



# Megasporogenesis, megagametogenesis, and embryogenesis in *Maxillaria crassifolia* (Lindl.) Rchb.f. (Cymbidieae, Orchidaceae)

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## Abstract

*Maxillaria crassifolia* (Lindl.) Rchb.f. belongs to the polyphyletic genus *Maxillaria* Ruiz & Pav., which currently is the subject of several taxonomic research. There are conflicting descriptions of megasporogenesis, megagametogenesis, and embryogenesis in orchids from the tribe Cymbidieae, in general, and in the genus *Maxillaria*, in particular. In the present report, all stages of embryonic development of *M. crassifolia* were examined using confocal fluorescence microscopy. Some features of the development of the ovule and embryo, which distinguish *M. crassifolia* from other species of the tribe Cymbidieae were identified. The T-shaped arrangement of megaspores is formed by dividing the micropylar megaspore of the dyad. The megagametophyte develops according to the modified Polygonum-type with an unstable number of nuclei in the embryo sacs. The nucleus of the central cell varies in composition and may include unfused micropylar and chalazal nuclei and daughter nuclei formed during their division. The sequence of embryonal divisions is strictly structured. A special variant of embryogenesis, the Cymbidium-type *Maxillaria*-variant, has been described. Its characteristic features are the strictly apical nature of embryonic divisions, the absence of basal cell (*cb*) division, the formation of one to three pairs of tubular suspensor cells, and the localization of all suspensor cells within the inner integument.

**Keywords** Orchidaceae · Embryogenesis · Suspensor · Confocal microscopy

## Introduction

The genus *Maxillaria* Ruiz & Pav. (Cymbidieae, Orchidaceae) is polyphyletic and is currently the subject of taxonomic research. The taxonomic affiliation of *M. crassifolia* (Lindl.) Rchb.f., as well as other species from the subtribe Maxillariinae, has been repeatedly revised, and the species itself has changed both generic and species names (Dressler 1981; Carnevali 1991; Senghas 2002; Christenson 2002; Chase et al. 2003; Ojeda Alaion 2003; Ojeda Alaion et al. 2003; Cabral et al. 2006; Blanco et al. 2007; Whitten et al.

2007; Szlachetko et al. 2012; Moraes et al. 2016). In particular, in several modern studies, this species appears as *Heterotaxis sessilis* (Sw.) Barros (de Barros 2002) and *H. gatunensis* (Schltr.) Szlach. & Sitko (Szlachetko et al. 2012). In this study, we have investigated the embryonic development of *Maxillaria crassifolia* to determine important diagnostic characters that can help in defining this group along with the molecular data.

Genus *Maxillaria* belongs to the tribe Cymbidieae, which consists of 10 subtribes, including the subtribes Coeliopsidiinae, Cymbidiinae, Eulophiinae, Maxillariinae, Oncidiinae, and Stanhopeinae (Chase et al. 2015). It was hypothesized that embryogenesis in all Cymbidieae species is similar and corresponds to embryogenesis of Cymbidium-type or V-type with an irregular division of both basal (*cb*) and apical (*ca*) cells, filamentous proembryo, and multicellular suspensor with several tubular cells (Swamy 1949b; Clements 1999).

Ovule growth of 16 species of orchids from the tribe Cymbidieae is described in the literature (Treub 1879; Afzelius 1916, 1918; Swamy 1942, 1943, 1946; Veyret 1965; Abe 1967; Nagashima 1982; Vij and Sharma 1986; Zee and Ye 1995; Yeung et al. 1994, 1995, 1996; Huang et al. 1998;

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Clements 1999; Mayer et al. 2011), but data are fragmentary (Online Resource). The most detailed developmental data were obtained for *Cymbidium bicolor* Lindl. (Swamy 1942, 1946) and *C. sinense* (Yeung et al. 1994; Zee and Ye 1995; Huang 1998) from the subtribe Cymbidiinae; *Gomesa flexuosa* (G.Lodd.) M.W.Chase & N.H.Williams (synonym, *Oncidium flexuosum* G.Lodd.) (Mayer et al. 2011) from the subtribe Oncidiinae; and *Eulophia epidendraea* (J.Koenig ex Retz.) C.E.C.Fisch. (Swamy 1943) and *Geodorum densiflorum* (Lam.) Schltr. (Swamy 1949b) from the subtribe Eulophiinae.

The existence of several types of megasporogenesis in the tribe Cymbidieae was described. Bisporic (Yeung et al. 1994, 1995; Zee and Ye 1995) and monosporic (Swamy 1943; Mayer et al. 2011) embryo sacs were identified in different genera. In *Eulophia dabia* (D.Don) Hochr., embryo sacs of both the Polygonum-type and Allium-type were found (Vij and Sharma 1986). The megasporogenesis of orchids from the genus *Maxillaria* has not been studied yet.

We found only three original works discussing the embryogenesis of the genus *Maxillaria* (Fleischer 1874; Veyret 1965, 1974), and their conclusions are contradictory. Veyret (1965, 1974) studied the initial stages of embryo development of 4 species from the tribe Cymbidieae—*Eulophia bouliawongo* (Rchb.f.) J.Raynal (synonym, *E. oedoplectron* Summerh.), *E. cucullata* (Afzel. ex Sw.) Steud., *Stanhopea costaricensis* Rchb.f., and *Maxillaria variabilis* Bateman ex Lindl. She found that *M. variabilis* has a different type of embryo development than the species *Stanhopea* Frost. ex Hook., *Eulophia* R.Br. ex Lindl., *Cymbidium* Sw., and *Geodorum* Jacks. At the octant stage, she revealed in *Maxillaria variabilis* almost the same organization of the 12-celled embryo as in *Corallorhiza* Gagnebin, but without elongated suspensor

cells. Based on these data, the embryogenesis of *Maxillaria variabilis* should be attributed to the Calypso-type or Cypripedioid-type according to the Clements classification (1999), and not to the Cymbidium-type.

Thus, there are conflicting descriptions of embryogenesis in orchids from the tribe Cymbidieae, in general, and in the genus *Maxillaria*, in particular. This allows us to put forward a hypothesis that there are several variants of embryogenesis of the Cymbidium-type with different genesis and specialization of the embryonic and suspensor cells.

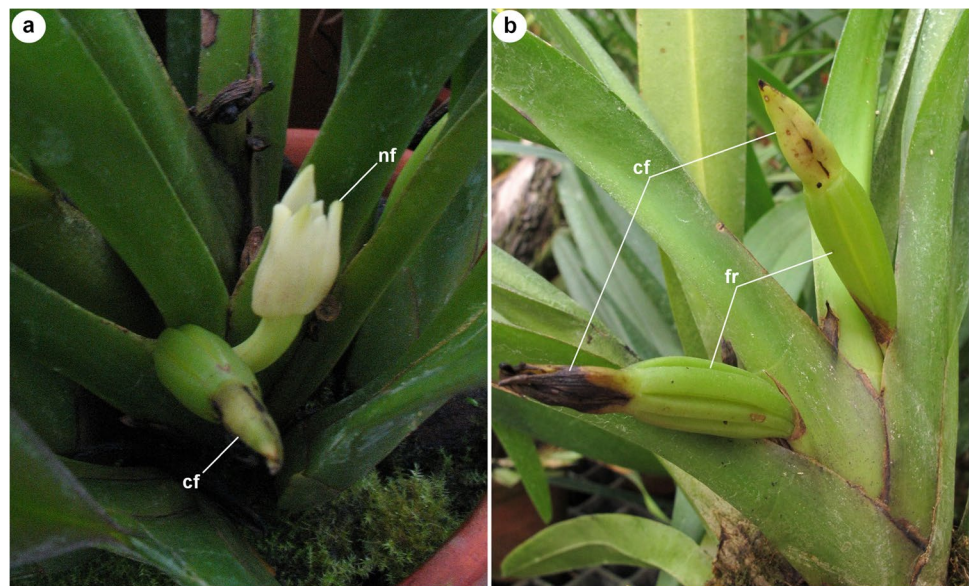
## Materials and methods

### Plant material

We used *M. crassifolia* plants from the collection of the Stock Greenhouse of the Main Botanical Garden of the Russian Academy of Sciences (Moscow, Russia). Plants were grown under conditions of warm temperature (winter night temperature of 16–18 °C) and relative humidity of 70–80% in natural light. The flowering and fruit formation occurred from autumn to the end of winter; fruits were tied spontaneously by auto-pollination (Fig. 1a, b). The studies were conducted in 2017–2020.

The fragments of immature fruits of different ages were placed in 2% paraformaldehyde in 0.05 M phosphate buffer pH 7.4 in a refrigerator at 4 °C for fixation and subsequent storage until use (at least 1 week).

**Fig. 1** *Maxillaria crassifolia*. **a** Plant with cleistogamous and normal flowers. **b** Fruits at different ripening periods. Abbreviations: *nf*, normal flower; *cf*, cleistogamous flower



## Confocal microscopy

Ovules were stained on a glass slide for 1–2 h in a humid chamber to prevent drying out. For staining, water solutions of each of the fluorescent dyes were used: 100 µg/ml berberine (BRB, Sigma-Aldrich, USA), 1–5 mg/ml Calcofluor (CF, Fluorescent Brighter 28, Sigma-Aldrich), 2.5 mg/ml Direct Yellow (DY, Sigma-Aldrich), 1 mg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), or 50 µg/ml propidium iodide (PI, Sigma-Aldrich). Dipyradamole (DPM, Sigma, USA) was also used as a 10–12 mg/ml solution in 5% acetic acid. The stained samples were washed several times with distilled water on a glass slide, placed in 50% (w/v) glycerol, covered with a cover glass, and stored at 4 °C until use (usually at least 1–2 weeks). Samples stained with dipyradamole were washed with 50% (w/v) glycerol. Autofluorescence (Auto) was studied on fixed, unstained preparations embedded in 50% (w/v) glycerol. Seeds were incubated for 1–2 h in a 3 M urea solution, placed in 50% (w/v) glycerol, and squashed by pressing a cover glass.

Unfixed ovules immediately after extraction from the fruits were stained for 0.5–1 h on a glass slide with 0.4 mg/ml 2',7'-dichlorofluorescein diacetate (DCFH, Serva, Germany), 1 mg/ml resazurin (RZ, 3H-phenoxazin-3-one, 7-hydroxy-, 10-oxide, sodium salt, Sigma-Aldrich, USA, staining in the dark) or fluorescein diacetate (FDA, 50 µg/ml in 50% glycerol, Sigma-Aldrich, USA), washed with distilled water, placed in 50% glycerol, and studied for the next 1–2 h.

The samples were viewed in a confocal laser scanning microscope (Olympus FV1000D, Japan). Lasers with a wavelength of 405 nm, 473 nm, and 560 nm were used individually or in combination according to the absorption spectra of the dyes to excite fluorescence. The stained samples were examined using 5–20% laser intensity. When studying autofluorescence, the laser intensity was increased to 50% maximum power output. The signal was registered in the blue (425–460 nm), green (485–530 nm), and red (560–660 nm) channels that corresponded to the settings of the channel parameters for DAPI (4,6-diamidino-2-phenylindole), Alexa Fluor 488, and rhodamine, respectively. The figures show confocal fluorescence images alone or merged with differential interference contrast (DIC).

## Cell numbering

We used the following abbreviations for embryo cells: *ca*, the apical cell of the first generation; *cb*, the basal cell; *ca1*, the chalazal daughter cell from the *ca* cell; *z*, the micropylar cell from the *ca* cell; *ca2*, chalazal daughter cell from the *ca1* cell; *y*, the micropylar cell from the *ca1* cell; *ca3*, the chalazal daughter cell from the *ca2* cell; *x*, the micropylar cell from the *ca2* cell; *w*, the micropylar cell from the *ca3*

cell; and *ca4*, the chalazal daughter cell from the *ca3* cell and the first cell of the embryo proper.

## Results

### Pollination

Flowers of *Maxillaria crassifolia* are predominantly cleistogamous (Fig. 1a, b). Auto-pollination occurs due to the stop of lip growth and the simultaneous growth of the column. Inside the still unopened flower, the curled edges of the immovable dying lip mechanically displace the pollinia onto the stigma, transferring them through the rostellum barrier. In closed cleistogamous flowers, obligate fruit setting and the formation of full-fledged seeds occur. In open but not pollinated flowers, the ovaries do not develop, and the primordia of the ovules on the placenta die.

Similar to other species from the tribe Cymbidieae, the placenta has three bilobed placental ridges. Before pollination, primordia of ovules on fertile valves appear as tiny tubercles. After pollination, ovule primordia elongate and branch (Fig. 2a, b).

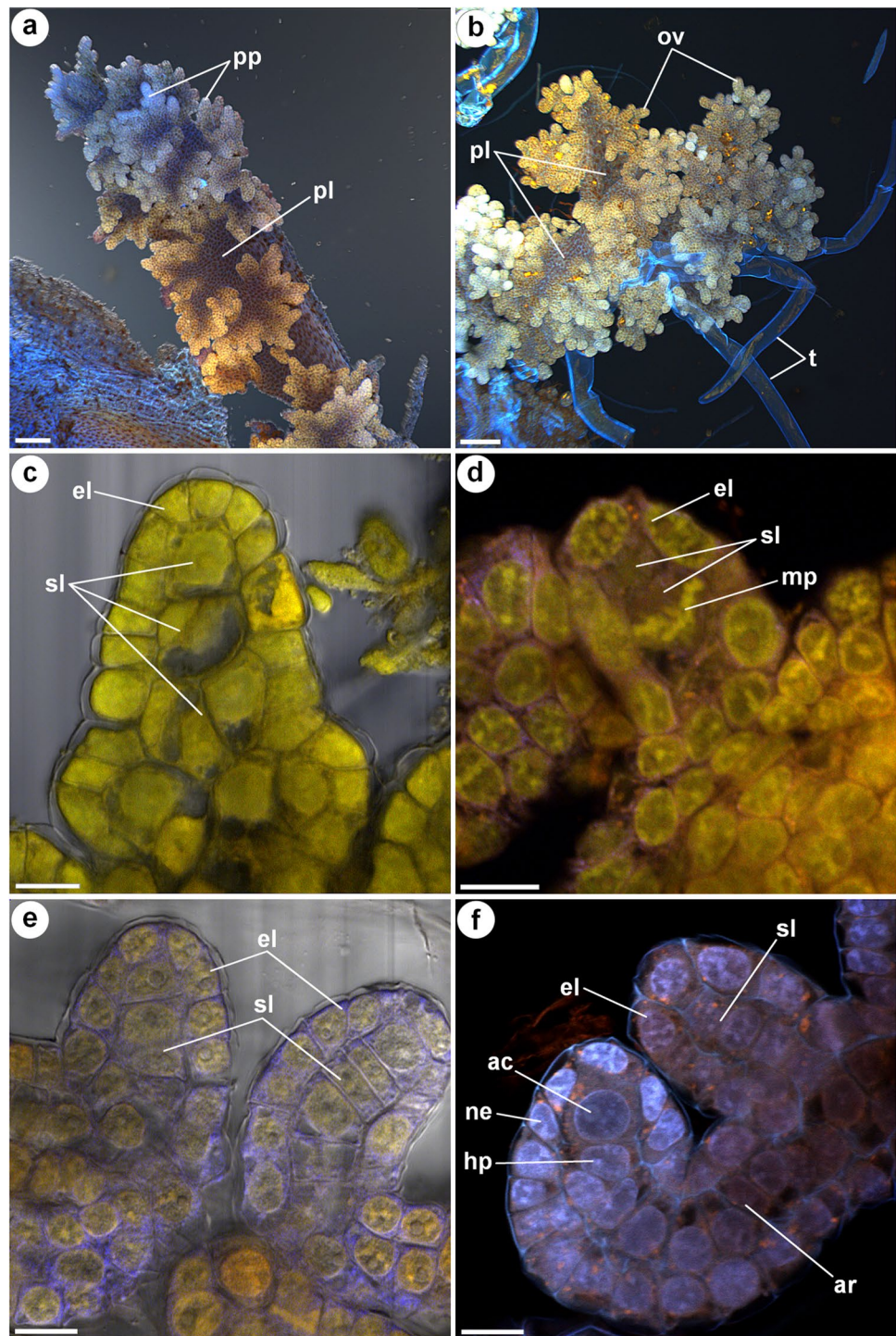
### Stage of the nucellar filament (from the formation of the ovular primordia to the differentiation of the archesporial cell)

The subepidermal cells of the ovular primordia divide transversely and tangentially (Fig. 2c). Transverse divisions form cells of the axial row (Fig. 2d). Tangential divisions give rise to new primordia. Each nucellar filament consists of an axial row of cells surrounded by a layer of epidermal cells (Fig. 2e, f).

### Development of anatroypous ovule (from the appearance of the inner integument initials to the onset of megasporogenesis)

The upper cell of the axial row differentiates into an archesporial cell and then into a megasporocyte, and five or six surrounding epidermal cells become the epidermis of the nucellus. The underlying row of epidermal cells divides periclinally and forms the initials of the inner integument (Fig. 3a). At the same time, there is a tangential division of the second cell of the axial row, which is located directly behind the archesporial cell. The division of this cell is a prerequisite for the development of the anatroypous ovule. In addition to the archesporial cell, the axial row at this stage includes one hypostase cell, one or two chalaze cells, and continues with the central cell file of the funiculus (Fig. 3b).

**Fig. 2** A nucellar filament formation. **a** Placental ridges with proliferative papillae (DAPI). **b** Ovule primordia (DAPI). **c** Periclinal divisions of subepidermal cells and anticlinal divisions of epidermal cells in ovule primordium (Brb). **d** The formation of a three-celled axial row: division of a cell located under the archesporium (Brb). **e** Four-celled axial row (Brb). **f** The second cell of a four-celled axial row of nucellar filament is divided by an oblique septum, one of the daughter cells shifts to the dermal layer (DAPI). Abbreviations: *ac*, archesporial cell; *ar*, axial row of cells; *el*, epidermal layer; *f*, funiculus; *mp*, metaphase plate; *hp*, hypostase; *ne*, nucellar epidermis; *ov*, ovule primordia; *pl*, placental ridges; *pp*, proliferative papillae; *sl*, subepidermal layer; *t*, trichome. Scale bars: **a**, **b** = 100  $\mu$ m; **c**, **f** = 10  $\mu$ m

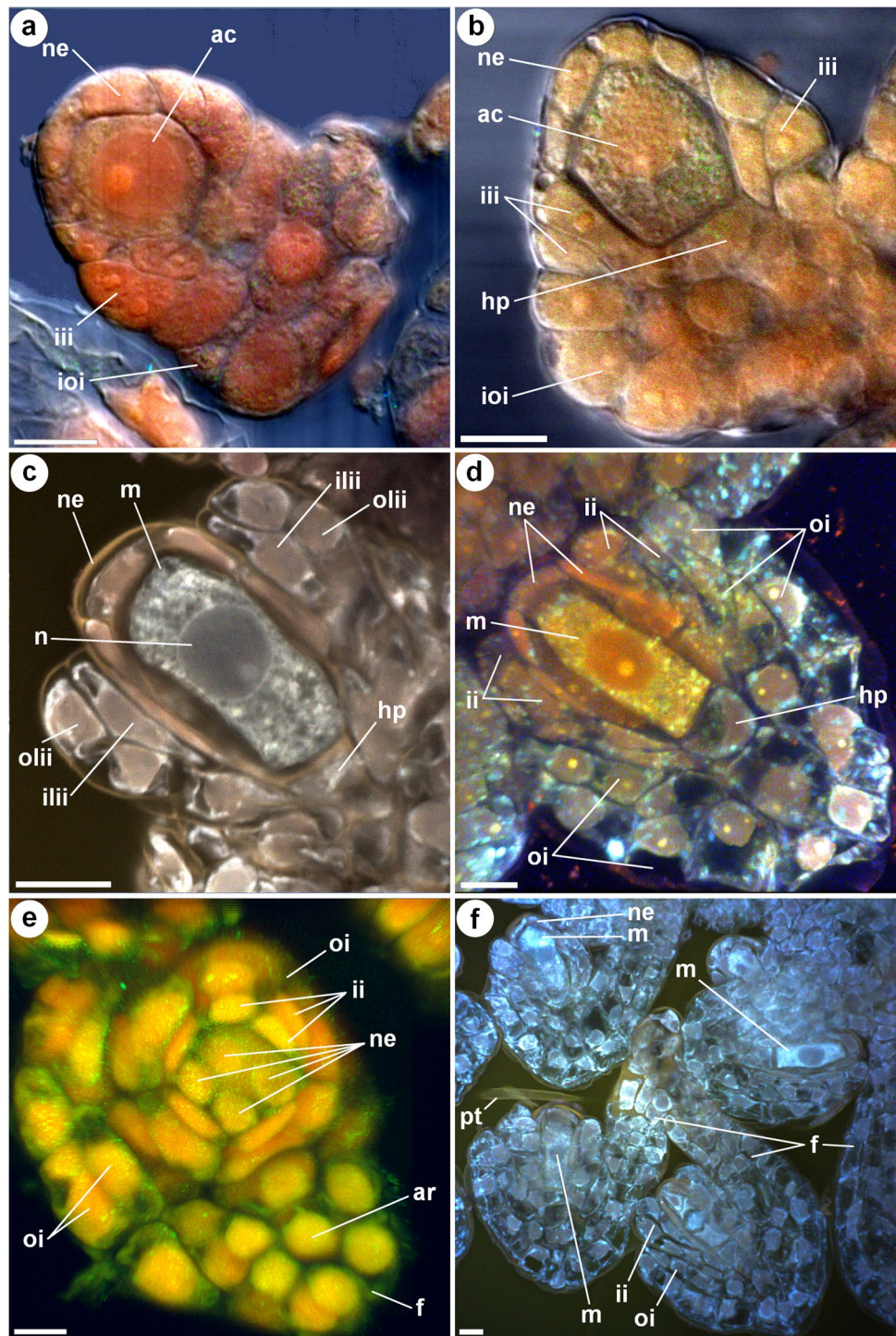


When the initials of the outer integument appear, the inner integument consists of three cells and then gradually lengthens due to several oblique cell divisions up to the middle of the archesporial cell (megasporeocyte) (Fig. 3c). At this stage, the ovule turns toward the placental side of the ovary into an anatropous position. The funiculus is well defined (Fig. 3e, f).

### Megasporogenesis

As in other orchids, the transformation of the archesporium cell into a megasporeocyte, and then into the mother cell of megaspores, occurs without the separation of parietal (integumentary) cells. Before meiosis I, the megasporeocyte greatly increases in size (Fig. 3c, d). The first division occurs before the inner integument surrounds the

**Fig. 3** The differentiation of initials of integuments and curvature of the ovule. **a** The first divisions of the initials of the inner integument (Auto). **b** The first divisions of the initials of the outer integument; initial curvature of the ovule (Auto). **c** The archesporial cell turns into a large megaspore mother cell, a two-layer inner integument is formed. When staining with DPM, the thickened cell walls of the cells of the nucellus and inner integument are visible (DPM). **d** The megaspore mother cell increased before meiosis I, the outer integument was almost equal in length to the inner integument, and the ovule became anatropous. **e** An axial section of the anatropous ovule through the apical part of the nucellus and funiculus (Auto). **f** Anatropous ovules at the stage of megasporogenesis (DPM). Abbreviations: *ac*, archesporial cell; *ar*, axial row of cells; *f*, funiculus; *hp*, hypostase; *ii*, inner integument; *iii*, initials of the inner integument; *ilii*, inner layer of inner integument; *olii*, outer layer of inner integument; *ioi*, initials of the outer integument; *m*, megaspore mother cell; *n*, nucleus; *ne*, nucellar epidermis; *oi*, outer integument; *pt*, pollen tube. Scale bars = 10  $\mu$ m

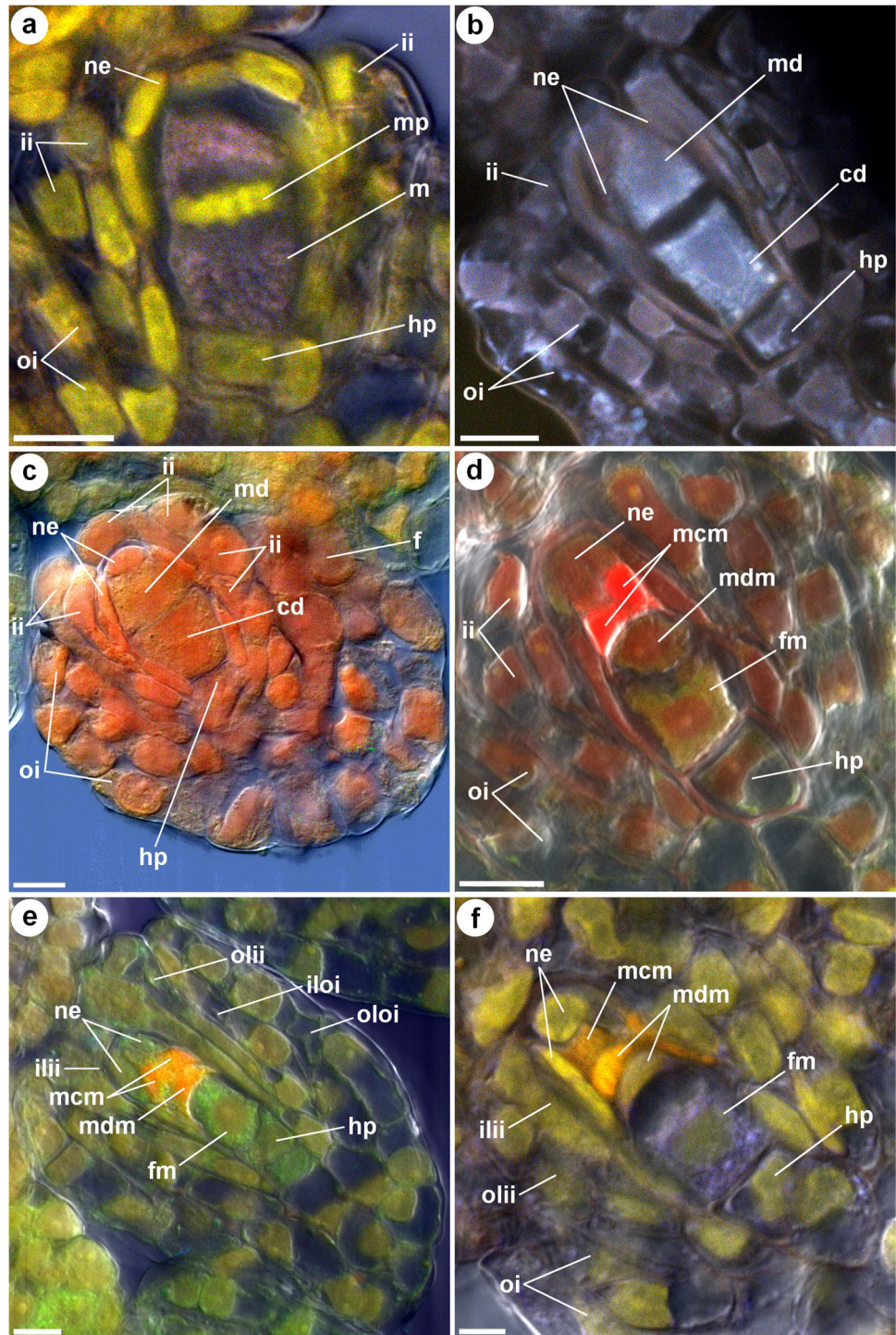


ovule. As a result of meiosis I, two cells of the dyad are formed (Fig. 4a, b).

Each of the two cells of the dyad undergoes one division (Fig. 4c) with the formation of 4 megaspores. The micropylar cell usually divides by an oblique septum and forms a T-shaped tetrad of megaspores (Fig. 4d, e). A linear tetrad of megaspores was occasionally observed (Fig. 4f). Daughter cells of the micropylar dyad die simultaneously.

The micropylar daughter cell of the chalazal dyad dies, and the chalazal cell becomes a female gametophyte. Sometimes, apoptosis also involves cells of the nucellar epidermis at the micropylar end. The fluorescence of the nuclear material of degenerating megaspores is brighter than that of the functional megaspore and increases with degradation. T-shaped dying megaspores fluoresce strongly in red (Fig. 4d), or yellow (Fig. 4e, 5a), and dying epidermis of

**Fig. 4** Megasporogenesis. **a** The first meiotic division of the megaspore mother cell (Brb). **b** Dyad stage (DAPI). **c** Division of the chalazal dyad with the formation of a functional megaspore (Auto). **d** Tetrads stage, micropylar megaspores separated by an oblique septum to form a T-shaped tetrad, dying cells fluoresce in red (PI). **e** T-shaped tetrad of megaspores, three dying cells fluoresce in yellow (Brb). **f** Linear tetrad of megaspores, ovule at the stage of completion of megasporogenesis (Brb). Abbreviations: *cd*, chalazal dyad; *fm*, functional megaspore; *hp*, hypostase; *ii*, inner integument; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *m*, megaspore mother cell; *mc*, micropyle; *mcm*, micropylar megaspore; *md*, micropylar dyad; *mdm*, middle megaspore; *mp*, metaphase plate; *ne*, nucellar epidermis; *oi*, outer integument; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument. Scale bars = 10  $\mu$ m

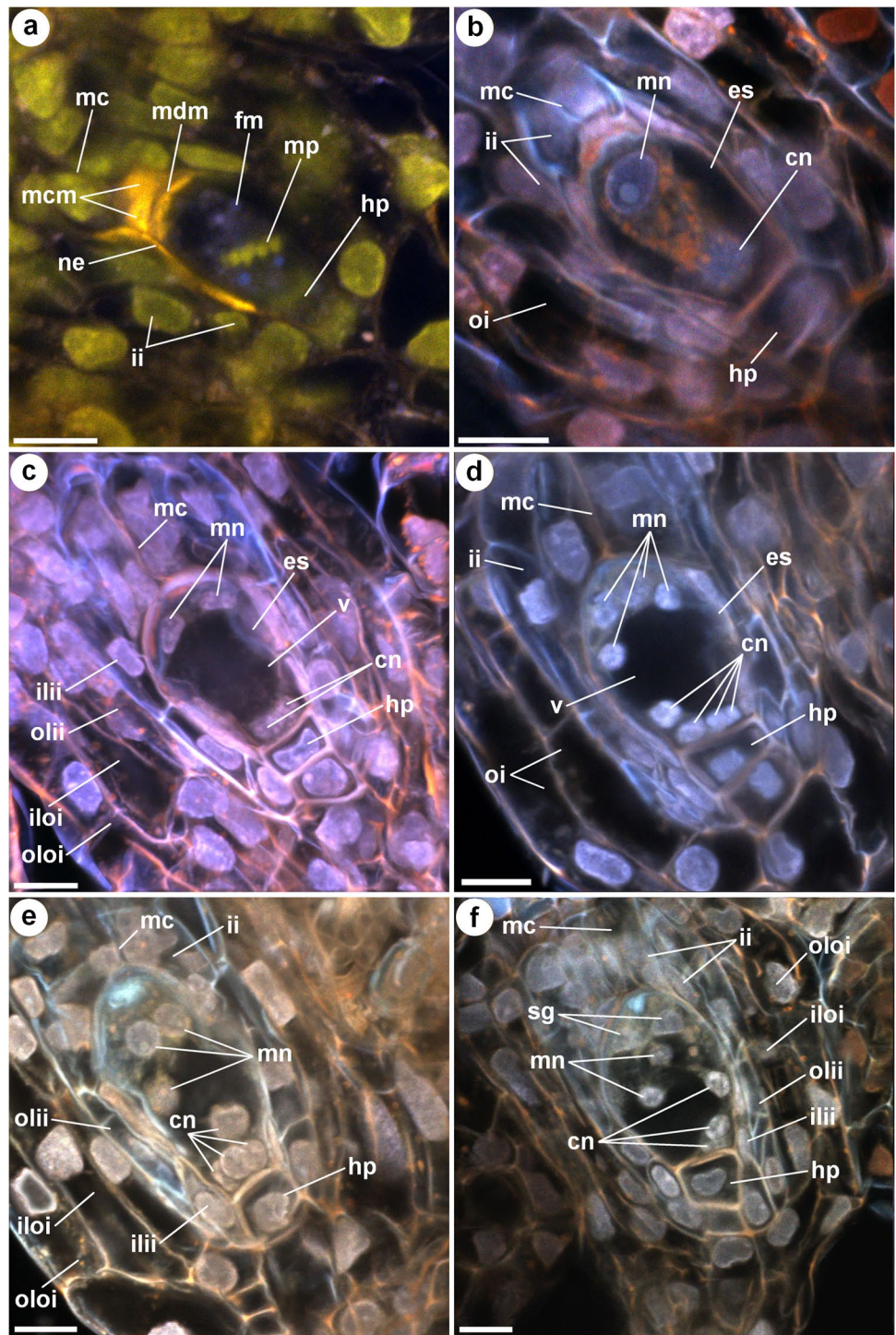


nucellus (yellow thin upper cells) fluoresces in the same way (Fig. 5a). At the stage of the binucleate embryo sac, the nuclei diverge to the poles (Fig. 5a, b). A large central vacuole then forms in the centre (Fig. 5c). Thus, the embryo sac of *M. crassifolia* is monosporic and develops from the chalazal cell of the tetrad of megaspores.

### The structure of the female gametophyte

The female gametophyte of *M. crassifolia* corresponds to the modified Polygonum-type. We found six-, seven-, or eight-nucleated embryo sacs (Figs. 5d, e, f and 6a, b). Eight nuclei were observed only in less than 1/4 of the embryo sacs.

**Fig. 5** Megagametogenesis. **a** The first mitotic division of the functional megaspore, the metaphase plate is visible (Brb). **b** Binucleated embryo sac (DAPI). **c** Four-nucleated embryo sac (DAPI). **d** Eight-nucleated embryo sac (DAPI). **e** Seven-nucleated embryo sac with three micropylar nuclei (DAPI). **f** Seven-nucleated embryo sac with three chalazal nuclei (DAPI). Abbreviations: *cn*, chalazal nucleus; *es*, embryo sac; *fm*, functional megaspore; *hp*, hypostase; *ii*, inner integument; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *mc*, micropyle; *mcm*, micropylar megaspore; *mdm*, median megaspore; *mp*, metaphase plate; *cn*, chalazal nuclei; *mn*, micropylar nuclei; *ne*, nucellar epidermis; *oi*, outer integument; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument; *sg*, synergids; *v*, vacuole. Scale bars = 10 μm



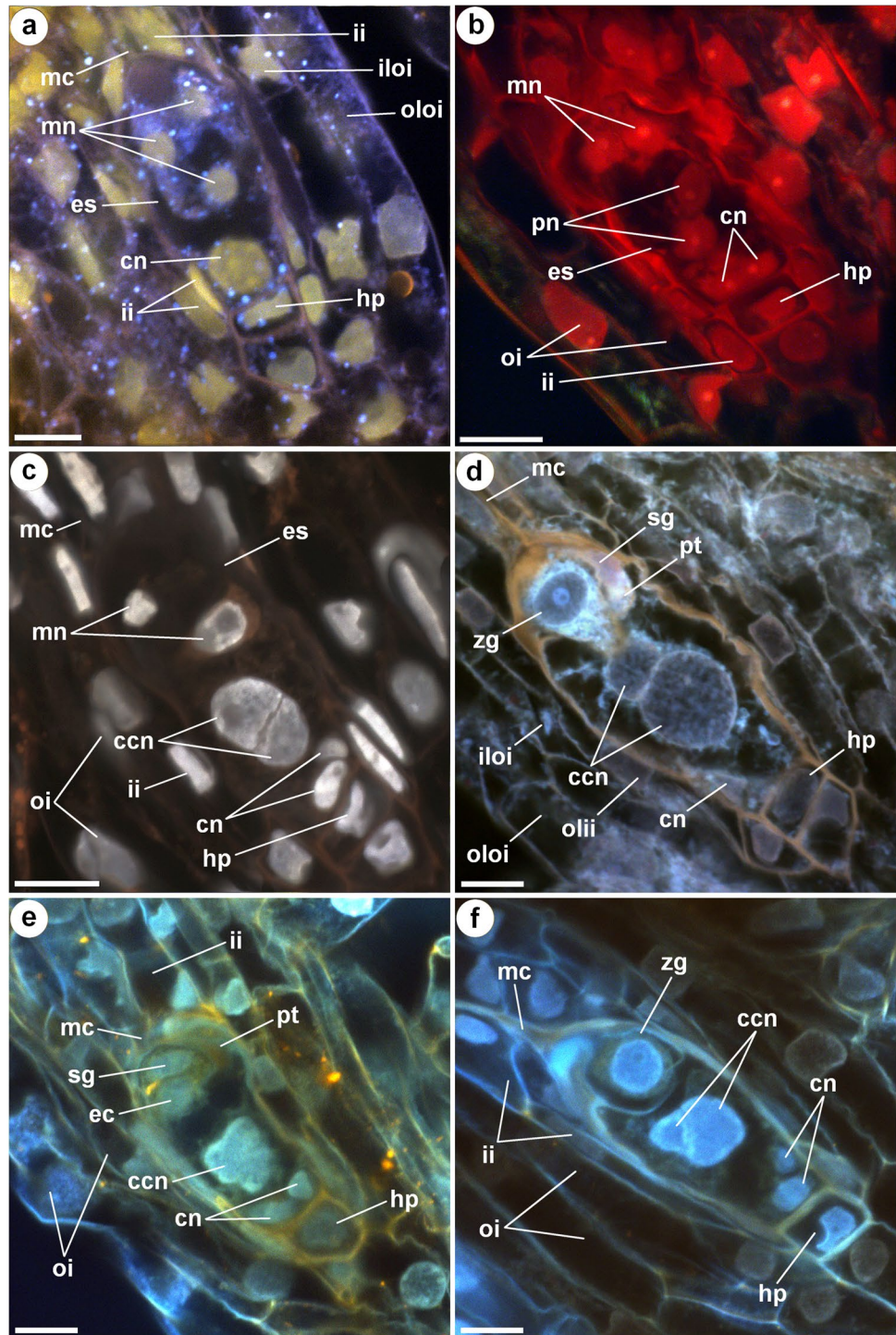
Two polar nuclei form a compound nucleus of the central cell of two connected but not fused haploid nuclei. In *M. crassifolia*, one of them is usually larger (Fig. 6d). In some cases, more complex central nuclei of three or more nuclei were observed as a result of (1) connection of polar nuclei with one male nucleus (Fig. 7f), (2) attachment of one of the chalazal nuclei (Fig. 6c), or (3) division of one of the polar nuclei (Fig. 6e, f). A compound central nucleus and chalazal

nuclei can be seen within the embryo sacs of *M. crassifolia* up to the eight-nine-celled embryo stage (Figs. 8 and 9e, f).

**Fertilization**

Fertilization in *M. crassifolia* is porogamous: the pollen tube (Fig. 7a) enters the micropylar canal. The content of the pollen tube has a specific bright yellow fluorescence

**Fig. 6** Variations in the number of nuclei in the complex of nuclei of the central cell at different stages of development. **a** Four-nucleated embryo sac (BRB). **b** Connection of two polar nuclei (PI). **c** The nucleus of the central cell consists of two similar polar nuclei (BRB). **d** The nucleus of the central cell consists of connected small micropylar and large chalazal polar nuclei (fertilization stage) (DPM). **e** The multinucleated nucleus of the central cell, a zygote and a single synergid in the micropylar end of the embryo sac, and two chalazal nuclei in the chalazal end of the embryo sac (DAPI). **f** The multinucleated nucleus of the central cell at the zygote stage (DAPI). Abbreviations: *ccn*, central cell nucleus; *cn*, chalazal nuclei; *ec*, egg cell; *es*, embryo sac; *hp*, hypostase; *ii*, inner integument; *iloi*, inner layer of outer integument; *mc*, micropyle; *mn*, micropylar nuclei; *oi*, outer integument; *oloi*, outer layer of outer integument; *pn*, polar nuclei; *pt*, pollen tube; *sg*, synergids; *zg*, zygote. Scale bars = 10  $\mu$ m



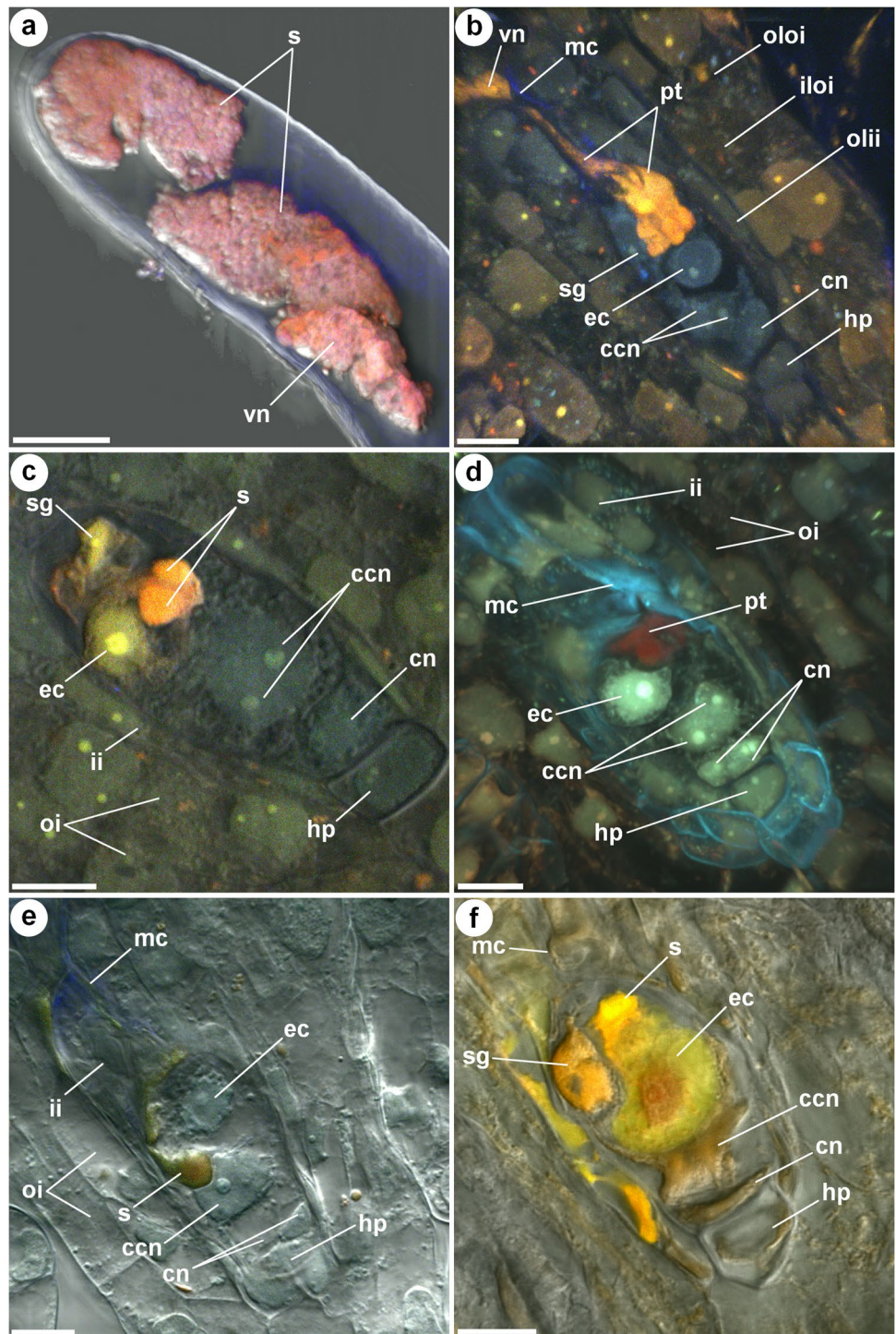
(Fig. 7b, c, e, f). This makes it possible to observe the process of penetration of sperm into the embryonic sac. When stained with Calcofluor, the coat of pollen tube fluoresces blue, while that of the sperm fluoresces dark red (Fig. 7d). One of the sperm fertilizes an egg, forming a zygote. The second sperm nucleus can sometimes be observed in the region of the compound nucleus of the central cell (Fig. 7e).

## Embryogenesis

The zygote is divided by a transverse (Fig. 8a) or oblique septum (Fig. 8b). In both cases, two cells of almost the same size are formed. One of them (cell *ca*) is located closer to the chalazal end, and the second (*cb*) is closer to the micropylar end of the embryo sac. The *cb* cell does not divide further. As a result of cell division *ca*, a three-celled embryo forms



**Fig. 7** Fertilization. **a** The tip of the pollen tube with two sperm nuclei and a vegetative nucleus (DY). **b** The content of the pollen tube penetrates the embryo sac, the vegetative nucleus outside the micropyle (DY). **c, d** One or two sperms are visible, the nucleus of synergids, the egg cell, the nucleus of the central cell, composed of two connected nuclei (**c**, Auto; **d**, CF). **e** Fusion of sperm with the nucleus of the central cell (DY). **f** Fusion of sperm with the egg cell (DCF). Abbreviations: *ccn*, central cell nucleus; *cn*, chalazal nuclei; *ec*, egg cell; *es*, embryo sac; *hp*, hypostase; *ii*, inner integument; *iloi*, inner layer of outer integument; *mc*, micropyle; *oi*, outer integument; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument; *pt*, pollen tube; *s*, sperm; *sg*, synergids; *v*, vacuole; *vn*, vegetative nuclei. Scale bars = 10  $\mu$ m

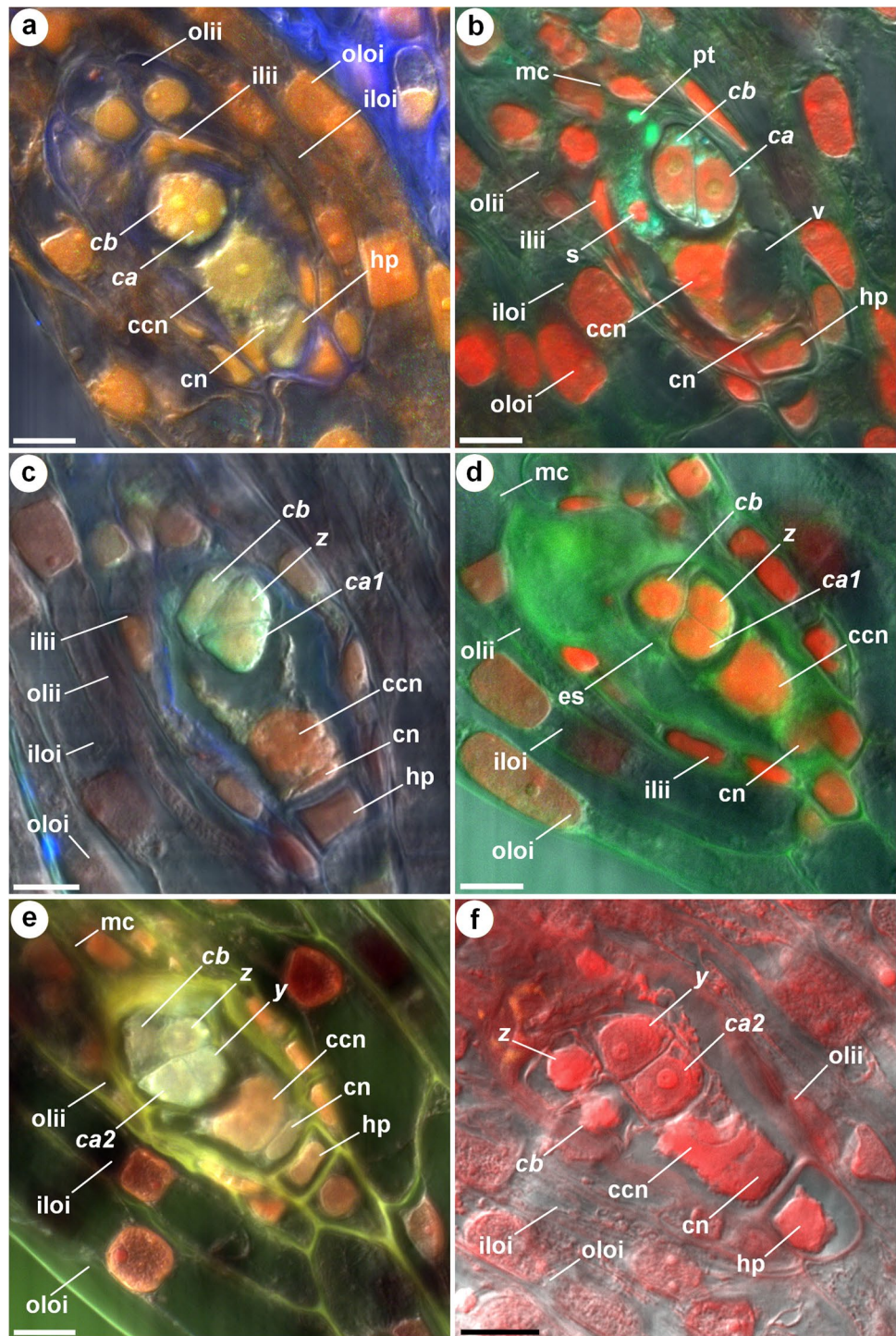


with the formula  $cb+z+ca1$  (Fig. 8c, d, 10). Then the apical cell *ca1* is divided by an oblique septum, forming cells *y* and *ca2*. The nuclei of daughter cells remain connected in the apical part of the embryo for some time and then move away from each other as the cytoplasm volume increases. This difference in the distances between the nuclei makes it possible to establish the sequence of cell division. After the oblique division of the *ca1* cell, its lower derivative (cell *y*) and the

*ca2* cell shift relative to each other and form a zigzag, as a result of which the four-celled embryo is a pseudoisobilateral tetrad (Fig. 8e, f). The formula for a four-celled embryo is  $cb+z+y+ca2$ , and the formula for a five-celled embryo is  $cb+z+y+x+ca3$ .

Before the five-celled embryo stage, only cell division occurred in the apical position, i.e. only cells *ca*, *ca1*, and *ca2* divided sequentially, while their lower daughter cells

**Fig. 8** A sequence of cell division in the early stages of embryonic development (from the zygote stage to the tetrad stage). **a** Division of a rounded zygote into two equal cells *ca* and *cb* (CF). **b** Division of the oval-shaped zygote by an oblique septum (PI). **c, d** The formation of a three-celled embryo during the division of the *ca* cell by an oblique septum (**c**, CF; **d**, PI). **e** Formation of a pseudoisobilateral four-celled embryo, the *cb* cell is vacuolated (DPM). **f** Formation of a pseudoisobilateral four-celled embryo, beginning of lobe development in *cb* and *z* cells (PI). Abbreviations: *ca*, *ca* cell; *ca1*, *ca1* cell; *ca2*, *ca2* cell; *cb*, *cb* cell; *ccn*, central cell nucleus; *cn*, chalazal nuclei; *es*, embryo sac; *hp*, hypostase; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *mc*, micropyle; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument; *pt*, pollen tube; *s*, sperm; *v*, vacuole; *y*, *y* cell; *z*, *z* cell. Scale bars = 10  $\mu$ m

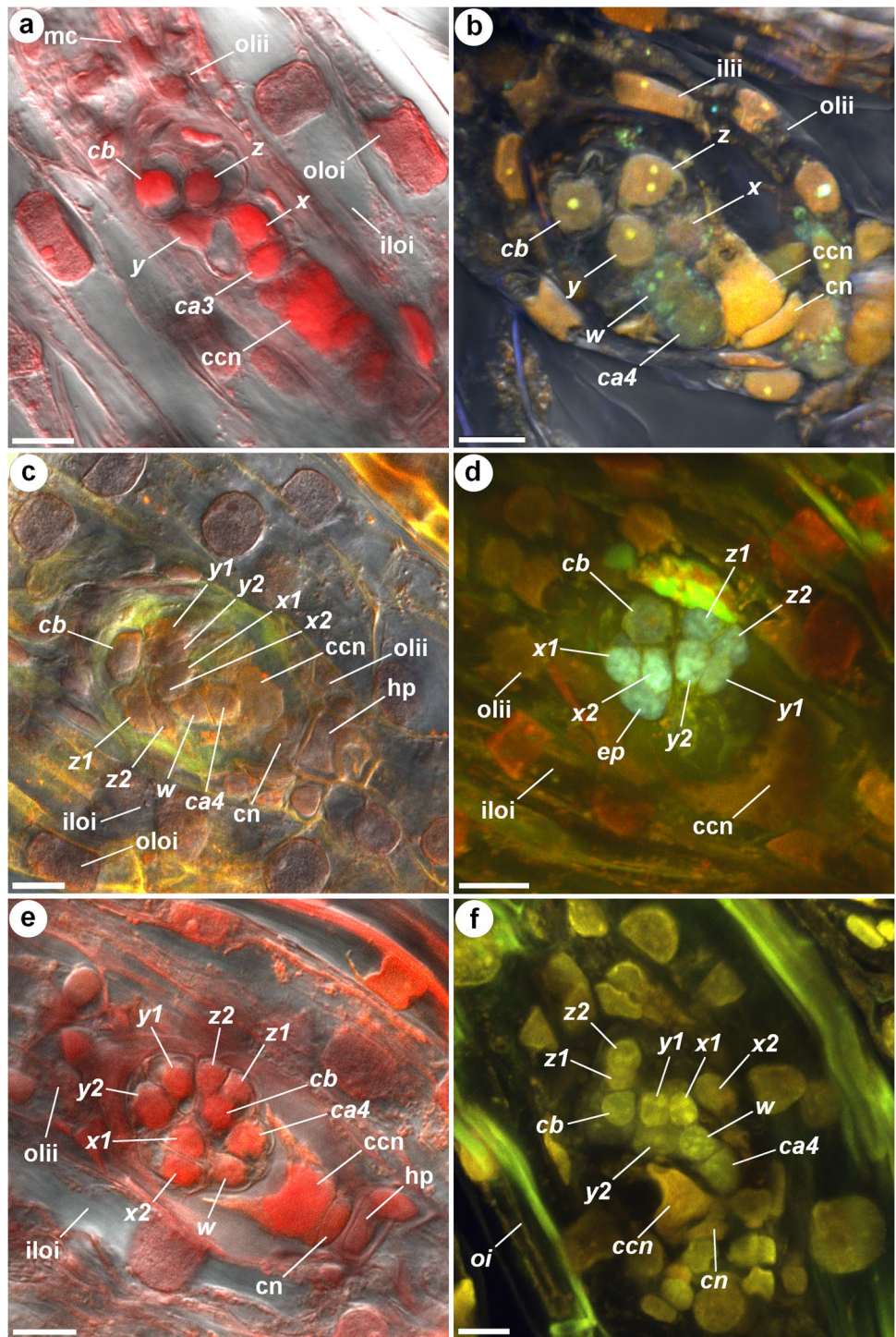


*z*, *y*, and *x* did not divide (Fig. 9a, b, 10). The apical cell divides by an oblique septum. With a lack of space in the embryo sac, the resulting zigzag structure of several cells connected in series with each other folds in a more complex manner.

Subsequently, two variants of suspensor cell formation were observed:

1) Three internal cells of a five-celled embryo *x*, *y*, and *z* divide simultaneously by oblique septa, forming three two-celled layers (Figs. 9d, e and 10). The suspensor becomes seven-celled and includes layers *cb*, 2*z*, 2*y*, and 2*x*. The formula for an eight-celled embryo is  $cb + 2z + 2y + 2x + ca3$ .

**Fig. 9** A sequence of the division of embryonic cells from the tetrad stage to the quadrant stage. **a** A five-celled embryo (PI). **b** A six-celled embryo (DY). **c, d** An eight-celled embryo (**c**, DAPI; **d**, DPM). **e, f** A nine-celled embryo (**e**, PI; **f**, Brb). Abbreviations: *ca3*, *ca3* cell; *ca4*, *ca4* cell; *cb*, *cb* cell; *ccn*, central cell nucleus; *cn*, chalazal nuclei; *ep*, embryo proper; *hp*, hypostase; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *mc*, micropyle; *oi*, outer integument; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument; *w*, *w* cell; *x*, *x* cell; *x1*, *x1* cell; *x2*, *x2* cell; *y*, *y* cell; *y1*, *y1* cell; *y2*, *y2* cell; *z*, *z* cell; *z1*, *z1* cell; *z2*, *z2* cell. Scale bars = 10 μm



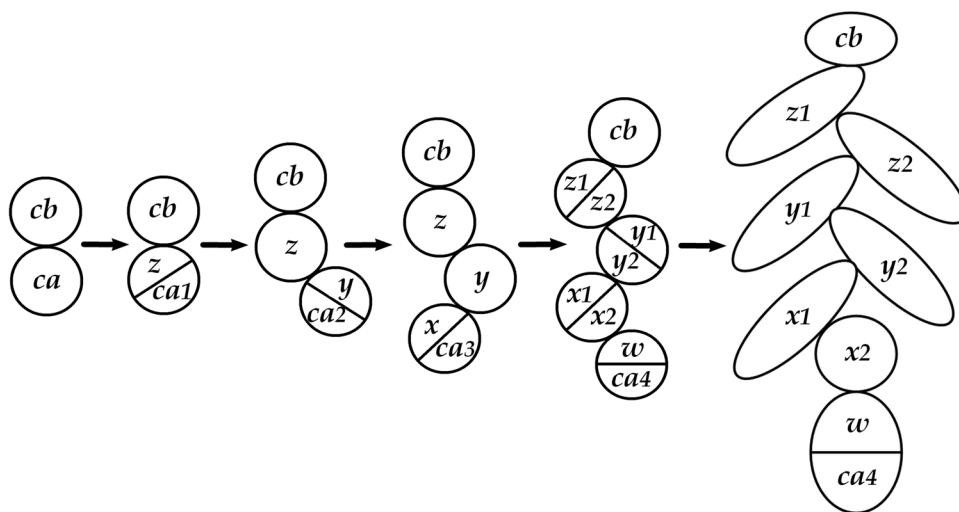
2) Only *z* and *y* cells divide. The *x* cell does not divide. That leads to a decrease in the number of suspensor blades. The formula for a seven-celled embryo is  $cb + 2z + 2y + x + ca3$ . Figure 11a and c show suspensors with four (2*z* and 2*y*) and six blades.

Further division of the *ca3* cell forms the last suspensor cell (*w*) without tubular elongation and the *ca4*

cell, which gives rise to the embryo proper (Figs. 9c, e, f and 10). The formula for a nine-celled embryo is  $cb + 2z + 2y + 2x + w + ca4$ .

The suspensor cells *cb*, 2*z*, 2*y*, and 2*x* form tubular elongations, which are directed along the body of the embryo proper towards the chalazal end (Fig. 11a, b). Sometimes the first signs of the onset of tubular growth appear already in the four-five-celled embryo (Fig. 8f,

**Fig. 10** Diagram of cell division during the formation of a suspensor. Abbreviations: *ca*, *ca* cell; *ca1*, *ca1* cell; *ca2*, *ca2* cell; *ca3*, *ca3* cell; *ca4*, *ca4* cell; *cb*, *cb* cell; *w*, *w* cell; *x*, *x* cell; *x1*, *x1* cell; *x2*, *x2* cell; *y*, *y* cell; *y1*, *y1* cell; *y2*, *y2* cell; *z*, *z* cell; *z1*, *z1* cell; *z2*, *z2* cell



9a). In *M. crassifolia*, cells *x* or cells *x* and *y* often lack tubular lobes.

All cells of the suspensor are specialized and do not subsequently divide. At the stage of the globular embryo, the tubular cells do not emerge either in the micropyle or the integumental space of the outer integument. They closely surround the embryo proper inside a dense shell formed by the inner layer of the inner integument (Fig. 11a, b). As the tubular cells elongate, their nuclei move closer to the ends (Fig. 11e). Figure 11b shows the suspensor blades and three sequentially located cells of the embryo proper.

The formation of tubular cells of the suspensor is ahead of the development of the embryo proper. At the stage of a multicellular embryo, a complex of tubular cells connected to the cells of the embryo proper through one (*x2*) or two (*x2*, *w*) cells located in series, which do not have tubular elongations (Fig. 11d, e).

At the stage of the multicellular embryo, the shells of the embryo proper in the presence of urea exhibit strong blue-green autofluorescence, while the suspensor shell does not fluoresce (Fig. 11f). At this stage, the tubular cells of the suspensor become very thin. When pressed on cover glass, the embryos emerged through the bursting chalazal part of the ovule with the body of the embryo forward. In this case, the suspensor lobes were preserved. This indicates that, although in the developing seed of *M. crassifolia*, the suspensor adjoins the micropyle, its lobes do not extend beyond the inner integument.

As in other orchids, at the stage of a mature globular embryo, the suspensor and cells of all integuments die off. The seed coat forms from the dead cells of the outer layer of the outer integument.

## Seed coats

*M. crassifolia* has a tenuinucellate short-lived nucellus without a pedestal. The micropylar part of the nucellar epidermis begins to die off by the time of the first division of the functional megaspore (Fig. 5a). The chalazal part is preserved until the beginning of the formation of the embryo sac. At the stage of mature female gametophyte and fertilization, nucellus is already eliminated.

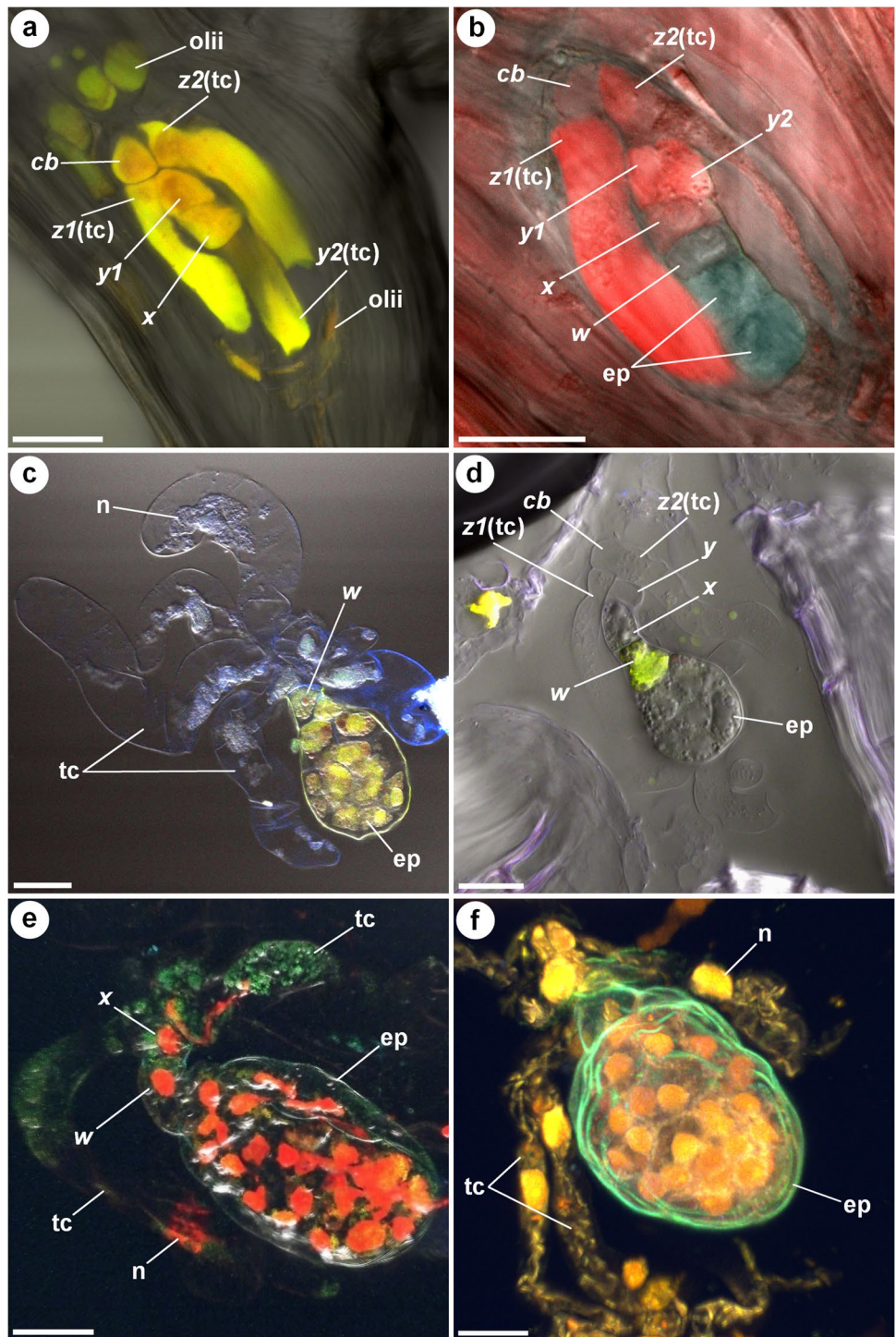
The inner integument consists of 2 cell layers. Before the start of the division of the megaspore mother cell, it grows faster than the outer integument (Fig. 3c). At the stage of the first meiotic division of the megaspore mother cell, both integuments are aligned in length (Fig. 3d). The outer integument has two layers on the frontal side and three layers on the funicular side (continuation of the funiculus). The funiculus is formed from the basal part of the nucellar filament; therefore, the number of cells in the cross-section at the funicular and archesporium level is the same (Fig. 3f).

The inner layer of the inner integument is initially thinner than the outer layer. At the stage of mature female gametophyte, the inner layer of the inner integument forms a cutinized layer, which fluoresces red when stained with dipyrindamole (Fig. 6d).

At the zygote stage, the chalazal part of the inner integument is strongly degraded, and the cells become thinner firstly in the medial region of the embryo sac and then in its chalazal region. The cells of the inner integument near the micropyle remain alive and form the inner part of the micropyle (Fig. 6f).

The features of early embryogenesis of *M. crassifolia* (embryonic cells division order, paired suspensor tubular cells development and their localization within the inner

**Fig. 11** Tubular suspensor at the stage of the globular embryo. **a** Six tubular suspensor cells, fluorescent yellow, inside an unstained membrane formed by an inner integument (DCF). **b** Suspensor tubular cells fluorescent red, two cells of the embryo proper are not stained (RZ). **c** The membrane of the embryo proper fluoresces in white, the suspensor membrane fluoresces in blue (Brb). **d** Tubular cells are connected in series with each other and attached to the embryo proper through a yellow-stained cell *w* (FDA). **e** The fluorescent red nuclear material is located at the ends of the suspensor tubular cells (DCF, PI). **f** When urea is added, the membrane of a mature embryo fluoresces green, the suspensor membrane does not fluoresce, and the nucleus of each tubular cell is displaced to the distal end of the deformed lobes (Auto, urea). Abbreviations: *cb*, *cb* cell; *ep*, embryo proper; *es*, embryo sac; *n*, nucleus; *olii*, outer layer of inner integument; *tc*, tubular cell; *w*, *w* cell; *x*, *x* cell; *y*, *y* cell; *y1*, *y1* cell; *y2*, *y2* cell; *z*, *z* cell. Scale bars = 20 μm



integument) indicate a special variant of Cymbidium-type embryogenesis, which was not previously mentioned in the literature. We named it Cymbidium-type *Maxillaria*-variant.

### Discussion

*Maxillaria crassifolia* is an epiphytic or lithophytic orchid widely distributed in Central and South America, Florida,

and the West Indies. It is a sympodial herbaceous perennial with small flattened laterally univalent pseudobulbs, several fleshy leaves at the base of the pseudobulbs, and yellow or white-yellow single flowers. The flowers are often cleistogamous, 1.5 cm in diameter, and the lip of the open flowers has trichomes for pollination by bees from the genus *Melipona*. The fruit is a capsule with small seeds. Like other orchids, *M. crassifolia* is a mycotrophic plant whose seeds germinate in nature with the help of endomycorrhizal fungi.

According to the classification of Ziegler (1981) and Barthlott and Ziegler (1981), four types of seeds were found in the species of the tribe Cymbidieae—Cymbidium-type, *Eulophia*-type, *Stanhopea*-type, and *Maxillaria*-type (Kolomeitseva et al. 2012). They differ in shape, ratio of length and width, sculpture of cell walls, and volume of air space inside the seed coat. The longest seeds of the Cymbidium-type are up to 1.5 mm. The shortest seeds of *Maxillaria*-type are in species of the genus *Maxillaria*. For example, in *M. crassifolia*, the seed length is  $0.27 \pm 0.03$  mm (Kolomeitseva et al. 2012).

Despite the difference in the structure of seeds in the tribe Cymbidieae, it was hypothesized that all representatives of this tribe have a common type of embryogenesis (Cymbidium-type), characterized by oblique cell division at the tetrad stage and the development of several tubular suspensor cells (Swamy 1949b; Clements 1999). However, the experimental data cover a small number of species, and the data on embryogenesis are fragmentary. This work is the first comprehensive study of all stages of embryonic development of the American genus *Maxillaria*.

## Nucellus

The functioning time of nucellus in orchid taxa close to *M. crassifolia* has not been previously discussed in the literature. However, Mayer et al. (2011) obtained photographs of the embryogenesis of *Gomesa flexuosa* (synonym, *Oncidium flexuosum*), on which tenuinucellate ovules can be identified. In *M. crassifolia*, we also observed tenuinucellate ovules. Micropylar cells of short-living nucellus survive longer than other cells and remain alive until the end of megasporogenesis.

## Megasporogenesis

The earliest study of megasporogenesis of orchids from the tribe Cymbidieae belongs to Afzelius (1916, 1918), who studied *Gomesa praetexta* (Rchb.f.) M.W.Chase & N.H.Williams (synonym, *Oncidium praetextum* Rchb.f.) from the subtribe Oncidiinae and revealed the instability of megasporogenesis in this species. He observed six-nucleated

embryonic sacs of both *Allium*-type and *Polygonum*-type, and also the modified *Allium*-type embryonic sacs, developing not from the chalazal, but the micropylar megaspore dyad.

Later, in orchids of the tribe Cymbidieae, the formation of monosporic and bisporic embryo sacs were revealed (Vij and Sharma 1986; Mayer et al. 2011). In the tribe Cymbidieae, five-, six-, seven-, and eight-nucleated embryonic sacs of the *Allium*-type and *Polygonum*-type were found (Online Resource). For example, in *Eulophia nuda* Lindl., *Polygonum*-type seven- and eight-nucleated sacs have been described (Swamy 1949a). In *Cymbidium bicolor* (Swamy 1942) and *C. sinense* (Yeung et al. 1994, 1995; Zee and Ye 1995), bisporic eight-nucleated, rarely six-nucleated *Allium*-type embryo sacs have been described. Mayer et al. (2011) revealed eight-nucleated *Polygonum*-type embryonic sacs in another orchid of the Cymbidieae tribe, *Gomesa flexuosa* (synonym, *Oncidium flexuosum*) (subtribe Oncidiinae).

In *M. crassifolia*, cytokinesis occurs in both cells of the dyad with the formation of a T-shaped tetrad of megaspores. Usually, the outer dyad is divided by an oblique septum. Then the three outer cells are eliminated, and a monosporic embryo sac is formed from the basal cell. This is consistent with Swamy (1943), who found *Polygonum*-type embryo sacs and T-shaped or linear tetrads of megaspores in *Eulophia epidendraea*. We have found a new anatomical characteristic of the tribe Cymbidieae: the presence of oblique division of the micropylar cell of the megaspore dyad (Fig. 4d, e).

## Megagametogenesis

Many orchids are characterized by a reduction in the number of nuclei in embryo sacs from eight to four (Savina 1978). The reduction in the number of nuclei at the chalazal pole of the embryo sac was called the “striking phenomenon” (Harling 1950). Moreover, sometimes the walls of the cells in the embryo sac undergo reduction (Savina 1974); therefore, in the family of orchids, antipodals are most often represented only by their nuclei. The reduction in the number of chalazal nuclei is more pronounced in bisporic embryo sacs (Abe 1977). According to Swamy (1942), the small antipodal nuclei in the embryo sacs of *Cymbidium bicolor* may indicate their limited functionality.

Usually, a reduction in the number of nuclei of the chalazal end is observed in the six-nucleated embryo sacs of orchids (Sood 1992; Shamrov 2008). In the five–six-nucleated embryo sac of *Calanthe Veitchii*, instability in the number of nuclei of the chalazal complex was revealed: either one nucleus was observed separately, or it was attached to the central nucleus of the embryo sac (Poddubnaya-Arnoldi 1958). In the tribe Cymbidieae, in addition to six- or eight-nucleated embryo sacs (Swamy 1942; Abe 1967; Nagashima 1982; Mayer et al. 2011), embryo sacs with five and seven

nuclei were identified (Swamy 1943, 1949a) (Online Resource).

The number of nuclei in the embryo sacs of *Maxillaria* has not yet been known. In most cases, we could observe six or seven nuclei in the *M. crassifolia* embryo sacs. The proportion of observed eight-nucleated embryo sacs was less than 1/4. Thus, the number of nuclei in the female gametophyte of *M. crassifolia* may be unstable. The diversity in the number of nuclei also includes variants previously found in other species of the tribe Cymbidieae.

### The compound nucleus of the central cell

Many orchids are characterized by varying degrees of reduction of double fertilization and endosperm formation, which is due to a decrease in seed size. In *Cymbidium sinense* from the tribe Cymbidieae (Yeung et al. 1996; Huang et al. 1998), the polar nuclei join but do not fuse, the nuclei of the primary endosperm do not divide, and their elimination occurs after the embryo reaches the globular stage. A fusion of three nuclei and the formation of a triploid endosperm nucleus or fusion of sperm with only one of the polar nuclei has been described in orchids from other tribes (Poddubnaya-Arnoldi 1958). A more complex four-nucleated polar-antipodal complex, consisting of two connected polar nuclei, antipodes and sperm, was found in the embryo sacs of *Calanthe Veitchii* (Poddubnaya-Arnoldi 1958).

In *M. crassifolia*, we observed a certain variability in the formation of the compound nucleus of the central cell, which is formed in different ways: (i) connection of two polar nuclei, (ii) connection of two polar nuclei and sperm, (iii) connection of two polar nuclei and chalazal nuclei, and (iv) from two to three divisions of the connected nuclei. Two-nucleated compound central cells nuclei were found in less than half of all embryonic sacs examined. More than a quarter of the nuclei of the central cell had four or five nuclei. In all cases, these nuclei were closely connected into a single structure, but not fused. A wide variety of the number of nuclei may indicate that this indicator is not critical for normal development. Despite individual cases of the division of nuclei in the nucleus of the central cell, none of the described variants can be called endosperm.

Thus, the megagametogenesis of *M. crassifolia* has the following features: (1) the number of nuclei in the embryo sacs is unstable; (2) the central cell nucleus can be two-nucleated or multinucleated; (3) the chalazal nuclei do not undergo cytokinesis.

### Embryogenesis

Using *Cymbidium bicolor* as an example, Swamy (1942, 1949b) was the first to describe a special type of

embryogenesis—Cymbidium-type or V-type, which is characterized by irregular cell division at the initial stages of embryo development and the formation of several tubular non-branching suspensor cells. The number of tubular cells in different genera varies from three to eight, and the formation of a suspensor precedes the embryo proper.

Yeung et al. (1996) and Huang and Zee (1998) found structured (regular) cell division in the embryo of *Cymbidium sinense* in the early stages of development. These authors concluded that the division of the *ca* and *cb* cells and the formation of four (Huang and Zee 1998) or five (Yeung et al. 1996) tubular suspensor cells is characteristic of cymbidiums. One of these cells, called the micropylar suspensor cell by the authors, is longer than other tubular cells and extends into the micropylar space. The other tubular cells are directed not towards the micropylar but the chalazal part of the ovule.

The suspensor of the *Maxillaria crassifolia* studied consisted of several tubular cells directed toward the chalazal end and surrounding the embryo proper. However, we did not find in *M. crassifolia* either a strong elongation of anyone tubular suspensor cell in comparison with others or the penetration of any suspensor cells into the micropyle. Probably, even in rather mature embryos of *M. crassifolia*, such penetration is difficult or even impossible for tubular cells due to the dense layer of the inner integument. This layer is so tightly closed at the micropylar end that even with mechanical action on the seed, it breaks not in the micropyle, but in the chalazal region.

In the tribe Cymbidieae, variation in the number of tubular cells, the methods of their formation (including the division pattern), and the time of elimination are still almost unexplored. It is known that in *Cymbidium*, *Eulophia*, and *Geodorum*, the number of suspensor tubular cells varies between 3 and 8 and differs even within the same ovary (Swamy 1949b). The first and only mention of the embryogenesis of our experimental species is in the work of Fleischer (1874), who discovered a long suspensor. According to Veyret (1965, 1974), another species of the genus *Maxillaria*, *M. variabilis*, had a one- or two-celled suspensor, and its embryogenesis corresponded to the Calypso-type or Cyripedioid-type, according to Clements (1999). In the embryos of other species of the tribe Cymbidieae studied by her, *Eulophia bouliawongo* (synonym, *E. oedoplectron*) and *Peristeria elata* Hook., no Cymbidium-type embryogenesis was also detected (Veyret 1965). In almost all studied species from the tribe Cymbidieae, she noted the regularity of the first embryonic divisions, except for *Eulophia bouliawongo* with irregular cell division. In *E. bouliawongo*, *E. cucullata*, and *Maxillaria variabilis*, nothing similar to the Cymbidium-type suspensor described in this work (Veyret 1965).

According to our studies, the embryogenesis of *M. crassifolia* differs significantly from all the orchid tribe

Cymbidieae described above by the absence of *cb* cell division throughout embryogenesis. In *M. crassifolia*, up to the stage of the five-celled embryo, only the apical cell division occurred, and then three internal cells underwent a single division with the formation of a seven-celled suspensor (*cb*-cell + 3 pairs of tubular cells *z*, *y*, and *x*) (Fig. 10). The development of the embryo proper proceeded from one apical cell of an eight-celled embryo and began only after the formation of a suspensor with several tubular cells, which is characteristic of *Cymbidium*-type embryogenesis (Swamy 1949b; Clements 1999; Yeung et al. 1996).

The collected data make it possible to describe the *Cymbidium*-type *Maxillaria*-variant of embryogenesis, the distinctive features of which are absence of the *cb*-cell division, strictly apical character of embryonic divisions, formation of paired suspensor tubular cells, and localization of all suspensor's cells within the inner integument.

### Seed coats

The protective layer of dead cells of the inner integument plays a significant role in the primary dormancy of orchid seeds (Arditti 1967), but its formation in species of the tribe Cymbidieae is not well understood.

In *Eulophia* the outer integument is known to be single-layered, and the embryo at a certain stage of development goes beyond the inner integument and continues to develop in the space of the outer integument (Swamy 1943). As the cells of the inner integument are destroyed, the tubular cells of the suspensor can enter the integumental space of the outer integument, but not outside the seed coat (Swamy 1949b). In *Maxillaria crassifolia*, we found a two-layer inner integument and a two–three-layer outer integument. In this case, the elimination of living cells of the inner integument of *M. crassifolia* did not begin with the chalazal part, as in *Dendrobium nobile* Lindl. (Vasudevan and van Staden 2010; Kolomeitseva et al. 2021), and from the medial part of the embryo sac. A feature of the destruction of the inner integument in *M. crassifolia* is the early (at the stage of megagametogenesis) elimination of its inner layer with long-term preservation of living micropylar cells of the outer layer of the inner integument.

The authors studied the embryogenesis in the genus *Cymbidium* (Swamy 1949b; Yeung et al. 1996; Huang and Zee 1998) and reported the penetration of the terminal suspensor cell into the micropyle. Besides, Swamy (1949b) reported elongating suspensor cells breaking through degenerating cells of the inner integument and eventually spreading in any direction within the space defined by the outer integument. In *Maxillaria crassifolia*, we found no exit of tubular cells outside the inner integument.

An interesting fact of the embryogenesis of *Cymbidium bicolor* was the observation by Swamy (1946) that

the polarity of the embryo inside the outer integument can change by 180 degrees, while the embryo turns the suspensor toward the chalaza. Such an inverted embryo can maintain its viability only in those orchids whose nutrition does not depend on the release of the suspensor into the integumental space of the outer integument or the placental cavity. Therefore, theoretically, in *Maxillaria crassifolia* such embryos could reach the globular stage and form the mature seed. However, we did not find embryos with a suspensor directed towards chalaza in the studied species. This is probably due to the insufficient space inside the seed coat of *M. crassifolia*, which is less than in *Cymbidium* (71% and 91–96% of the seed volume, respectively) (Kolomeitseva et al. 2012).

At the stage of the globular embryo, the membrane of the *M. crassifolia* embryo proper fluoresces differently than the membrane of the suspensor (Fig. 11c, f). This indicates a difference in their chemical composition and, in particular, the possible existence of a cuticular layer in the embryo proper. The cuticular layer has been described in the embryos of other species of the tribe Cymbidieae, *Cymbidium sinense* (Yeung et al. 1996) and *Gomesa flexuosa* (synonym, *Oncidium flexuosum*) (Mayer et al. 2011). These researchers believe that the cuticular substance around the embryo proper protects it from moisture loss and mechanical effects and also contributes to the formation of a pressure gradient between the embryo proper and the suspensor, due to which non-cutinized suspensor cells supply nutrients and water to the cells of the embryo proper.

### Conclusion

Megasporogenesis, megagametogenesis, and embryogenesis of *Maxillaria crassifolia* have both common feature characteristic of the tribe Cymbidieae and several peculiar features that distinguish it from other species of the tribe. General signs are tenuinucellate ovule, bitegmal, with a well-defined funiculus and without vascular tissues. The archesporial cell does not divide and directly transforms into a megasporocyte. The megagametophyte develops from the chalazal megaspore of the tetrad. In terms of the structure and dynamics of the tubular suspensor formation, the embryogenesis of *M. crassifolia* is close to the *Cymbidium*-type. The tubular cells of the suspensor are facing the chalazal end of the ovule. The development of the suspensor is ahead of the development of the embryo proper.

Along with the general ones, we found the following specific features of *M. crassifolia*. Megasporogenesis ends with the formation of a T-shaped tetrad of megaspores by an oblique division of the micropylar cell of the dyad. The megagametogenesis of *M. crassifolia* is unstable in the number of nuclei of the embryo sac and their subsequent



differentiation. The embryo sacs of the modified Polygonum-type are four-, six-, seven- or eight-nucleated, with a predominance of the seven-nucleated. The compound nucleus of the central cell may include two attached but unfused polar nuclei, daughter nuclei formed during the division of one of these nuclei, sperm nucleus and chalazal nuclei.

The sequence of cell division during embryogenesis is strictly structured. The embryogenesis of *M. crassifolia* differs from the Cymbidium-type in the absence of division of the basal cell *cb*. The three-celled embryo is formed by the transverse or oblique division of the *ca* cell. Then, only the division of the apical derivatives of the *ca* cell (*ca1-ca3*) occurs up to the *ca4* layer with the formation of a filamentous row of several cells. At the five-celled embryo stage, cells *z*, *y*, and *x* undergo a single tangential division to form three two-cell layers. The most developed nine-celled embryo has a suspensor consisting of 7 tubular cells (1 *cb* cell + 2 *z* cells + 2 *y* cells + 2 *x* cells) and a non-tubular *w* cell; the *ca4* cell gives rise to the embryo proper. In less developed embryos, the number of tubular cells varies from three to five if the *x* cells *x* or the *x* and *y* cells are not tubularized. The tubular elongations of the suspensor cells facing the chalazal end cover the embryo proper but do not go beyond the inner integument. At the stage of a multicellular embryo, the membrane of the embryo proper and the membrane of the suspensor fluoresce differently, which probably indicates the existence of a cuticular substance only around the embryo proper.

Thus, the initial stages of *M. crassifolia* embryogenesis differ significantly from the previously studied species of the tribe Cymbidieae. We have described Cymbidium-type Maxillaria-variant of embryogenesis with different genesis and specialization of embryonic and suspensor cells for the first time. Its distinctive features are structuring the embryo at the stage of first cell divisions, absence of *cb*-cell division, strictly apical character of embryonic divisions, formation of one to three pairs of suspensor tubular cells, and localization of all tubular cells within the inner integument.

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**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors declare no competing interests.

## References

- Abe K (1967) Contributions to the embryology of the family Orchidaceae. I. Development of the embryo sac in *Cymbidium goeringii* Reichb.f. Science Reports of the Tōhoku University, Ser. 4, (Biology) 33:79–82.
- Abe K (1971) Contributions to the embryology of the family Orchidaceae, IV. Development of the embryo sac in *Bletilla striata*. Science Reports of the Tōhoku University, Ser. 4, (Biology) 35:213–218.
- Abe K (1972) Contributions to the embryology of the family Orchidaceae, VI. Development of the embryo sac in 15 species of orchids. Science Reports of the Tōhoku University, Ser. 4, (Biology) 36: 135–178.
- Abe K (1977) Development of the embryo sac in *Amitostigma kinoshitae* (Makino) Schltr. (Orchidaceae). Ann Bot 41:897–899. <https://doi.org/10.1093/oxfordjournals.aob.a085367>
- Afzelius K (1916) Zur Embryosackentwicklung der Orchideen. Sven Bot Tidskr 10:183–262
- Afzelius K (1918) Zur Embryosackentwicklung Der Orchideen Botanisches Centralblatt 138:172
- Arditti J (1967) Factors affecting the germination of orchid seeds. Bot Rev 33:1–97. <https://doi.org/10.1007/BF02858656>
- Atwood JT, Mora de Retana DE (1999) Flora Costaricensis. Tribe Maxillarieae: Subtribes Maxillariinae and Oncidiinae. Fieldiana 40:1–84
- Barthlott W, Ziegler B (1981) Mikromorphologie der Samenschalen als systematisches Merkmal bei Orchideen. Berichte Der Deutschen Botanischen Gesellschaft 94:267–273. <https://doi.org/10.1111/j.1438-8677.1981.tb03402.x>
- Blanco MA, Carnevali G, Whitten WM, Singer RB, Koehler S, Williams NH, Ojeda I, Neubig KM, Endara L (2007) Generic realignments in Maxillariinae (Orchidaceae). Lankesteriana 7:515–537. <https://doi.org/10.15517/lank.vi.7935>
- Cabral JS, Felix LP, Guerra M (2006) Heterochromatin diversity and its co-localization with 5S and 45S rDNA sites in chromosomes of four *Maxillaria* species (Orchidaceae). Genet Mol Biol 29:659–664. <https://doi.org/10.1590/S1415-47572006000400015>
- Carnevali G (1991) The cytology and the pollinaria in the Maxillariinae (Orchidaceae). PhD Thesis, University of Missouri, St. Louis, Missouri, USA.
- Chase MW, Cameron KM, Freudenstein JV, Pridgeon AM, Salazar G, Van den Berg C, Schuitman A (2015) An updated classification of Orchidaceae. Bot J Linn Soc 177:151–174. <https://doi.org/10.1111/boj.12234>
- Chase MW, Freudenstein JV, Cameron KM (2003) DNA data and Orchidaceae systematics: a new phylogenetic classification. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ (eds) Orchid conservation. Natural History Publications, Sabah, Malaysia, Kota Kinabalu, pp 69–89
- Christenson EA (2002) Vue d'ensemble du genre *Maxillaria*. Richardiana 2:41–62.
- Clements MA (1999) Embryology In: Pridgeon AM, Cribb JC, Chase MW, Rasmussen FN, eds. Genera Orchidacearum. General Introduction, Apostasioideae, Cyrtopodioideae. New York: Oxford University Press, pp 38–58.

- Davies KL, Winters C, Turner MP (2000) Pseudopollen: its structure and development in *Maxillaria* (Orchidaceae). *Ann Bot* 85:887–895. <https://doi.org/10.1006/anbo.2000.1154>
- Dressler RL (1981) The orchids: natural history and classification. Harvard University Press, Cambridge
- Dressler RL (1993) Phylogeny and classification of the orchid family. Dioscorides Press, Portland
- De Barros F (2002) Notastaxonômicas para espécies brasileiras dos generos *Epidendrum* e *Heterotaxis* (Orchidaceae). *Hoehnea* 29:112
- Fleischer E (1874) Beiträge zur Embryologie der Monokotylen und Dikotylen. *Flora* 27:417–432
- Garay LA (1997) The identity of the type drawings of *Maxillaria* Ruiz & Pavon. *Maxillarieae* 1:3–5
- Harling G (1950) Embryological studies in the Compositae. Part 1. Anthemideae- Anthemidinae *Acta Horti Bergiani* 15:135–168
- Hoehne FC (1953) *Maxillaria crassifolia*. In: *Flora Brasília*. Instituto de Botanica, São Paulo. 12:250.
- Huang B-Q, Ye X-L, Yeung EC, Zee SY (1998) Embryology of *Cymbidium sinense*: the Microtubule organization of early embryos. *Ann Bot* 81:741–750. <https://doi.org/10.1006/anbo.1998.0628>
- Kolomeitseva GL, Antipina VA, Babosha AV, Ryabchenko AS (2014) Generative propagation of auto-pollinated hothouse orchids. *Bulletin of the Nizhny Novgorod University named after N.I. Lobachevsky* 3:43–48 (in Russian)
- Kolomeitseva GL, Antipina VA, Shirokov AI, Khomutovskij MI, Babosha AV, Ryabchenko AS (2012) Orchid seeds: development, structure, germination. Publishing house «Geos», Moscow (in Russian)
- Kolomeitseva GL, Babosha AV, Ryabchenko AS, Tsavkelova EA (2021) Megasporogenesis, megagametogenesis, and embryogenesis in *Dendrobium nobile* (Orchidaceae). *Protoplasma* 258:301–317. <https://doi.org/10.1007/s00709-020-01573-2>
- Mayer JLS, Camello-Guerreiro SM, Appezzato-da-Gloria B (2011) Anatomical development of the pericarp and seed of *Oncidium flexuosum* Sims (Orchidaceae). *Flora* 206:601–609. <https://doi.org/10.1016/j.flora.2011.01.009>
- Moraes AP, Olmos Simões A, Ojeda Alaion DI, De Barros F, Forn-Martins ER (2016) Detecting mechanisms of karyotype evolution in heterotaxis (Orchidaceae). *PLoS ONE* 11(11):e0165960. <https://doi.org/10.1371/journal.pone.0165960>
- Nagashima T (1982) Studies on the seed germination and embryogenesis in the *Cymbidium goeringii* Rchb. f. and *Paphiopedilum insigne* var. *sanderæ* Rchb. f. *Journal of the Japanese Society for Horticultural* 51:94–105. <https://doi.org/10.2503/jjshs.51.94>
- Ojeda Alaion DI (2003) Filogenia del complejo *Heterotaxis* Lindley (Orchidaceae): evolution de la arquitectura vegetative y de los syndromes de polinizacion. PhD Thesis, Merida, Yucatan, Mexico.
- Ojeda Alaion DI, Carnevali G, Williams NH, Whitten WM (2003) Phylogeny of the *Heterotaxis* Lindley complex (Maxillariinae): evolution of the vegetative architecture and pollinations syndrome. *Lankesteriana* 7:45–47
- Poddubnaya-Arnoldi VA (1958) Investigation of the process of fertilization in some angiosperms on living material. *Botanicheskii Zhurnal* 43:178–193 (in Russian)
- Rao AN, Chua LG (1978) Fruit and seed development in certain local orchids. In: Alphonso AG, Seng PY, Rao AN, Soon TE, Jin GC (eds) *Proceedings of the symposium on orchidology*, The Orchid Society of South-East Asia, Singapore, pp 24–42.
- Savina GI (1974) Fertilization in Orchidaceae. In: Linskens HF (ed) *Fertilization in higher plants*. North-Holland, Amsterdam, pp 197–204
- Savina GI (1978) Certain peculiarities in the embryology of orchids. *Proc Indian Natl Sci Acad* 44:141–145
- Shamrov II (1997) Principles of classification of embryogenesis types. In: Batygina TB (ed) *Embryology of flowering plants. Terminology and concepts*. 2. Seed, St. Petersburg: Peace and Family-95, pp 493–508. (In Russian)
- Shamrov II (2008) Ovule of flowering plants: structure, functions, origin. Batygina TB (ed) Moscow: KMK Scientific Press Ltd. (In Russian)
- Shamrov II, Anisimova GM (2003) Critical stages of ovule and seed development. *Acta Biol Cracov Bot* 45:167–172
- Schuiteman A, Chase M (2015) A reappraisal of *Maxillaria* (Orchidaceae). *Phytotaxa* 225:1–78. <https://doi.org/10.11646/phytotaxa.225.1.1>
- Senghas K (2002) *Maxillaria* (Orchidaceae), un genre chaotique. *Richardiana* 2:29–38
- Sood SK (1992) Embryology of *Malaxis saprophyta*, with comments on the systematic position of *Malaxis* (Orchidaceae). *Plant Syst Evol* 179:95–105. <https://doi.org/10.1007/BF00938022>
- Swamy BGL (1942) Female gametophyte and embryogeny in *Cymbidium bicolor* Lindl. *Proceeding of the Indian Academy of Science* 15:194–201
- Swamy BGL (1943) Gametogenesis and embryogeny of *Eulophia epidendreae* Fischer. *Proc Indian Natl Sci Acad* 9:59–65
- Swamy BGL (1946) Some notes on the embryo of *Cymbidium bicolor* Lindl. *Curr Sci* 15:139–140
- Swamy BGL (1949a) Embryological studies in the Orchidaceae, I. Gametophytes. *Am Midl Nat* 41:184–201. <https://doi.org/10.2307/2422025>
- Swamy BGL (1949b) Embryological studies in the Orchidaceae, II. Embryogeny. *Am Midl Nat* 41:202–232. <https://doi.org/10.2307/2422026>
- Szlachetko DL, Sitko M, Tukało P, Mytnik-Ejsmont J (2012) Taxonomy of the subtribe Maxillariinae (Orchidaceae, Vandoideae) revised. *Biodiversity Research and Conservation* 25:13–38. <https://doi.org/10.2478/v10119-012-0017-2>
- Tohda H (1968) Development of the embryo of *Bletilla striata*. *Science Reports of the Tôhoku University* 34:125–131
- Treub M (1879) Notes sur l'embryogénie de quelques orchidées. PI. I to VIII. *Verhandelingen der Koninklijke Nederlandsche Akademie van Wetenschappen. Afdeling Natuurkunde* 1 9: 1–50.
- Vasudevan R, van Staden JV (2010) Fruit harvesting time and corresponding morphological changes of seed integuments influence in vitro seed germination of *Dendrobium nobile* Lindl. *Plant Growth Regul* 60:237–246. <https://doi.org/10.1007/s10725-009-9437-1>
- Veyret Y (1965) Embryogénie comparée et blastogénie chez les Orchidaceae-Monandreae. Paris: *O.R.S.T.O.M.* (France)
- Veyret Y (1974) Development of the embryo and the young seedling stages of orchids. In: Withner CL (ed) *The orchids: scientific studies*. John Wiley and Sons, New York, pp 223–265
- Vij SP, Sharma M (1986) Embryo sac development in Orchidaceae. In: Vij SP (ed) *Biology, conservation and culture of orchids*. Affiliated East-West Press, New Delhi, India, pp 31–48
- Yeung ECY, Law SK (1997) Ovule and megagametophyte development in orchids. In: Arditti J, Pridgeon AM (eds) *Orchid biology: reviews and perspectives*, vol 7. Kluwer Academic Publishers, pp 31–73
- Yeung ECY, Zee SY, Ye XL (1994) Embryology of *Cymbidium sinense*: ovule development. *Phytomorphology* 44:55–63

- Yeung ECY, Zee SY, Ye XL (1996) Embryology of *Cymbidium sinense*: embryo development. *Ann Bot* 78:105–110. <https://doi.org/10.1006/anbo.1996.0101>
- Whitten WM, Blanco MA, Williams NH, Koehler S, Carnevali G, Singer RB, Endara L, Neubig KM (2007) Molecular phylogenetics of *Maxillaria* and related genera (Orchidaceae: Cymbidieae) based on combined molecular data sets. *Am J Bot* 94:1860–1889. <https://doi.org/10.3732/ajb.94.11.1860>
- Zee SY, Ye XL (1995) Changes in the pattern of organization of the microtubular cytoskeleton during megasporogenesis in *Cymbidium sinense*. *Protoplasma* 185:170–177. <https://doi.org/10.1007/BF01272857>
- Ziegler B (1981) Mikromorphologie der Orchideensamen unter Berücksichtigung taksonomischer Aspekte. PhD Thesis, Ruprecht Karls Universität, Heidelberg.

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