruits are scarce, although these organs vary greatly in shape, size, texture and ornamentation (Dressler, 1993; Rasmussen and Johansen, 2006). Most of the ontogenetic studies are concentrated on ovules and seeds and little is known about the structure and physiology of the fruits (Rasmussen and Johansen, 2006).

The flowers of the Orchidaceae are epigynous and the floral parts are adnate with the ovary in its full extension (Dressler, 1993). It is currently accepted that the ovary of orchids is composed of three carpels, although this organ has been interpreted in different ways over the years. For Lindley (1830–1840, 1847), Saunders (1923) and Arber (1925), the ovary of orchids was composed of six carpels, three with a placenta and three without a placenta. Brown (1831), apud Rasmussen and Johansen (2006), interpreted the ovary as being composed of only three carpels. Duncan and Curtis (1943), Swamy (1949a), Cribb (1999) and Rasmussen and Johansen (2006) supported the interpretation of Brown (1831), stating that the ovary in the family would be syncarpous and tricarpellate, showing a pattern of six valves in cross section: three fertile and three sterile. The sterile valves correspond to the bases of the sepals and the fertile valves correspond to petal base and two carpel-halves, carrying one marginal placenta from each (Rasmussen and Johansen, 2006).

The fruit is a capsule and is rarely described in studies, which is likely due to either a lack of knowledge or the belief of researchers that the fruits have little diagnostic value (Cribb, 1999). The capsules are generally dehiscent, and dehiscence usually occurs preferentially as a rupture along the midline of each carpel, and later between the carpels, generating three wide, fertile valves and three narrow, sterile valves. The six half-carpels remain joined at the apex in most species, but in some *Maxillaria* Ruiz & Pavón and *Lockhartia* Hooker taxa the carpels separate completely at the apex (Cribb, 1999; Dressler, 1993).

In this family, proliferation of the placenta and formation of the ovules generally occur only after pollination. The periods of time between pollination, fertilization and formation of the seeds are varying among species, even within the same genus (Duncan and Curtis, 1942; Swamy, 1949a). Pollination in Orchidaceae can be considered to have a dual effect: the first is to stimulate the enlargement of the ovary and the maturation of the ovules, and the second is to promote fertilization (Hildebrand, 1863; apud Duncan and Curtis, 1942).

Orchids produce large amounts of seeds, and each capsule can contain up to four million seeds (Arditti and Ghani, 2000). However, in natural conditions, few seeds successfully germinate because of the lack of both an endosperm and the ability to directly use nat-

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ural substrates thus requiring mycorrhizal associations (Withner, 1974). Most orchid species have small seeds with undifferentiated embryos, that is, a mass of undifferentiated cells. These can be compared to the globular stage of the embryos from dicotyledons. However, most orchid seeds exhibit acotyledonous embryos (Arditti, 1992; Veyret, 1974).

The genus *Oncidium* Sw. *sensu lato* contains more than 400 species (Chase et al., 2009), and its delimitation is controversial, as demonstrated by studies of chromosome numbers (Chase, 1986, 1987; Félix and Guerra, 2000) and of molecular systematics (Chase and Palmer, 1992). Studies of the molecular phylogeny show that the genus *Oncidium* is clearly polyphyletic, and several changes of the intrageneric relationships are currently being proposed (Chase, 1986; Chase et al., 2009).

Based on the few available studies of ovary and fruit structures of Orchidaceae the interpretation of the anatomy of the carpels is therefore still controversial, making it difficult to elucidate the development and dehiscence of the fruits of orchids. Moreover, there are few studies about the ontogeny of fruit, ovule and seed in this family. The present study investigated the anatomical development of the fruit and seed of *Oncidium flexuosum* Sims to identify important diagnostic characteristics that, along with molecular data, can assist in characterizing this genus. The species belongs to the subfamily Epidendroideae and occurs naturally in Brazil in the remaining fragments of the Atlantic Forest (Pabst and Dungs, 1977).

Materials and methods

For light microscopy and scanning electron microscopy (SEM), we collected samples of flower buds, flowers during anthesis, and fruits in different developmental stages from *O. flexuosum*. Artificial pollination was performed in ten individual cultures. A voucher of the investigated species (# 150303) was deposited in the UEC Herbarium, Brazil.

For light microscopy analysis, samples were fixed in Karnovsky (Karnovsky, 1965; modified by preparation in phosphate buffer pH 7.2) for 24 h, dehydrated in a graded ethanol series and embedded in Leica Historesin® (Heraeus Kulzer, Hanau, Germany). Serial sections (5 μm thick) were cut on a rotary microtome, stained with toluidine blue O (Sakai, 1973), and mounted in Entellan® synthetic resin (Merck, Darmstadt, Germany).

The chemical natures of the substances found in the embryos were determined using the following histochemical tests: Lugol's iodine solution to identify starch (Berlyn and Miksche, 1976), Sudan IV to identify lipid compounds (Pearse, 1985) and Aniline blue black (Fisher, 1968) to identify total protein. Photomicrographs were taken with a Leica® DM LB photomicroscope equipped with a Leica® DC 300F camera (Leitz, Wetzlar, Germany).

For scanning electron microscope analysis, samples of the *O. flexuosum* fruits were fixed in Karnovsky (Karnovsky, 1965; modified by preparation in phosphate buffer pH 7.2) for 24 h, dehydrated in a graded ethanol series and critical point-dried with CO_2 (Horridge and Tamm, 1969). The samples were attached to aluminum stubs and coated with gold (30–40 nm). Finally, the samples were examined under a LEO VP435 (Zeiss, Oberkochen, Germany) scanning electron microscope at 20 kV.

Results

Fruit morphology

The development of the *O. flexuosum* fruit from the 3rd to the 110th day after the artificial pollination can be seen in Fig. 1 and Table 1. Five days after the pollination of the flowers, the petals and sepals wither and the region of the inferior ovary begins to stretch. This expansion of the ovary continues until 40–45 d after pollination, when the fruit starts to increase in diameter. At 90–110 d after pollination, while the capsule is still green, the valves separate longitudinally, initially at the distal end. However, they remain attached at the apex and base when the seeds are released.

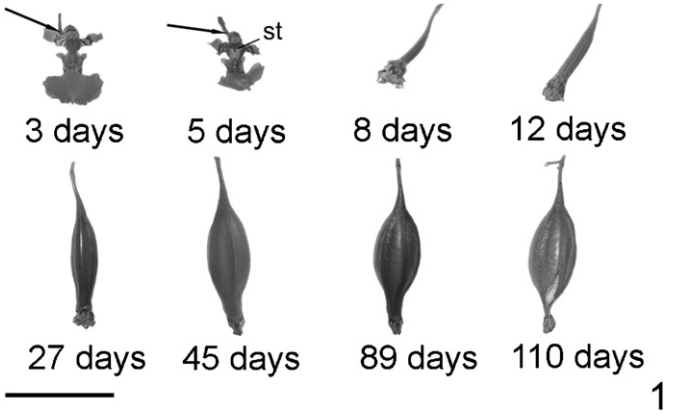


Fig. 1. Development of the fruit of *Oncidium flexuosum* Sims. Age refers to the period after the artificial pollination of the flowers. The arrows indicate the fruit. Region of the stigma: st. Scale bar = 2 cm.

Anatomical structure of the ovary

In a cross section of the ovary of the flower during anthesis, there are three carpels divided into six valves: three are fertile, with the presence of the placenta region, and three are sterile (Fig. 2). The outer epidermis of the ovary that contains stomata is single layered with cells that are elongated in the radial direction, in both cross and longitudinal sections, each with an obvious central nucleus and dense cytoplasm. The fundamental tissue has compact isodiametric parenchyma cells, and the cells near the outer epidermis are larger than those near the inner epidermis (Figs. 2 and 3). Each fertile valve has 14–16 layers of parenchyma cells (Fig. 2), and each sterile valve has 12–14 layers (Fig. 2). The fertile valves have one vascular bundle, and the sterile valves have two vascular bundles. The bundles in both the fertile and sterile valves are located near the inner epidermis, and idioblasts containing raphides are observed (Fig. 2). In the fertile valves, the placenta does not present fully developed ovules, only small projections composed by cells containing very dense cytoplasm (Figs. 2 and 3).

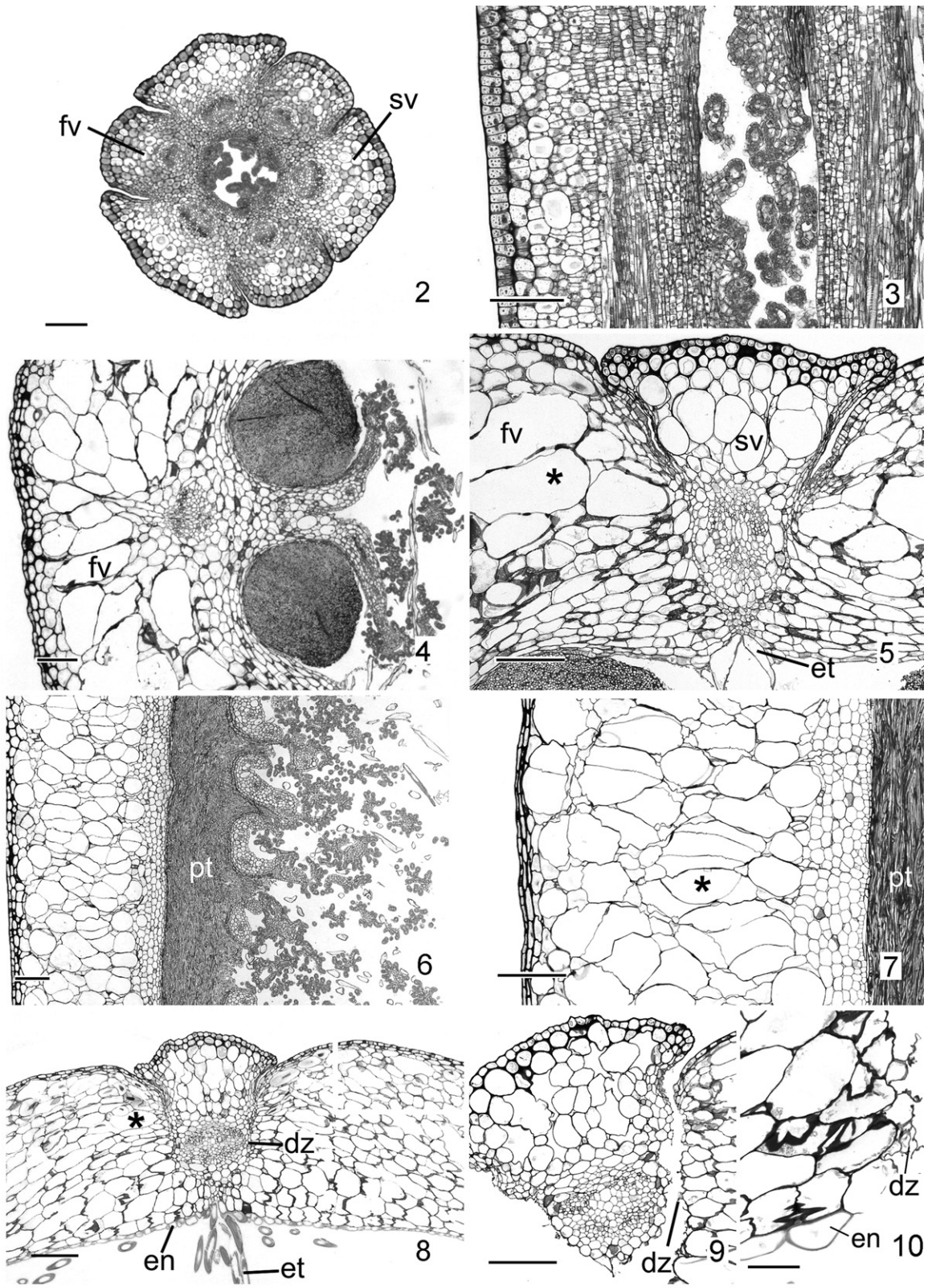
Anatomical development of the pericarp

Young fruit I: 23 d after pollination (Figs. 4–6)

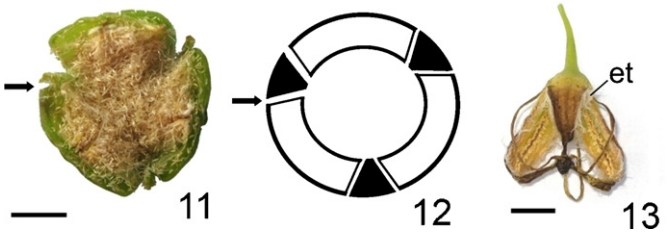
Fertile valve: The exocarp is single layered, with small cells slightly elongated in the longitudinal direction (Fig. 6). The meso-

Table 1
Morphological and anatomical changes observed during the development of the *Oncidium flexuosum* Sims fruit after pollination.

3–5 d	Petals and sepals wither Growth of the margin of the column, sealing the stigmatic cavity Proliferation of the placenta Germination of the pollen grains
5–45 d	Growth in the length of the fruit Elongation of the pollen tubes
35–50 d	Enlargement of the fruit diameter Differentiation of the ovules
50–65 d	Fertilization of the ovules Degeneration of the pollen tubes
90–110 d	Dehiscence of the fruit



Figs. 2–10. Structure of the flower ovary during anthesis and the pericarp of *Oncidium flexuosum* Sims. (2) Cross section of the middle region of the ovary; sterile valve: sv, fertile valve: fv. (3) Longitudinal section of the middle region of the ovary. (4, 5 and 8, 9) Cross-section and (6 and 7) longitudinal structure of the pericarp in the middle region of the fertile valve. (4–6) Fruit, 23 d after pollination. (7) Fruit, 60 d after pollination. (8) Mature fruit, 110 d after pollination. (9–10) Region of dehiscence of the fruit. Parenchyma cells showing hypertrophy (*); dehiscence zone (dz), endocarp (en), endocarpic trichomes (et) and pollen tube (pt). Scale bars = 100 μm (2 and 3, 10) and 200 μm (4–9).



Figs. 11–13. Fruit of *Oncidium flexuosum* Sims. (11 and 12) Cross section of the mature fruit. (12) Black portions correspond to the sterile valves, and white portions correspond to the fertile valves. (13) Appearance of the fruit after seed release. Location of dehiscence of the fruit (arrow) and endocarpic trichomes (et). Scale bars = 0.5 cm (13) and 2.5 cm (11).

carp contains two distinct regions: one is formed by two to three layers of small subepidermal cells, with thick walls and a compact arrangement, and five to seven layers of large cells with thin walls and large vacuoles and intercellular spaces. The other is formed by three to seven layers of small parenchyma cells with thin walls and a compact arrangement (Fig. 6). There is a single vascular bundle immersed within the parenchyma tissue. The endocarp is single layered with small isodiametric thin-walled cells. There is a formation of trichomes in the endocarp of the margins of the fertile valve next to the sterile valve (Figs. 5 and 8). In each fertile valve, the proliferation of the placental cells forms two projections that consist of parenchyma cells and two to three reduced vascular bundles. The ovule differentiation begins at the apex of each projection (Fig. 4).

Sterile valve: The exocarp is, similar to the fertile valve, single layered with small cells that are slightly elongated in the radial direction. The mesocarp is composed of two to three layers of small subepidermal cells, with thick walls and a compact arrangement, and five to seven layers of voluminous cells, with thin walls and large vacuoles and wide intercellular spaces; there are two vascular bundles inside of these layers. Three to five layers of very small fertile valve cells are evident under the sterile valve.

During this phase, the precursor cells of the dehiscence line are present in the edge between the sterile valves and the fertile valves. The precursors of the dehiscence line become evident as a layer of small cells. They are similar to a sheath that isolates the vascular bundles and the parenchyma cells of the mesocarp from the sterile valve and the two adjacent fertile valves (Fig. 5).

Young fruit II: 60 d after pollination (Fig. 7)

Changes occur only in the mesocarp, while the exocarp and endocarp remaining unchanged in both valves.

In the fertile valves, the edges between the regions of the mesocarp become more evident in this phase. The cells of the most interior layers, in both valves, remain small in both diameter and length (Fig. 7), while there is a pronounced enlargement of the cellular volume and intercellular space in the most exterior layers.

Mature fruit: 110 d after pollination (Figs. 9–13)

A pronounced radial elongation of the parenchyma cells of the mesocarp occurs in the fertile valve (Fig. 8). However, there is no increase in the number of cells, and there is only a small change in the cell volume and lytic formation of spaces in the mesocarp of the sterile valve (Fig. 9). Therefore, there is a reversal of the size proportions between the sterile and fertile valves in the fruit (Figs. 11–13) when compared to that observed in the ovary.

At this stage, the fruit reaches its final size and starts the ripening process. There are no modifications in the exocarp when compared to the previous phase. The endocarp cells, including the trichomes, become lignified in mature fruits. The fruit begins to open at the endocarp, opposite of the sterile valve. This is progressing along the dehiscence line by rupture of the cells there (Figs. 9–13). Lignified endocarp and remnants of cell walls in the dehiscence region of the fruit are seen in Fig. 10.

Anatomical development of the ovule and the seed

Shortly after pollination, the growth of the pollen tubes through the style begins (Figs. 14 and 15), and the proliferation of the placenta occurs by intense mitotic activity (Fig. 18). The ovule differentiation is not synchronous, not even within the same fruit, and it starts at around 35–50 d after pollination (Fig. 16–17). The ovule is initiated in a single subdermal cell which by periclinal divisions gives rise to an axial row of cells covered by a dermal layer.

The subdermal terminal cell differentiates into an archesporial cell with an evident nucleus (Fig. 19). The cell divisions of the dermal layer lead to the formation of the inner and outer integuments and to the curvature of the ovule (Fig. 20). The archesporial cell does not divide, but directly becomes the mother cell of the megaspore (Fig. 21). The nucellar epidermis has only one layer of cells at the micropylar end (Fig. 21). The ovule is tenuinucellate because the megaspore mother cell lies directly below the nucellar epidermis by absence of parietal cells. At the end of its development, the ovule is anatropous and bitegmic (Fig. 21).

The archesporial cell elongates prior to meiosis (Fig. 21), and its first division results in a dyad of similar-sized cells (Fig. 22). The second meiotic division gives rise to a functional chalazal megaspore and three micropylar megaspores that degenerate (Fig. 23). In the beginning of this meiotic stage, the integuments do not encompass the nucellar epidermis yet (Fig. 22).

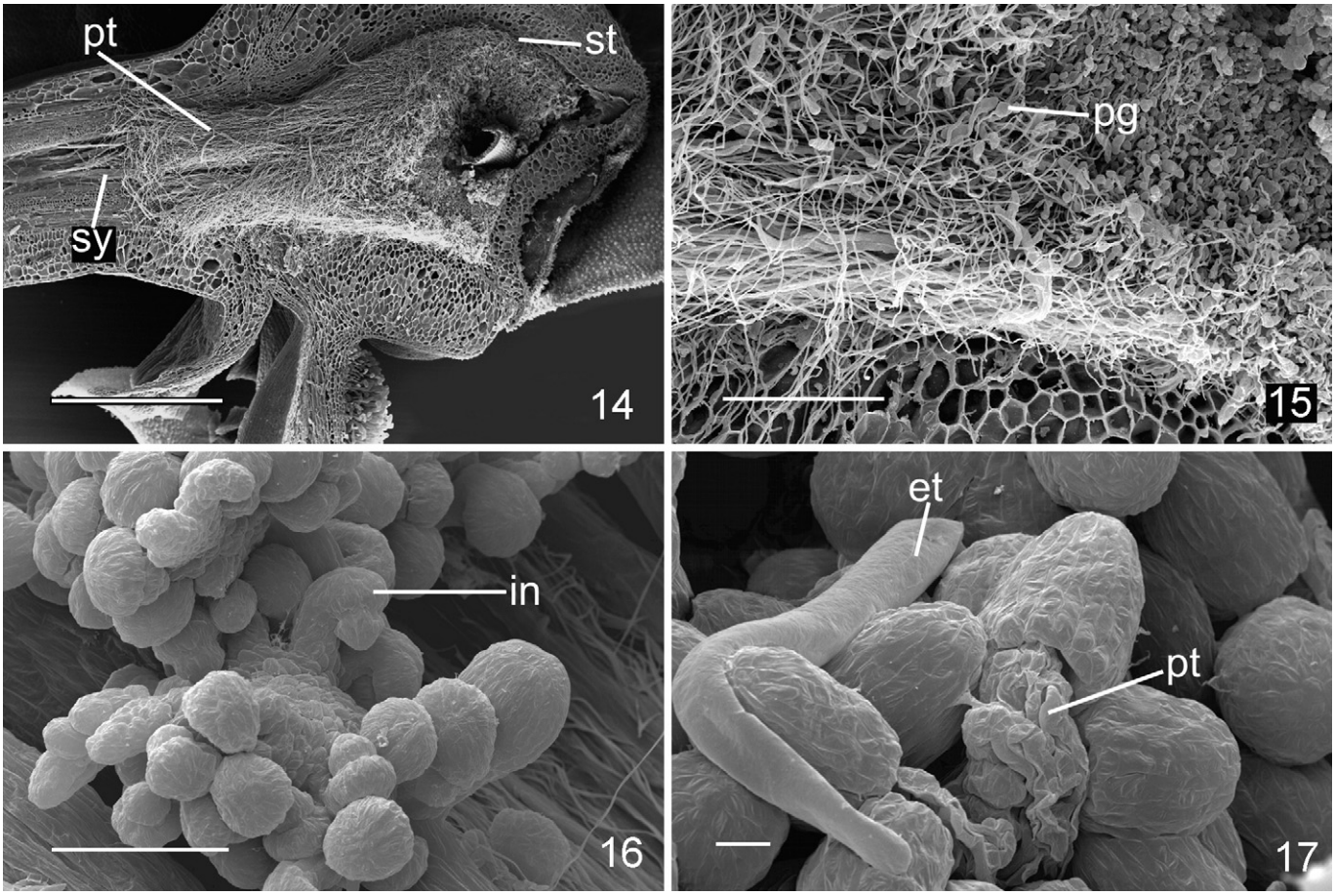
At the end of the formation of the functional megaspore, the inner integument elongates and its margins begin to cover the nucellar epidermis (Fig. 24). The chalazal megaspore divides by mitosis, forming the binucleate embryo sac. The migration of the two nuclei to the opposite poles of the cell occurs because of a large central vacuole (Fig. 24). These nuclei simultaneously undergo the second mitotic division, and the embryo sac becomes tetranuclear (Fig. 25). After the third mitotic division, the mature embryo sac presents eight nuclei (Fig. 26). It was not possible to observe the division of the cell wall, even when using a phase contrast microscope. The megagametophyte that is formed is monosporic, similar to the *Polygonum* type.

Fertilization occurs 50–65 d after pollination, and the outer integument elongates and undergoes no internal change. The first mitotic division of the zygote is asymmetric, generating a smaller apical cell and a larger basal cell (Fig. 27). The apical cell will form the embryo, and the basal cell will form the suspensor at the micropylar pole (Fig. 28). The apical cell of the embryo is transversally divided and remains in this stage until the full elongation of the outer integument of the ovule. The polar nuclei fuse to the nucleus of the male gamete (Fig. 27), forming the endosperm, which is not divided.

The outer integument forms the seed coat (testa) because the inner integument degenerates (Figs. 27–31). In the outer integument, the cells of the first layer have walls thickened and lignified; the cells of the second layer have thin walls (Fig. 30). At this stage, the embryo apical cell divides longitudinally (Fig. 29). The basal cell divides transversely, forming additional suspensor cells.

The cells of the suspensor in *O. flexuosum* are tubular and highly vacuolated, with thin cell walls and an absence of impregnated lipophilic substances (Fig. 33). These cells occupy the whole internal space, involving completely the embryo and remaining in close contact with the inner layer of the testa (Figs. 29 and 30). With the development of the embryo, the suspensor degenerates and the cells of the inner layer of the outer integument collapse (Fig. 31).

The embryo of the mature seed does not present differentiation of either the meristems or the cotyledons; however, it cannot be



Figs. 14–17. Development of pollen tube and ovule of *Oncidium flexuosum* Sims. (14 and 15) Fruit five days after pollination. (14) Overview of the region of the stigma and style. (15) Pollen grains germinating. (16 and 17) Fruit, 60 d after pollination. (16) Detail of the formation of the integuments of the ovule. (17) Details of the ovules and pollen tubes. Endocarp trichomes (et), integuments (in), pollen grain (pg), pollen tube (pt), stigma (st) and style (sy). Scale bars = 20 μm (17), 100 μm (16), 200 μm (15), and 1000 μm (14).

considered as a mass of undifferentiated cells. There is a clear difference between the cells of the chalazal pole (apical) and those of the micropylar pole (basal): Fig. 31. Throughout development, the embryo contains protein substances as reserves (Fig. 32). A lipophilic substance was identified that covered only the protoderm of the embryo from the beginning of development until the formation of the embryo in the mature seed (Fig. 33). Starch grains were not observed in the embryo either during development or in the mature seed.

Discussion

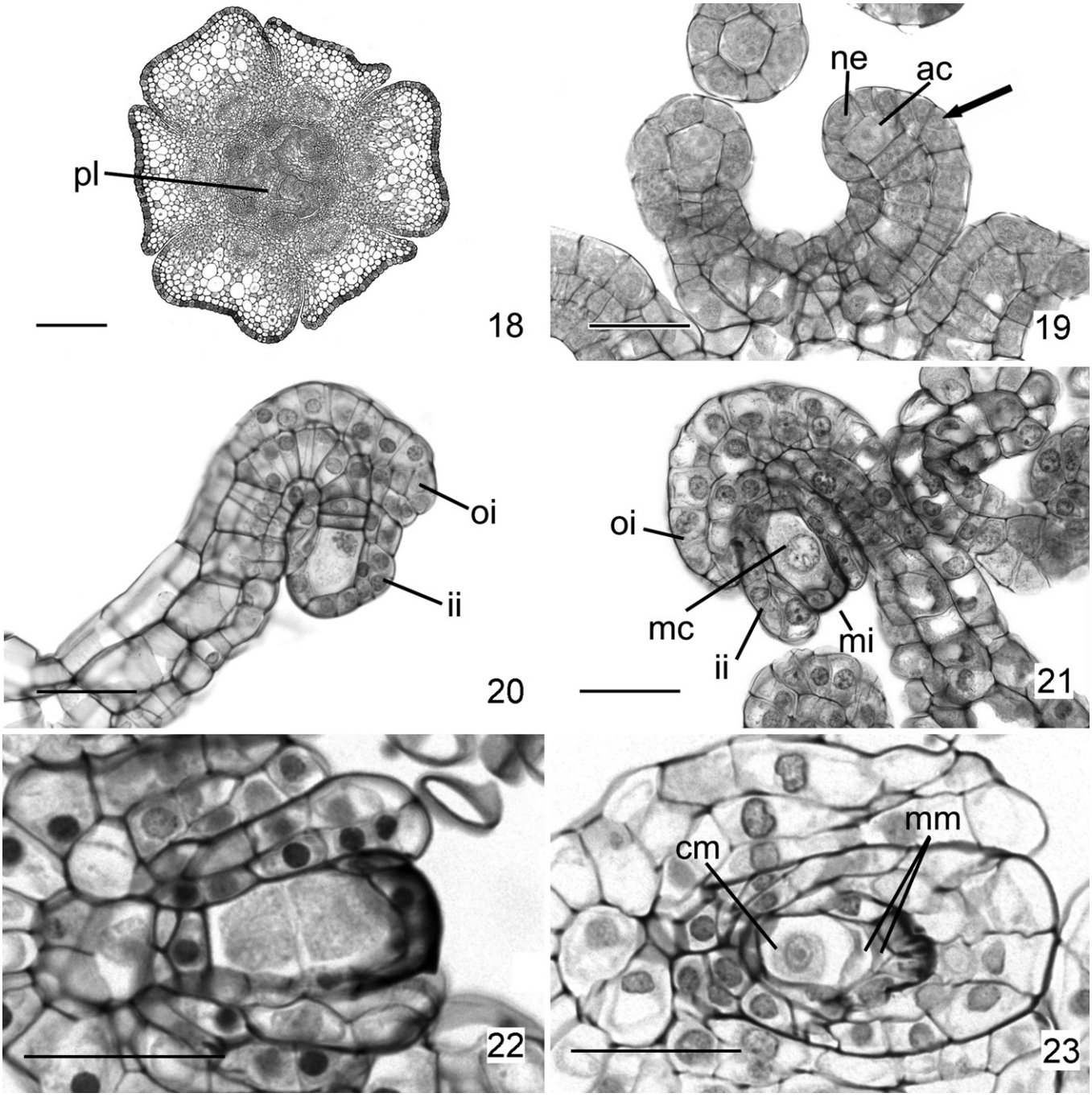
The ovary of *O. flexuosum*, similar to most orchids, is composed of six valves: three are fertile and three are sterile. According to Rasmussen and Johansen (2006), the six lobes in the ovary of the orchid originate from the bases of the sepals and petals. The sterile valves correspond to the bases of the sepals, and the fertile valves correspond to petal base and two carpel-halves. By applying the model proposed by Rasmussen and Johansen (2006) for the structure of the ovary of *O. flexuosum*, we find that the region formed by the four to six inner cell layers that line the locule would correspond to the carpels. Therefore, in this species, the fusion of the ovary wall with the hypanthium is extreme, resulting in carpels reduced to a few cell layers. Still, according to the model suggested by the authors, the two projections formed in each fertile valve after pollination would correspond to an edge of each carpel, and the bundle opposite to the dorsal sterile valve would be absent.

Few studies address the structure and development of the fruits of Orchidaceae, despite the high number of species occurring in the family. Among these works are the studies by Rao and Rao (1984), Sood and Rao (1986, 1988), Rao and Sood (1987) and Sood (1989, 1992).

The increase in the fruit diameter results mainly from the increased volume of mesocarp cells and not from the number of cell layers. In other species, such as *Crepidium saprophytum* (King & Plantl.) A.N. Rao [syn. *Malaxis saprophyta* (King & Plantl.) Tang & F.T. Warg in Sood (1992)], *Liparis paradoxa* (Lindl.) Rchb.f., and *L. rostrata* Rchb.f. (Sood, 1989), an increase in the number of pericarp layers in both the fertile and sterile valves was observed.

In the mature fruit of *O. flexuosum*, the exocarp and mesocarp are parenchymatous. Only a few cells near the vascular bundles in the sterile valves and the single layer of the endocarp and its trichomes are lignified; this differs from other species of Orchidaceae in which all or several layers of the pericarp become sclerenchymatous (Rao and Sood, 1987; Sood, 1989, 1992; Sood and Rao, 1986, 1988).

There are no literature references on details of the anatomical features of cells located in the region of dehiscence of the Orchidaceae fruit. The dehiscence of the fruit of *O. flexuosum* begins with the fruit still being green colored. It is related to the formation of a dehiscence line consisting of small cells with thin walls that are located at the edge between the sterile and the fertile valve. This differs from what has been described for other species of Orchidaceae: according to some authors, the endocarp cells of the sterile valves are arranged longitudinally, and those of the

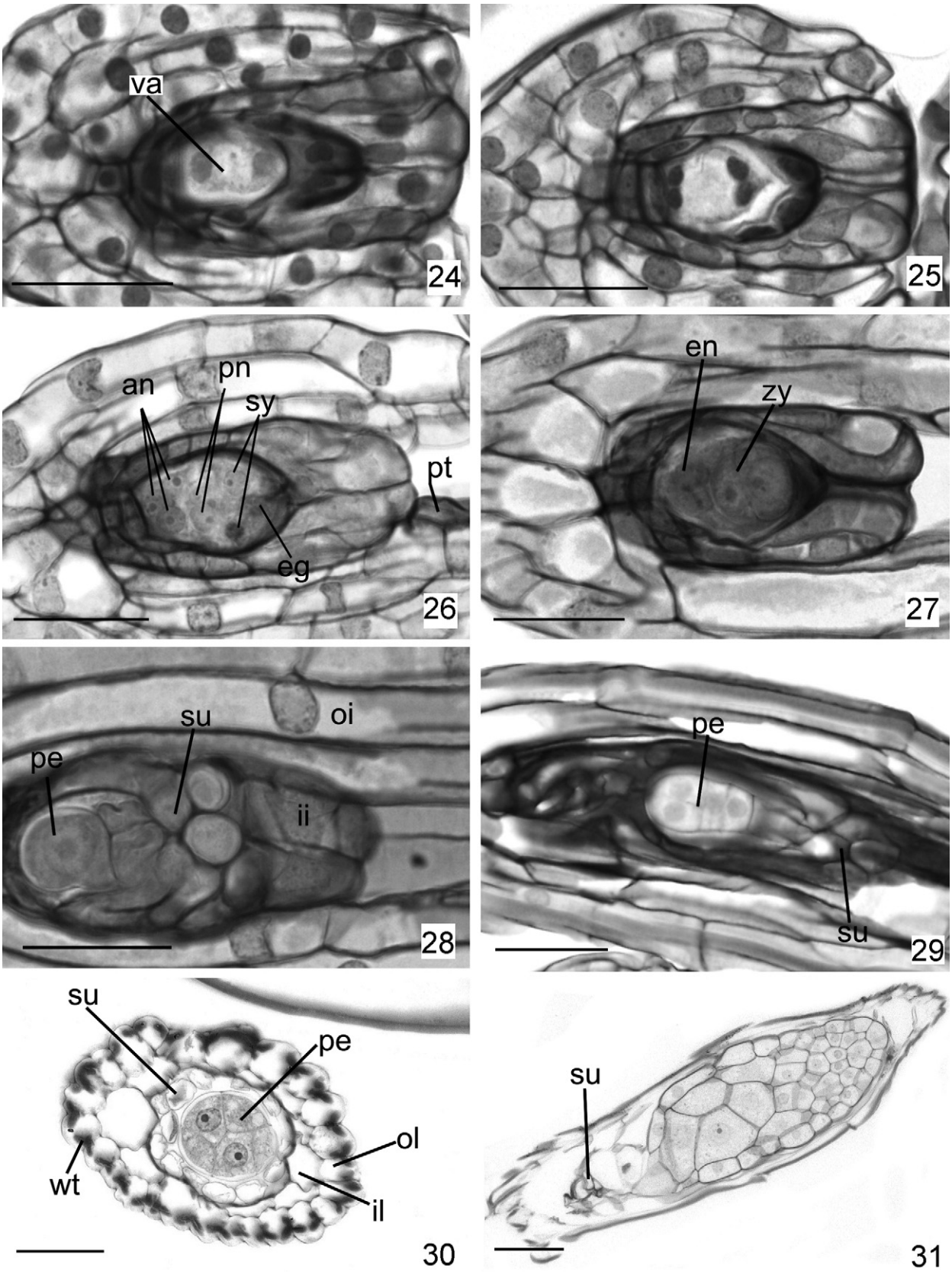


Figs. 18–23. Development of the ovule of *Oncidium flexuosum* Sims. (18) Cross-section of the fruit five days after pollination. (19) Differentiation of the initial archesporial cell and the first divisions of the dermal layer (arrow). (20) Formation of integuments. (21) Differentiation of the megaspore mother cell 50 d after pollination. (22) Dyad of megaspores. (23) Functional chalazal megaspore and the degenerate micropylar megaspores. Initial archesporial cell (ac), chalazal megaspore (cm), inner integument (ii), megaspore mother cell (mc), micropyle (mi), micropylar megaspores (mm), nucellar epidermis (ne), outer integument (oi) and placenta (pl). Scale bars = 25 μm (19–23) and 200 μm (18).

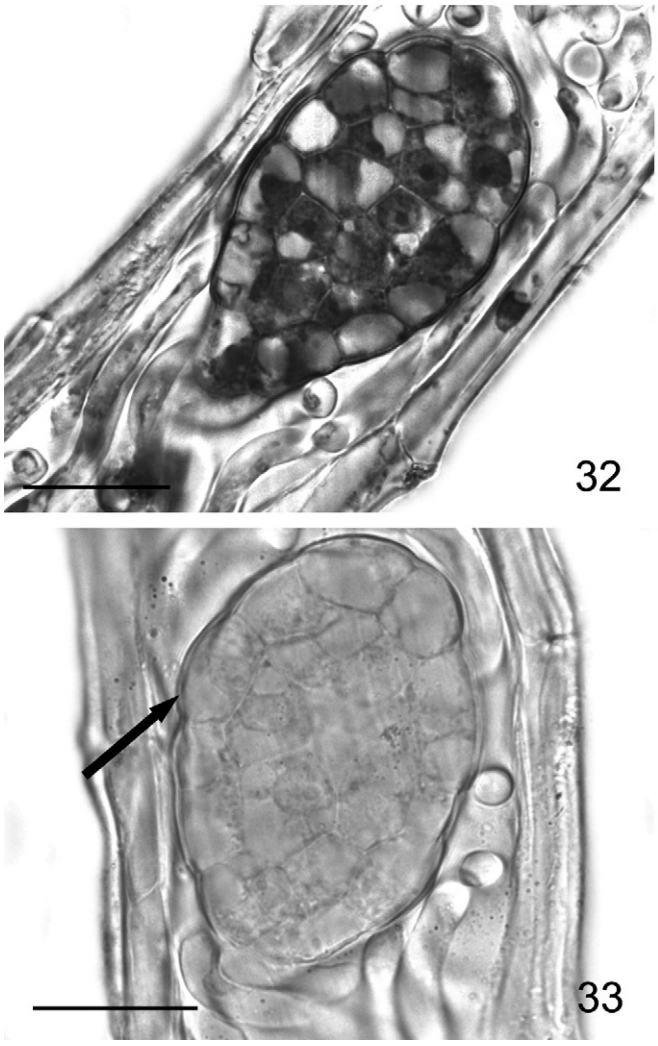
fertile valves are arranged transversely; when the capsule dehydrates, the fertile and sterile valves contract in opposite directions, resulting in a longitudinal rupture (Rao and Rao, 1984; Rao and Sood, 1987; Sood and Rao, 1986, 1988). Moreover, the absence of sclerification of the cells near the dehiscence line observed in *O. flexuosum* differs from the description for other orchids, in which a broad sclerenchyma tissue participates in the process of dehiscence, as is found in the dehiscent fruits from other families also (Fahn and Zohary, 1955; Liljegren et al., 2004; Meakin and Roberts, 1990).

Considering the model proposed by Rasmussen and Johansen (2006), mentioned above, the line of dehiscence of the fruits of *O. flexuosum* would be equivalent to the margins of the sepals forming the sterile valves. The opening of the fruit would occur along the dorsal line.

Mature long trichomes with thick cell walls are formed in the endocarp, in the region of the fertile valve opposite the sterile valve, during the development of the fruit of *O. flexuosum*. According to Cribb (1999), epiphytic orchids often have trichomes, also termed elaters, within the capsule that are elongated and hygro-



Figs. 24–31. Development of ovule and seed of *Oncidium flexuosum* Sims. (24) Two-nucleate embryonic sac. (25) Four-nucleate embryonic sac. (26) Eight-nucleate embryonic sac. (27) Zygote with two cells. (28) Embryo with multiple cells. (29) Embryo proper with four cells. (30) Cross section showing the thickening of the testa cell wall. (31) Mature seed. Antipodes (an), egg cell (e.g.), endosperm (en), inner integument (ii), inner layer of the testa (il), outer integument (oi), outer layer of the testa (ol), embryo proper (pe), polar nucleus (pn), pollen tube (pt), suspensor (su), synergids (sy), vacuole (va), wall thickening (wt) and zygote (zy). Scale bars = 20 μm (29), 25 μm (24–28, 30) and 50 μm (31).



Figs. 32–33. Histochemical tests in zygotic embryos of *Oncidium flexuosum* Sims. (32) Presence of proteins revealed by reaction with Aniline blue black. (33) Reaction with Sudan IV, observe the cuticle (arrow). Scale bars = 50 μm (32) and 25 μm (33).

scopic. It is believed that the movement of these trichomes helps in the release of the seeds. In *Cymbidium bicolor* Lindl., during the last stages of development, the trichome cell wall becomes thicker and the nucleus and cytoplasm degenerate (Swamy, 1949b). In this study we did not observe any such degeneration, only parietal thickening.

The ovule development of *O. flexuosum* follows the pattern of Orchidaceae forming an anatropous, bitegmic and tenuinucellate ovule (Johri et al., 1992). The smallest ovule of Orchidaceae does have not even a trace of any provascular tissue in the raphe (Johri, 1984).

Observing the mature embryo sac of *O. flexuosum* is difficult because of its reduced size and the fact that the nuclei are present in different planes. The only description found for the genus was given by Afzelius (1916) for *O. praetextum* Rchb.f. This author described the embryonic sac as being composed of six nuclei, which differs from the eight nuclei found in *O. flexuosum*. Studies involving various genera of the family showed both embryonic sacs containing eight nuclei (Sood and Rao, 1986; Sood and Sham, 1987; Sood, 1986) and embryonic sacs with smaller numbers of nuclei (Law and Yeung, 1989; Maheshwari, 1950; Poddubnaya-Arnoldi, 1960, 1967; Sood and Rao, 1988; Swamy, 1945). Swamy (1949a,b) and Law and Yeung (1989) suggest that there is a tendency for the reduction of the number of embryonic sac nuclei in Orchidaceae, with several tribes containing six, five or only four nuclei. However, Fredrikson (1990, 1991, 1992) affirms that the most common condition for the family is the presence of eight nuclei in the embryonic sac. This author confirmed that in species of the genus *Epipactis*, there were eight nuclei in the embryonic sac and not six, as had been described in previous works.

The embryonic development of *O. flexuosum* occurs without the development of an endosperm and with formation of a long and elaborate suspensor. This form of embryonic development is similar to that called by Clements (1999) the '*Cymbidium* type', which has been described in the genera *Bletilla*, *Cymbidium*, *Dipodium*, *Eulophia*, *Geodorum*, *Grammatophyllum*, *Oeceoclades* and *Stanhopea*. The structure of the suspensor may be characteristic for each particular genus (Nikitcheva, 2006), and the embryonic development pattern is important for phylogenetic studies of the family (Clements, 1999).

The cells of the suspensor in *O. flexuosum* are similar to those observed in the species *Cymbidium sinense* (Yeung et al., 1996) and *Phalaenopsis amabilis* var. *formosa* (Lee et al., 2008). In both *O. flexuosum* and *C. sinense* (Yeung et al., 1996), these cells occupy the entire inner space and remain in intimate contact with the inner layer of the testa. The close contact between the suspensor cells and the inner surface of the integument indicates the possibility of apoplastic transport (Yeung et al., 1996).

The main reserve substance in Orchidaceae embryos appears to vary according to the species and the stage of development. In *O. flexuosum*, the reserve substance is composed of protein throughout embryonic development, and the accumulation of starch grains was not observed at any stage. Protein bodies and starch grains were deposited in the embryo of *Phalaenopsis amabilis* (L.) Blume early in development. As it approaches maturity, the starch grains disappear and lipids accumulate in the cytoplasm (Lee et al., 2008). Embryos from mature seeds of *Guarianthe aurantiaca* (Bateman ex Lindl.) Dressler & W.E. Higgins (=*Cattleya aurantiaca* (Bateman ex Lindl.) P.N. Don) presented both protein bodies and lipids as reserve material (Harrison, 1977).

The presence of a cuticle on the embryo of *O. flexuosum*, as observed in *Cymbidium sinense* (Yeung et al., 1996) and *Paphipedilum delenatii* (Lee et al., 2006), may be related to the protection of the embryo against anticipated desiccation, as the seed has a thin integument and lacks an endosperm.

Embryos from mature seeds of *O. flexuosum* exhibit the protoderm externally, and internally they exhibit a gradient from small cells in the apical pole to larger cells in the basal pole. This structural difference between the apical and basal poles in the embryo may be related to the ease of germination (Lee et al., 2008). Species with a low germination capacity have embryos with similar-sized cells in the poles (Yeung and Law, 1992).

In agreement with the findings of other authors for epiphytic species, pollination is required for the differentiation of the ovules in *O. flexuosum*, and fertilization occurs only 50–65 d after that event. The embryonic development of *O. flexuosum,* with the degeneration of the endosperm and the formation of an elaborate suspensor, is similar to that of other species derived from the Epidendroideae (Clements, 1999). The present work illustrates some characters that are not yet described for the family, such as the line of dehiscence and the increase in the cell volume during the development of the fruit. It also describes some characteristics that differ from the descriptions currently available in the literature, such as the number of cells in the embryonic sac, the development of the suspensor and the parietal thickening of the pericarp cells.

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References

Afzelius, K., 1916. Zur Embryosackentwicklung der Orchideen. Sv. Bot. Tidskr. 10, 183–227.
Arditti, J., 1992. Fundamentals of Orchid Biology. John Wiley & Sons, New York.
Arditti, J., Ghani, A.K.A., 2000. Numerical and physical properties of orchid seeds and their biological implications. New Phytol. 145, 367–421.
Arber, A., 1925. Monocotyledons: A Morphological Study. Cambridge University Press, London.
Berlyn, G.P., Miksche, J.P., 1976. Botanical Microtechnique and Cytochemistry. The Iowa State Press, Ames.
Brown, R., 1831. Observations on the Organs and Mode of Fecundation in Orchideae and Asclepiadeae. Richard Taylor, London.
Chase, M.W., 1986. A reappraisal of the oncidioid orchids. Syst. Bot. 11, 477–491.
Chase, M.W., 1987. Systematic implications of pollinarium morphology in *Oncidium* Sw., *Odontoglossum* Kunth, and allied genera (Orchidaceae). Lindleyana 2, 8–28.
Chase, M.W., Palmer, J.D., 1992. Floral morphology and chromosome number in subtribe Oncidiinae (Orchidaceae): evolutionary insights from a phylogenetic analysis of chloroplast DNA restriction site variation. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants. Chapman and Hall, New York, pp. 324–339.
Chase, M.W., Williams, N.H., Faria, A.D., Neubig, K.M., Amaral, M. do C., Whitten, W.M., 2009. Floral convergence in Oncidiinae (Cymbidieae; Orchidaceae): an expanded concept of *Gomesa* and a new genus *Nohawilliamsia*. Ann. Bot. 104, 387–402.
Clements, M.A., 1999. Embryology. In: Pridgeon, A.M., Cribb, P.J., Chase, M.W., Rasmussen, F.N. (Eds.), Genera Orchidacearum: vol. 1: General Introduction, Apostasioideae, Cypripedioideae. Oxford University Press, Oxford, pp. 38–58.
Cribb, P.J., 1999. Morphology. In: Pridgeon, A.M., Cribb, P.J., Chase, M.W., Rasmussen, F.N. (Eds.), Genera Orchidacearum: vol. 1: General Introduction, Apostasioideae, Cypripedioideae. Oxford University Press, Oxford, pp. 13–23.
Dressler, L.R., 1993. Phylogeny and Classification of the Orchid Family. Cambridge University Press, Cambridge.
Duncan, R.E., Curtis, J.T., 1942. Intermittent growth of fruits of *Phalaenopsis* A correlation of the growth phases of an orchid fruit with internal development. Bull. Torrey Bot. Club 69, 167–183.
Duncan, R.E., Curtis, J.T., 1943. Growth of fruits in *Cattleya* and allied genera in the Orchidaceae. Bull. Torrey Bot. Club 70, 104–119.
Fahn, A., Zohary, M., 1955. On the pericarpial structure of legumen, its evolution and relation to dehiscence. Phytomorphology 5, 99–111.
Félix, L.P., Guerra, M., 2000. Cytogenetics and cytotaxonomy of some Brazilian species of Cymbidioid orchids. Genet. Mol. Biol. 23, 957–978.
Fisher, D.B., 1968. Protein staining of ribboned epon sections for light microscopy. Histochemie 16, 92–96.
Fredrikson, M., 1990. An embryological study of *Herminium monorchis* (Orchidaceae) using confocal scanning laser microscopy. Am. J. Bot. 77, 123–127.
Fredrikson, M., 1991. An embryological study of *Platanthera bifolia* (Orchidaceae). Plant Syst. Evol. 174, 213–220.
Fredrikson, M., 1992. The development of the female gametophyte of *Epipactis* (Orchidaceae) and its inference for reproductive ecology. Am. J. Bot. 79, 63–68.
Harrison, C.R., 1977. Ultrastructural and histochemical changes during the germination of *Cattleya aurantiaca* (Orchidaceae). Bot. Gaz. 138, 41–45.
Hildebrand, F., 1863. Die Fruchtbildung der *Orchideen*, ein Beweis für die doppelte Wirkung des Pollens. Bot. Zeitung 21 (329-333), 337–345.
Horridge, G.A., Tamm, S.L., 1969. Critical point drying for scanning electron microscopic study of ciliary motion. Science 163, 817–818.
Johri, B.M., 1984. Embryology of Angiosperms. Springer, Berlin, Heidelberg.
Johri, B.M., Ambegaokar, K.B., Srivastava, P.S., 1992. Comparative Embryology of Angiosperms, vol. 2. Springer, Berlin, Heidelberg.
Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27, 137–138.
Law, S.K., Yeung, E., 1989. Embryology of *Calypso bulbosa*. I. Ovule development. Am. J. Bot. 76, 1668–1674.
Lee, Y.-I., Yeung, E.C., Lee, N., Chung, M.-C., 2006. Embryo development in the Lady's Slipper Orchid, *Paphiopedilum delenatii*, with emphasis on the ultrastructure of the suspensor. Ann. Bot. 98, 1311–1319.
Lee, Y.-I., Yeung, E.C., Lee, N., Chung, M.-C., 2008. Embryology of *Phalaenopsis amabilis* var. *formosa*: embryo development. Bot. Stud. 49, 139–146.
Liljegren, S.J., et al., 2004. Control of fruit patterning in Arabidopsis by INDEHISCENT. Cell 116, 843–853.
Lindley, J., 1830–1840. The Genera and Species of Orchidaceous Plants. Ridgways, Piccadilly, London.
Lindley, J., 1847. The Vegetative Kingdom, 2nd ed. Bradbury and Evans, London.
Maheshwari, P., 1950. An Introduction to the Embryology of Angiosperms. McGraw-Hill, New York.
Meakin, P.J., Roberts, J.A., 1990. Dehiscence of fruit in oilseed rape. 1. Anatomy of pod dehiscence. J. Exp. Bot. 41, 995–1002.
Nikitcheva, Z.I., 2006. Suspensor. In: Batygina, T.B. (Ed.), Seed. Embryology of Flowering Plants, vol. 2. Science Publishers, New Hampshire, pp. 198–202.
Pabst, G.F.J., Dungs, F., 1977. Orchidaceae Brasilienses, vol. 2. Brücke-Verlag Kurt Schmersow, Hildesheim.
Pearse, A.G.E., 1985. Histochemistry, Theoretical and Applied, vol. II., 4th ed. Churchill Livingston, Edinburgh.
Poddubnaya-Arnoldi, V.A., 1960. Study of fertilization in the living material of some angiosperms. Phytomorphology 10, 185–198.
Poddubnaya-Arnoldi, V.A., 1967. Comparative embryology of the Orchidaceae. Phytomorphology 17, 312–320.
Rao, P.R.M., Rao, K.M., 1984. Embryology of *Habenaria pectinata*. Phytomorphology 34, 237–242.
Rao, P.R.M., Sood, S.K., 1987. Embryology of *Oreorchis foliosa* (Orchidaceae). Phytomorphology 37, 1–8.
Rasmussen, F.N., Johansen, B., 2006. Carpology of orchids. Selbyana 27, 44–53.
Sakai, W.S., 1973. Simple method for differential staining of paraffin embedded plant material using toluidine blue O. Stain Technol. 48, 247–249.
Saunders, E.R., 1923. A reversionary character in the Stock (*Matthiola Incana*) and its significance in regard to the structure and evolution of the gynoecium in the Rhoeadales, the Orchidaceae, and other families. Ann. Bot. 37, 451–482.
Sood, S.K., 1986. Gametogenesis, integuments initiation and embryogeny in three species of *Habenaria* (Orchidaceae, Orchideae). Proc. Indian Natl. Sci. Acad. 96, 487–494.
Sood, S.K., 1989. Embryology and systematic position of *Liparis* (Orchidaceae). Plant Syst. Evol. 166, 1–9.
Sood, S.K., 1992. Embryology of *Malaxis saprophyta*, with comments on the systematic position of *Malaxis* (Orchidaceae). Plant Syst. Evol. 179, 95–105.
Sood, S.K., Rao, P.R.M., 1986. Gametophytes, embryogeny and pericarp of *Microstylis wallichii* Lindl. (Orchidaceae). Bot. Mag. Tokyo 99, 351–359.
Sood, S.K., Rao, P.R.M., 1988. Studies in the embryology of the diandrous orchid *Cypripedium cordigerum* (*Cypripedieae*, Orchidaceae). Plant Syst. Evol. 160, 159–168.
Sood, S.K., Sham, N., 1987. Gametophytes, embryogeny and pericarp of *Rhynchostylis retusa* Blume (*Epidendreae*, Orchidaceae). Phytomorphology 37, 307–316.
Swamy, B.G.L., 1945. Embryo sac and fertilization in *Cypripedium spectabile*. Bot. Gaz. 107, 291–295.
Swamy, B.G.L., 1949a. Embryological studies in the Orchidaceae. I. Gametophytes. Am. Midl. Nat. 41, 184–201.
Swamy, B.G.L., 1949b. Embryological studies in the Orchidaceae. II. Embryogeny. Am. Midl. Nat. 41, 202–232.
Veyret, Y., 1974. Development of the embryo and young seedling stages of orchids. In: Withner, C.L. (Ed.), The Orchids: Scientific Studies. John Wiley & Sons, New York, pp. 223–265.
Withner, C.L., 1974. The Orchids: Scientific Studies. John Wiley & Sons, New York.
Yeung, E.C., Law, S.K., 1992. Embryology of *Calypso bulbosa*. II. Embryo development. Can. J. Bot. 70, 461–468.
Yeung, E.C., Zee, S.Y., Ye, X.L., 1996. Embryology of *Cymbidium sinense*: embryo development. Ann. Bot. 78, 105–110.