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ORIGINAL ARTICLE

Megasporogenesis, megagametogenesis, and embryogenesis in *Dendrobium nobile* (Orchidaceae)

Galina L. Kolomeitseva[1] · Alexander V. Babosha[1] · Andrey S. Ryabchenko[1] · Elena A. Tsavkelova[2]

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Abstract

The orchid reproductive strategy, including the formation of numerous tiny seeds, is achieved by the elimination of some stages in the early plant embryogenesis. In this study, we documented in detail the formation of the maternal tissues (the nucellus and integuments), the structures of female gametophyte (megaspores, chalazal nuclei, synergids, polar nuclei), and embryonic structures in *Dendrobium nobile*. The ovary is unilocular, and the ovule primordia are formed in the placenta before the pollination. The ovule is medionucellate: the two-cell postament and two rows of nucellar cells persist until the death of the inner integument. A monosporic eight-nucleated embryo sac is developed. After the fertilization, the most common central cell nucleus consisted of two joined but not fused polar nuclei. The embryogenesis of *D. nobile* is similar to the Caryophyllad-type, and it is characterized by the formation of all embryo cells from the apical cell (*ca*) of a two-celled proembryo. The only exception is that there is no formation of the radicle and/or cotyledons. The basal cell (*cb*) does not divide during the embryogenesis, gradually transforming into the uninuclear suspensor. Then the suspensor goes through three main stages: it starts with an unbranched cell within the embryo sac, followed by a branched stage growing into the integuments, and it ends with the cell death. The stage-specific development of the female gametophyte and embryo of *D. nobile* is discussed.

Keywords Orchids · Female gametophyte · Embryogenesis · Suspensor · Nucellus · Integument

Introduction

The genus *Dendrobium* Sw. of subfamily Epidendroideae (tribe Dendrobieae, subtribe Dendrobiinae) is one of the largest within the Orchidaceae family, comprising of 1184 species of epiphytes (Alrich and Higgins 2008) that are widely distributed in diverse habitats across the South and Southeast Asia regions as well as in Australia and the islands located in the Indian and Pacific Oceans. It is one of the most commonly cultivated and popular genera among the orchid amateurs and in the horticulture industry. However, the ongoing interest in the *Dendrobium* is explained not only by the ornamental features of the flowers (Hinsley et al. 2018) but also by diverse biologically active compounds, produced in stems and leaves of various *Dendrobium* species, in particular *Dendrobium nobile* Lindl., which is one of the basic herbs of the Chinese traditional medicine (Yang et al. 2007; Zhang et al. 2007; Hwang et al. 2010; Chen et al. 2013).

Together with many other wild orchid populations, these plants are threatened or endangered: apart from the climate changes, they are primarily suffering from the anthropogenic impact on the tropical rainforests and illegal poaching (Roberts and Dixon 2008; Swarts and Dixon 2009). Thus, the integrated conservation strategies, particularly realized through the botanic gardens that have traditionally been focused on the orchid biodiversity, should combine diverse knowledge and modern trends, including seed banking and conservation genetics, long-term ex situ germplasm storage and cryo- and low-temperature preservation (Swarts and Dixon 2009), the development of the protocols for the effective asymbiotic and symbiotic seed propagation (Teixeira da

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✉ Elena A. Tsavkelova
tsavkelova@mail.ru

[1] N.V. Tsitsin Main Botanical Garden of the Russian Academy of Sciences, Botanicheskaya 4, Moscow, Russia 127276

[2] Department of Microbiology, Faculty of Biology, Lomonosov Moscow State University, 1-12 Lenin's Hills, Moscow, Russia 119234

Silva et al. 2015a, b), and new studies on the orchid-fungal and orchid-bacterial associations (Tsavkelova et al. 2016) that could facilitate the reintroduction and ecological restoration. Nevertheless, the prospects of both the restoration of the native populations and the commercial use of *Dendrobium* orchids require fundamental studies on their reproductive biology. This knowledge is essential for effective seed germination of *Dendrobium* plants in vitro, as well as for the long-term ex situ germplasm storage.

The development of the female gametophyte has been described in at least nine species of the genus *Dendrobium* (Pastrana and Santos 1931; Swamy 1949; Poddubnaya-Arnoldi 1958, 1959; Rajan 1971; Chardard 1978; Govindappa and Karanth 1980; Vasudevan and van Staden 2010; Gurudeva 2016) and several hybrids (Niimoto and Sagawa 1961; Sagawa and Israel 1964; Israel and Sagawa 1965). Poddubnaya-Arnoldi (1958, 1959) noted that *D. nobile* has an embryo sac with five and six nuclei, whereas Rajan (1971) reported that *D. macrostachyum* Lindl. has an embryo sac with both six and eight nuclei. The mature embryo sacs of *D. aqueum* Lindl. (Govindappa and Karanth 1980) and *D. ovatum* (L.) Kraenzl. (Gurudeva 2016) were reported to have eight and six nuclei, respectively. Among *Dendrobium* species, the fertilization and the basic early events of the embryo development are partially described for *D. nobile* (Poddubnaya-Arnoldi 1958, 1959; Vasudevan and van Staden 2010).

The suspensor structure is essential to the development of the embryo. In Angiosperms, suspensors may have diverse sizes and shapes, such as columnar, filamentous, spherical, or be even irregular in shape (Yeung and Meinke 1993). Within the genus *Dendrobium*, different variations in the suspensor morphology and its development have been also described. In one of the earliest studies on the orchid embryogenesis (Swamy 1949), it was shown that the embryo of *D. barbatulum* Lindl. possessed a unicellular unbranched suspensor, extended through the inner integument into the outer integument. Other species, like *D. microbulbon* A.Rich. and *D. wightii* A.D.Hawkes & A.H.Heller (synonym, *D. graminifolium* Wight), possess a very tiny suspensor with a vacuolated basal cell of approximately equal size to the other cells of the embryo (Swamy 1949).

However, in the earlier studies, there was no description of the first stages of the ovular primordium development. There is still lacking detailed information regarding the processes of the formation, developmental modifications, and degeneration of the non-functional megaspores, the seed coats, the endosperm, and zygotic embryo. All previously obtained data showed the substantial variations in the certain peculiar features of the embryo sac and endosperm development, as well as in suspensor formation within the different species of the genus *Dendrobium*, thus, requiring further clarification. Such information is keenly needed not only for the fundamental knowledge of the embryological events but also for a better understanding of the evolutionary process in orchids. The objectives of this study were to document in detail the megasporogenesis, megagametogenesis, and embryogenesis of *D. nobile* with emphasis on the appearance and degradation of transitory embryonic structures.

Materials and methods

Plant material

The plants of *D. nobile* Lindl. were grown in the Stock Greenhouse of the Main Botanical Garden of the Russian Academy of Sciences (Moscow, Russia). The cultivation was performed under the natural light/dark cycles and moderately warm conditions, which were as follows: the temperature regime was 26–28/19–20 °C (day/night) for the summer period, and 25–28/14–16 °C (day/night) for the winter period under the relative humidity of 70–80%. The flowering period starts in January and continues until March. More than 50 flowers from 25 plants were artificially pollinated, and the fruits were harvested at the different stages of their development.

Confocal microscopy

The fragments of the immature fruits of different ages were fixed in 2% paraformaldehyde in 0.05 M phosphate buffer saline, pH 7.4, and stored at 4°C until use. The ovules were excised from ovaries and stained for approximately 1–2 h in a moist chamber. A water solution of each of the fluorescent dyes, i.e., acridine orange (5 mg/ml, Serva, Germany), aniline blue (20 mg/ml, Serva, Germany), calcofluor (1–5 mg/ml, Fluorescent Brighter 28, Sigma-Aldrich, USA), or ethidium bromide (1 μg/ml, Serva, Germany), was used. For the staining in dipyridamole ($C_{24}H_{40}N_8O_4$, Sigma-Aldrich, USA), an aliquot of 10–12 mg/ml was dissolved in 5% of acetic acid. Unfixed ovules were stained in a solution of fluorescein diacetate (50 μg/ml in 50% glycerol, Sigma-Aldrich, USA). In some experiments, unfixed ovules were stained with dipyridamole (10 mg/ml) or acridine orange (5 mg/ml). After the treatment, the samples were washed in distilled water several times and mounted in 50% glycerol under cover-glass at 4 °C until use. In the case of dipyridamole, the washing of the stained samples was performed with 50% glycerol. The autofluorescence of the cell structures and tissues was examined in the fixed but unstained samples, kept in 50% glycerol.

The samples were viewed in a confocal laser scanning microscope (Olympus FV1000D, Japan). To excite fluorescence, individual lasers with a wavelength of 405, 473, and

560 nm, or their combinations were used, following the dye absorption. 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. Acridine orange and ethidium bromide fluoresce in the red region of the spectrum, specifically staining the nucleic acids in the nuclei of cells (Nafisi et al. 2007). Similarly, calcofluor (Hoch et al. 2005) and aniline blue (Currier 1957) fluoresce in the blue region and specifically stain the cell wall polysaccharides. Fluorescein diacetate stains areas with esterase activity, since a green fluorescent product (fluorescein) is formed only when it is hydrolyzed by living cell esterases (Heslop-Harrison and Heslop-Harrison 1970). However, almost all the dyes used can non-specifically interact with the cell chemical compounds, changing the fluorescence spectrum of both the dyes themselves and the autofluorescence of cell structures. Thus, in order to analyze the morphological structures, we used the polychromatic images resulting from the summation of a dye-specific and non-specific fluorescence. The confocal fluorescence images alone or merged with differential interference contrast (DIC) were processed and analyzed using the ImageJ software (NIH, MD).

Scanning electron microscopy (SEM)

For the cryo-SEM studies, the seeds, as well as the freshly made sections of ovaries, were mounted to the copper plate, using a computer thermal paste AlSil-3 or KPT-8 (Russia). Then it was fixed on the cooling stage of a Deben Coolstage refrigerating unit (UK). The plant samples were viewed by the backscattered imaging, using LEO-1430 VP (Carl Zeiss, Germany) scanning electron microscope under the high vacuum mode and the temperature ranging between − 25 and − 30 °C at a 20-kV accelerating voltage and the working distance of 12–13 mm.

Cell numbering

For the case when the basal cell is not involved in the embryo proper formation, it is proposed to use the designation of the *cd* cell derivatives instead of the *cb* cell ones (Souèges 1939; Shamrov 1997). Thus, we used the following abbreviations for the different cells of the embryo: *ca*, the apical cell of the first generation; *cb*, the basal cell; *cc*, the chalazal daughter cell from the *ca* cell; *cd*, micropylar cell from the *ca* cell; *m*, chalazal daughter cell from the *cd* cell; *ci*, micropylar cell from the *cd* cell; *q*, quadrant stage; *l*, octant stage (upper-tier); *l′*, octant stage (low tier).

Results

Ovule development

The inferior ovary of *Dendrobium nobile* is unilocular (paracarpous). In the transverse sections, there are three sterile and three fertile valves in the ovary of the unpollinated flower (Figs. 1, 2a, 3a). The placentas are formed through the fusion of the adjacent carpels; thus, they have the bilobed placental ridges. The proliferative papillae (Figs. 2b, c and 3b), consisting of a small number of cells, initiate their development from the placenta region, located on the fertile valves. Initially, the neighboring placental lobes are tightly folded in pairs that resemble the folded leaves in the bud. After the pollination, the lobes start to straighten gradually with their length to shrink and their width to enlarge, resulting in the formation of new proliferative papillae.

Within 20 days after the pollination, about three to five upper subepidermal cells of ovules enter the stage of their first divisions and give rise to the ovule primordia (nucellar filaments) (Fig. 4a). The ovule primordia are formed due to the periclinal and anticlinal divisions of several subepidermal cells. The prevalence of the anticlinal divisions results in the increasing number of ovules, whereas the predominance of the periclinal divisions allows the elongation of the individual ovules.

Then the upper subepidermal cell at the distal end of the ovule divides by the periclinal division, and its upper daughter cell differentiates into the archesporial cell, whereas the inner cell forms four cells of the axial row (Fig. 4b). The two upper cells form a two-celled structure, called postament (Fig. 4b, c),

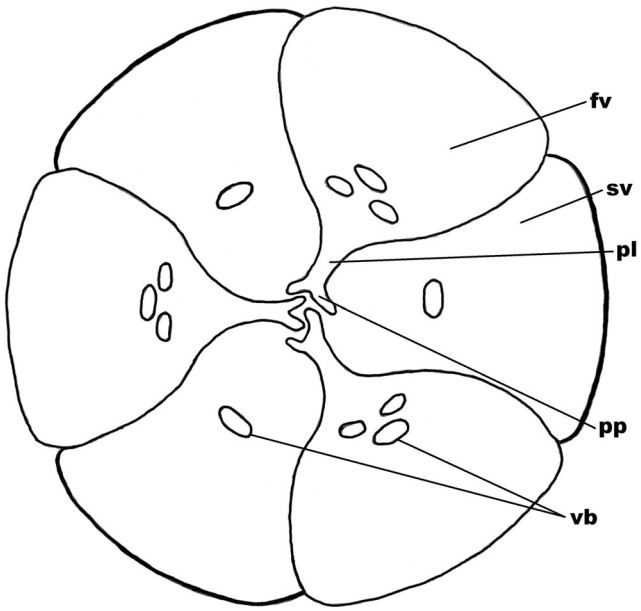

Fig. 1 The schematic organization of the unilocular ovary of *Dendrobium nobile*. *fv*, fertile valve; *pl*, placental ridges; *pp*, proliferative papillae; *sv*, sterile valve; *vb*, vascular bundle

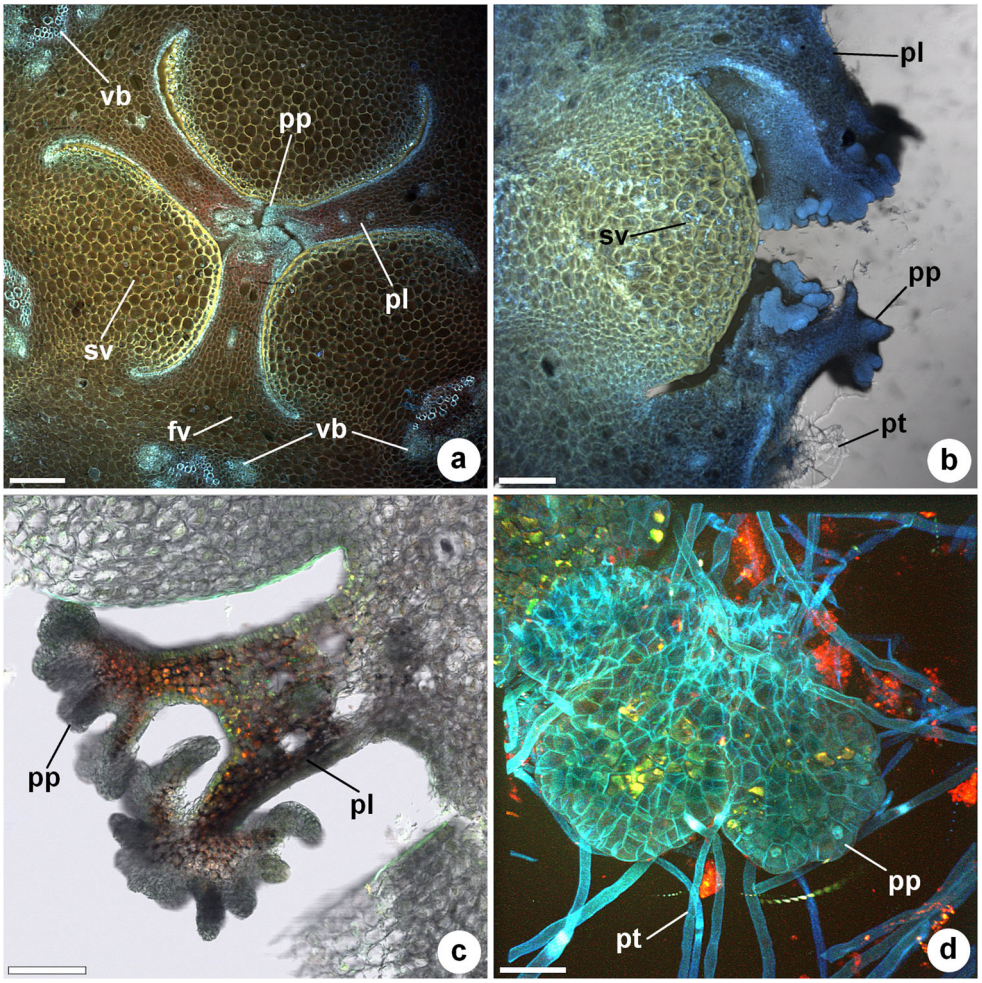

Fig. 2 The unilocular ovary of *Dendrobium nobile*. **a** The non-pollinated ovary after flower opening (dipyridamole staining). **b**, **c** The placenta at 20 days after pollination (dipyridamole staining). **d** The pollen tubes near the ovule primordia (aniline blue staining). Scale bars: **a**, **b** 150 μm; **c** 100 μm; **d** 30 μm. *fv*, fertile valve; *pl*, placental ridges; *pp*, proliferative papillae; *pt*, pollen tubes; *sv*, sterile valve; *vb*, vascular bundle

and the two lower cells form the central cells of hypostase. Thus, by the moment of the archesporial cell differentiation, the axial row of cells in *D. nobile* consists of four flattened and stacked cells, apart from the archesporial cell itself. The epidermal cells, adjacent to this structure, after the first anticlinal division, initialize the formation of the inner integument (Fig. 4c). In the axial section through the archesporial cell of the ovules, made on the stage of the inner integument formation, we can see the nucellar epidermis comprising seven rows of cells, whereas the inner integument consists of eight rows of cells. The pair arrangement of the cell rows of both the layers of the inner integument might be seen (Fig. 4d).

The ovules are attached to the placenta by a funiculus (Fig. 4e, f). The inner and outer integuments are formed from the epidermal cells in the hypostase region; they are two-layered, except a three-layered dorsal part of the outer integument (Fig. 4f). After staining of the fresh unfixed ovules with dipyridamole, the images of cells of the outer and inner integuments differed significantly. The funiculus of *D. nobile* has no vascular bundle, and it consists of three layers of short cells. Each of the three layers adjoins to the corresponding layer of the elongated cells on the dorsal side of the outer integument. However, when acridine orange was used on the unfixed ovules, funicular cells sharply differed in color from the outer integumentary cells (Fig. 4e).

The nucellus consists of a single layer of the flattened epidermal cells, surrounding the archesporium, and of both cells of the postament. Thus, at the archesporium stage, the ovule of *D. nobile* consists of a single archesporial cell (megasporocyte), two nucellar structures (single-layered nucellar epidermis and two-celled postament), hypostase cells, and two integuments.

Megasporogenesis and megagametogenesis

The megaspore mother cell undergoes meiosis I, and after the division, the two dyads are formed with a cell wall between them (Figs. 5, 6a). The further cytokinesis is suppressed in the micropylar dyad, whereas the chalazal dyad cell undergoes the second meiotic division (meiosis II). That is why in *D. nobile*, instead of a tetrad, a triad of cells is formed, where the only chalazal cell becomes a functional megaspore mother cell (Fig. 6b, Online Resource 1a, d).

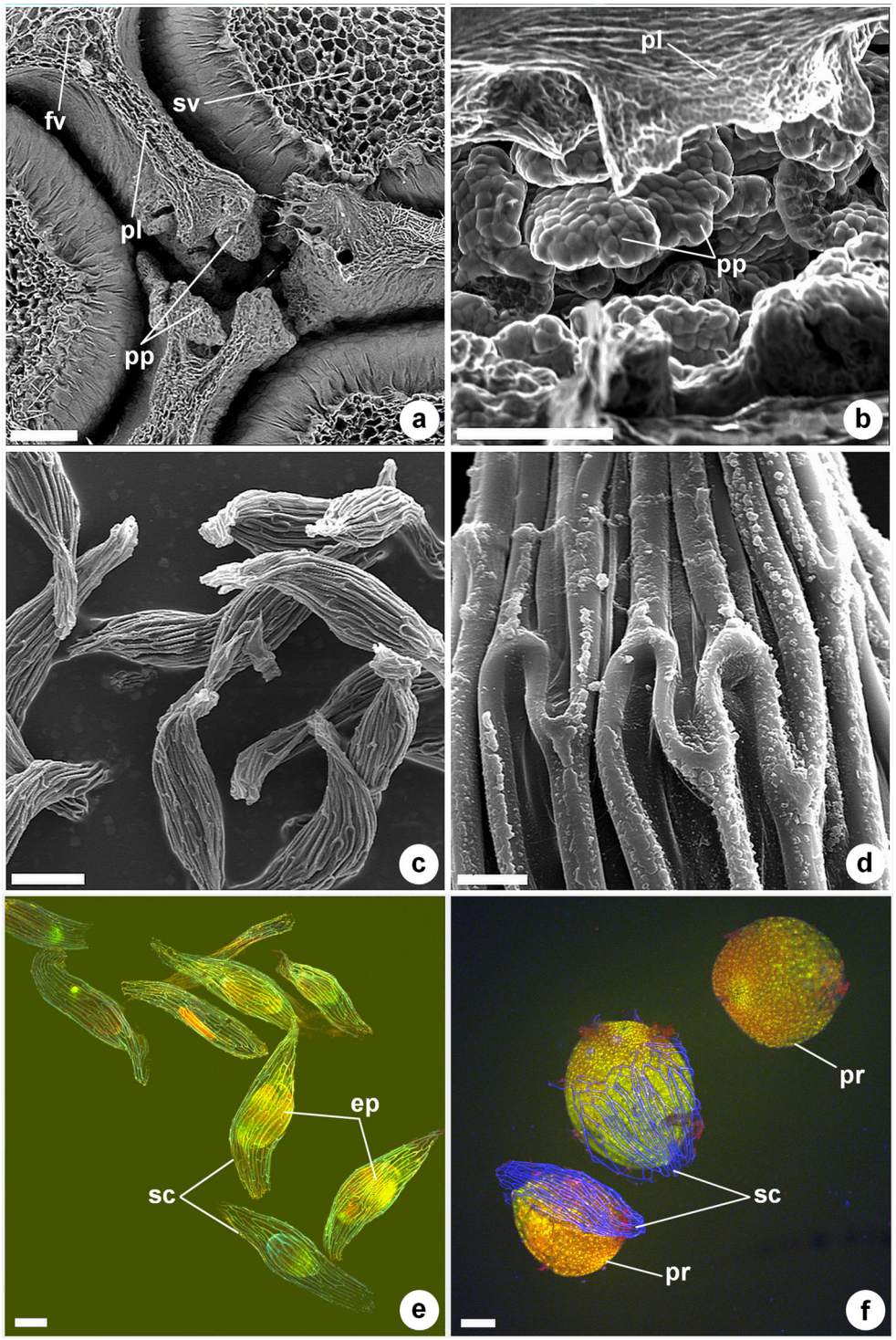

Fig. 3 The ovary and seeds of *Dendrobium nobile*. **a**, **b** The non-pollinated unilocular ovary and placental ridges after flower opening at different magnification (SEM). **c** The SEM image of the mature seeds (SEM). **d** The longitudinal seed coat ridges sculptured with verrucosities (SEM). **e** The confocal image of the mature seeds (fluorescein diacetate staining). **f** Protocorms with the splitted seed coat during their germination, 14 days after seeding (fluorescein diacetate staining). Scale bars: **a**–**c**, **e**, **f** 100 μm; **d** 10 μm. *ep*, embryo proper; *fv*, fertile valve; *pl*, placental ridges; *pp*, proliferative papillae; *pr*, protocorm; *sc*, seed coat; *sv*, sterile valve

Then the micropylar and the middle megaspores of the triad degenerate, and the remaining mononuclear haploid megaspore (Fig. 6b) develops further into the embryo sac. The nuclear material of the degenerated megaspores provides a strong and intense autofluorescence, increasing as it continues to degrade (Fig. 6c, Online Resource 1c, d). Thus, the monosporic embryo sac of *D. nobile* develops from the chalazal cell of the triad.

Between 85 and 90 days after pollination, the megaspore divides mitotically and forms a two-nucleated embryo sac (Fig. 6c). Then the second mitotic division occurs, and a four-nucleated embryo sac, consisting of two micropylar and two chalazal nuclei, appears (Fig. 6d). The third mitotic division of the nuclei results in the formation of the typical eight-nucleated embryo sac (Fig. 6e, f). One of the micropylar nuclei forms an egg cell, the other two nuclei are converted into

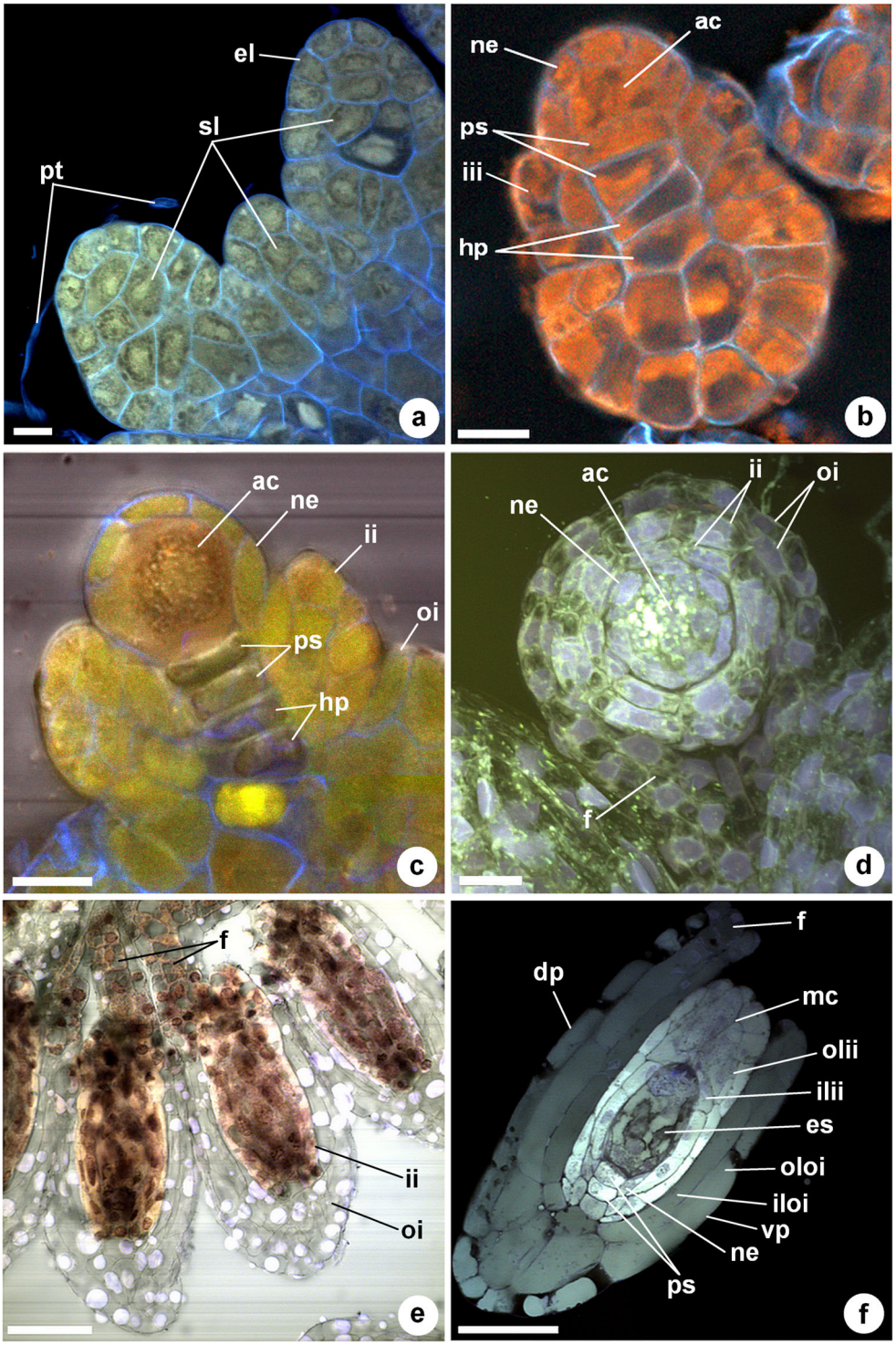

Fig. 4 An ovule formation. **a** The first divisions of the subepidermal cells of the ovule primordia 30–50 days after pollination (calcofluor staining). **b** The differentiation of the initials of the inner integument (double staining with dipyridamole, and then calcofluor). **c** The beginning of the formation of the inner integument (aniline blue staining). **d** An axial section of the ovule through the archesporial cell on the stage of the inner integument formation (dipyridamole staining). **e** A sagittal section of the three-layered funiculus attached to the three layers of the dorsal side of the outer integument (acridine orange staining). **f** Structural details of the inner and outer integuments on the sagittal section of the ovule at the stage of fertilization (dipyridamole staining). Scale bars: **a**–**d** 10 μm; **e**–**f** 40 μm. *ac*, archesporial cell; *dp*, dorsal part; *el*, epidermal layer; *es*, embryo sac; *f*, funiculus; *hp*, hypostase; *ii*, inner integument; *iii*, initials of the inner integument; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *ne*, nucellar epidermis; *mc*, micropyle; *oi*, outer integument; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument; *ps*, postament; *pt*, pollen tubes; *sl*, subepidermal layer; *vp*, ventral part

the synergids, and the fourth nucleus (the upper polar nucleus) remains membrane-free (Fig. 7). The chalazal set of nuclei consists of the lower polar nucleus and three chalazal nuclei.

In some cases, there was no clear separation between the nuclei, so they stayed very close together. Such a structure might be described as an embryo sac with fewer than eight nuclei, whereas this was a typical eight-nucleated stage. In Fig. 8f, the daughter nuclei formed after the division of one of the micropylar nuclei remained attached. One pair of such attached nuclei is located in the micropylar and chalazal parts of the embryonic sac (Online Resource 1f). These two nuclei tandem positions might be seen within the embryo sac up to the fertilization stage, and it is still questioned, whether the embryos with such developmental alterations are vital or not.

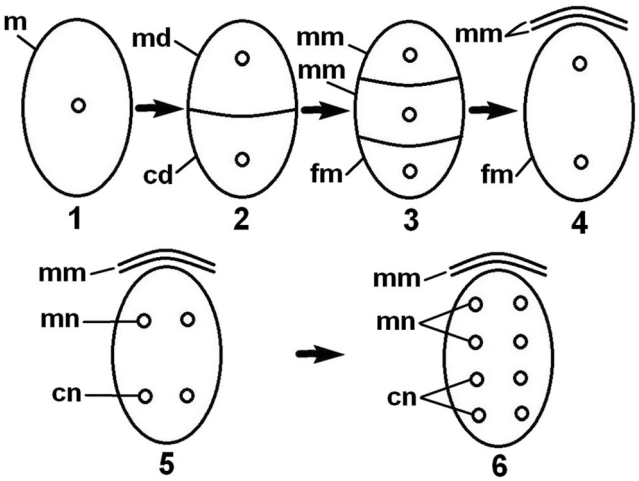

Fig. 5 Megasporogenesis and the development of the monosporic embryo sac of *Dendrobium nobile*. 1, mother megaspore cell (megasporocyte). 2, meiosis I (formation of micropylar and chalazal dyads). 3, meiosis II (a meiotic division of the chalazal dyad). 4, the death of both micropylar cells and the first mitotic division of the nucleus of the functional megaspore. 5, four-nucleated stage of the female gametophyte. 6, eight-nucleated stage of the female gametophyte. *cd*, chalazal dyad; *cn*, chalazal nuclei; *fm*, functional megaspore; *m*, megaspore mother cell; *md*, micropylar dyad; *mm*, the micropylar and middle megaspores; *mn*, micropylar nuclei

Sometimes, mitosis occurs in the micropylar region only, whereas in the chalazal region, a single nucleus is observed (Online Resource 1e).

At the time of the fertilization, the degeneration of the micropylar cells of nucellus leaves only a two-celled postament and a deformed layer, consisting of a two-cell row, adjacent to the chalazal zone of the embryo sac (Fig. 7). These structures can be observed up to the stage of the two- to four-celled embryo (Fig. 9d).

The fertilization and embryogenesis

In *Dendrobium nobile*, it takes 90–110 days from the pollination to fertilization (Fig. 7). This process can be observed most clearly due to the different color shades in the nuclei of the embryonic sac and pollen tube material when stained with ethidium bromide. The central part of the embryo sac is occupied by a large non-fluorescent vacuole (Fig. 8a, b; Online Resource 1e, f). The nuclei of the embryo sac and both integuments are colored in blue or brown (Fig. 8). The tiny nucleoli of red and yellowish color are visible inside the nuclei. The pollen tube content is colored in green with the reddish structures within it that are supposed to be the sperm's nuclear material. The pollen tube content penetrates the ovule and moves around the two synergids, bypassing them from the one side (Fig. 8a–d) or between them (Fig. 8e, f). The complex of 3 chalazal nuclei is visible at the fertilization stage, and it remains up to the stage of the quadrant formation (Fig. 9f).

This central cell nucleus may be observed in different positions, regarding the large central vacuole location. In some cases, it is in the central part of the embryo sac, closer to its micropylar part, but in most cases, it is in the chalazal part (Fig. 8b, d). It occurs more often when, after the fertilization, the sperm nucleus does not fuse with the polar nuclei, so no primary endosperm is produced.

We observed two (Fig. 8b–d) or three unfused nuclei (Online Resource 2), which were usually localized inside the embryo sac. Sometimes, polar nuclei remain attached even up to the stage of a multicellular embryo (Fig. 11c), until they are degraded and digested by the growing embryo. However, in other embryo sacs, only one large central nucleus is visible that may indicate nuclear fusion in the central cell (Fig. 9a, c).

The processes of the fertilization of the mature embryo sac and the development of the embryos in *D. nobile* do not occur simultaneously in all the ovules. Approximately 4.5 months after the pollination, we observed different stages of the embryogenesis from the zygote (Fig. 9a) to the multicellular embryo. The events of *D. nobile* embryogenesis are shown in Table 1.

In Fig. 9b, the larger cell is designated as *cb*, and the smaller cell is designated as ca. The nuclei of each cell are visible. The *ca* cell divides periclinally, generating a two-celled embryo proper ($cc + cd + cb$) (Fig. 9c).

The *cb* cell does not divide during the embryogenesis, gradually transforming into the hyper-elongated one-nucleated suspensor with the large vacuoles within. In Figs. 9c–e and 11a, the successive stages of *cb* cell development and its transformation into a suspensor can be seen. Upon the cell specialization, the amount of its cytoplasm is gradually increased and there is only one nucleus. On the contrary, both the nuclei and the cell walls of the embryo proper are quite distinguishable. In Fig. 9f, it is clearly seen that a 6-celled embryo proper has a unique nucleus in the suspensor cell, and it is hard to imagine that this cell with a huge vacuole is capable of division in the future. After the formation of the blades, only this single nucleus can be seen (Fig. 11e, f).

At the tetrad stage, there are a three-celled embryo proper and a one-celled elongated suspensor (*cb*), extended at the end. The nucleus of the suspensor cell moves to this distal end (Fig. 9e, f). The embryo, including the suspensor cell, remains within the embryo sac. For the formation of a five-cell embryo, the anticlinal divisions may take place, when the *cc* cell or the *m* cell divide, giving rise to a two-celled *cc*-layer (Fig. 9e) or a two-celled *m*-layer, respectively. The formula at this stage is as follows: $2cc + m + ci + cb$ or $cc + 2m + ci + cb$.

The variations in cell division patterns were also noticed at the stage of the seven- to the eight-cell embryo. The usual elongation of the suspensor cells and the formation of the upper layer quadrant according to the *cc* $(q + m + ci + cb)$ formula were observed (Fig. 9f). At this stage, the diminished polar nuclei as well as the two cells of the postament,

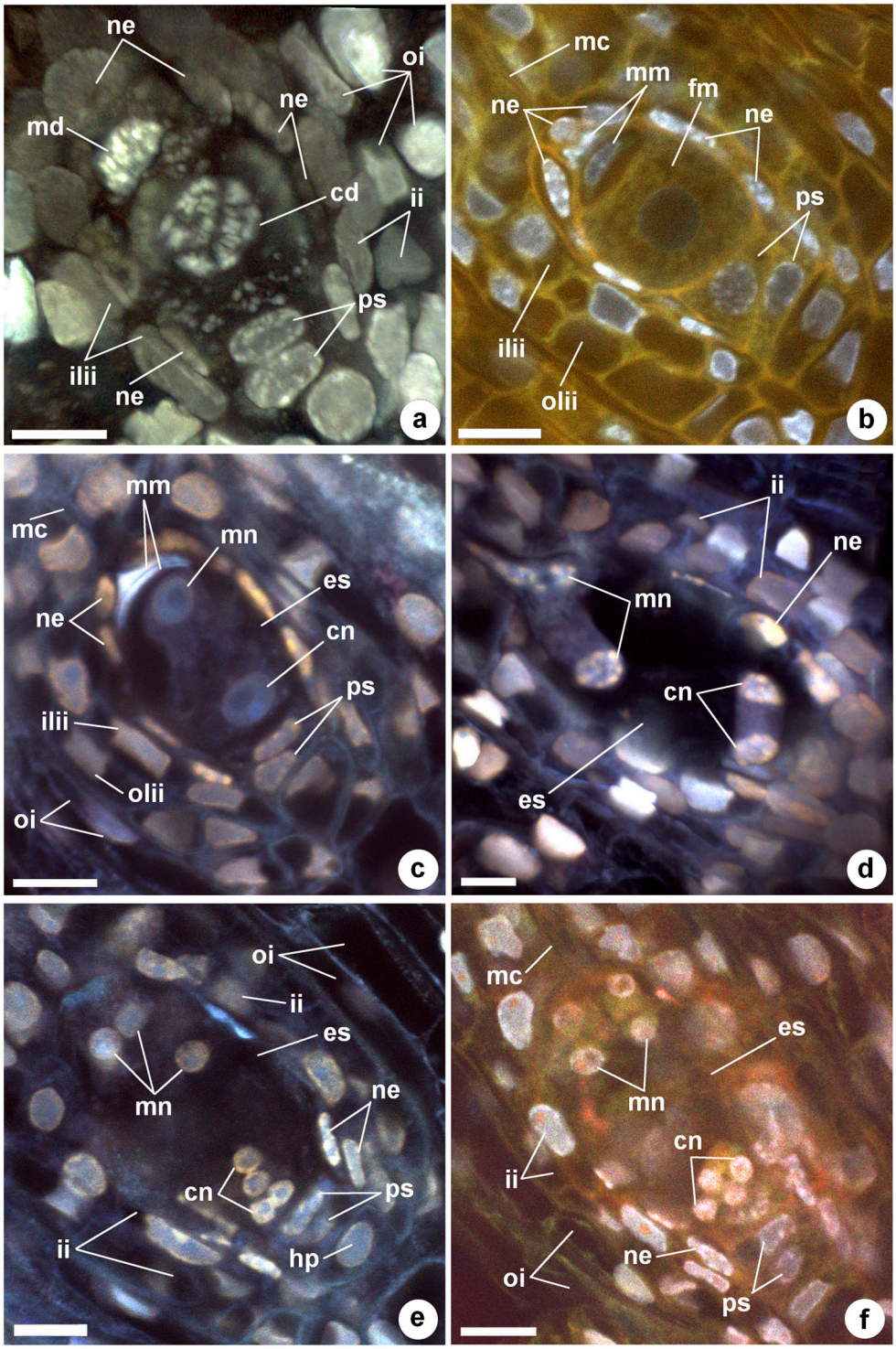

Fig. 6 Megasporogenesis (75–85 days after pollination) and megagametogenesis (85–90 days after pollination). **a** The first division of the mother megaspore cell (megasporocyte), meiosis I, and the beginning of degradation of the micropylar megaspore (autofluorescence). **b** Degeneration of the middle megaspore (dipyridamole staining). **c** Binucleated embryo sac, both nuclei are of the same size (calcofluor staining). **d** Nuclei division in the binucleated embryo sac (calcofluor staining). **e** Seven-nucleated embryo sac (calcofluor staining). **f** Eight-nucleated embryo sac (ethidium bromide staining). The micropylar end is in the upper left corner. Scale bar: **a**–**f** 10 μm. *cd*, chalazal dyad; *cn*, chalazal nuclei; *es*, embryo sac; *fm*, functional megaspore; *hp*, hypostase; *ii*, inner integument; *ilii*, inner layer of inner integument; *md*, micropylar dyad; *mc*, micropyle; *mm*, micropylar megaspore; *mn*, micropylar nuclei; *ne*, nucellar epidermis; *oi*, outer integument; *olii*, outer layer of inner integument; *ps*, postament

hypostase, and the remnants of the nucellar epidermis might be seen in the chalazal region of the embryo sac.

Although the cells of the inner integument keep alive, the widened apical end of the yet unbranched suspensor already enters the micropylar cavity (Figs. 10, 11b). The formula of the embryo at the octant stage is as follows: $l + l' + m + ci + cb$. At this stage, the *cb* suspensor cell elongates and grows out of the embryo sac, where it is transformed into an amoeba-like structure with several irregularly shaped blades (Figs. 10, 11c–e). The blades of suspensor are surrounded by a thin mantle, which can be seen by its autofluorescence, or when it is stained with certain dyes (Fig. 11b–f).

At the late globular stage, the cells of the inner integument are gradually collapsing and disappearing (Fig. 11c, d). The

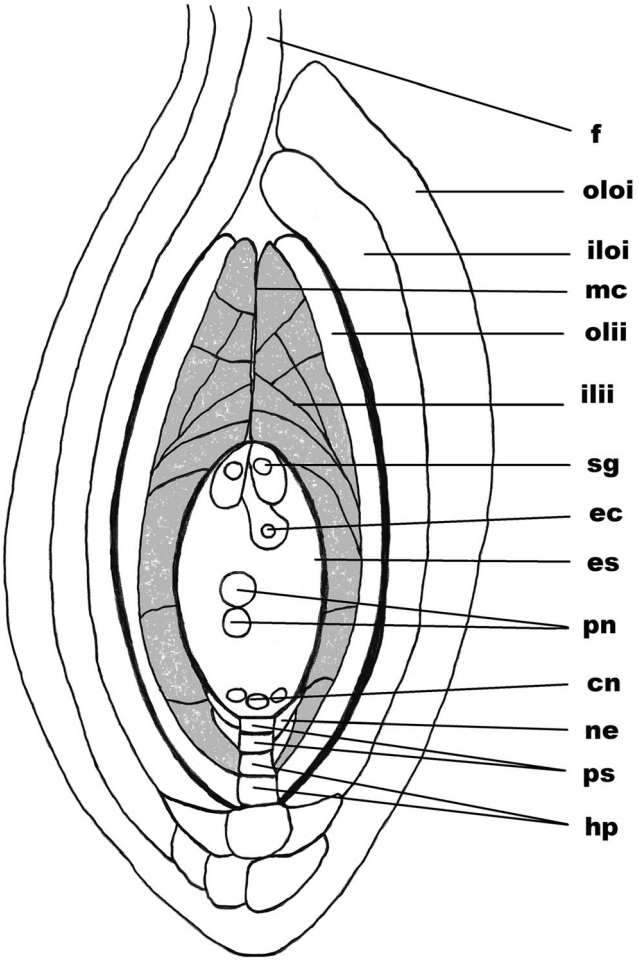

Fig. 7 The schematic organization of *D. nobile* ovule. *cn*, chalazal nuclei; *ec*, egg cell; *es*, embryo sac; *f*, funiculus; *hp*, hypostase; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument; *ne*, nucellar epidermis; *mc*, micropyle; *sg*, synergids; *pn*, polar nuclei; *ps*, postament

cells of the postament are completely crashed (Table 2). The *cb* cell of the suspensor remains alive until the inner layer of the outer integument is destroyed. Interestingly, the morphology of the *cb* cell changes greatly during its development; it is elongated and unbranched when the suspensor is inside the embryo sac, and it enlarges and branches into several blades when the suspensor is outside the embryo sac and the inner integument (Fig. 11c, d). An average lifespan of this *cb* cell is about 60 days. The *ci* cell (the lowest cell of the embryo proper) does not divide during the embryogenesis. After the death of the *cb* suspensor cell, the *ci* cell transforms into the basal cell of the embryo proper (Fig. 10).

At the stage of a 3-cell embryo, the longitudinal anticlinal cell walls of the inner layer of the inner integument get thickened. Without staining, the secondary material was only distinguished by autofluorescence of brownish hues (Online Resource 3a), and it was intensely red when the fluorescent dyes (calcofluor or ethidium bromide) were used (Fig. 11b, c, Online Resource 3b).

The polar nuclei were not lysed at this stage (Fig. 11c), and they persisted until the total degeneration of the embryo sac. In some cases, the suspensor remained unbranched and bladder-shaped, and it did not expand through the embryo sac (Fig. 11a). Thus, the lack of endosperm formation and the delayed embryogenesis characterize the seed development of *D. nobile*.

Seed coats

Approximately 45 to 60 days after pollination, the inner integument elongates up to the central part of the archesporium, and the ovule bends 180 degrees and becomes anatropous. The funiculus structure might be well seen (Fig. 4d–f). In *D. nobile*, the processes of megaspore mother cell differentiation, the formation of the inner integument primordia, and funicular formation are taking place fairly simultaneously. The inner integument appears before the outer one (Fig. 4b) and is resorbed before the inner layer of the outer integument. The outer integument appears later (Fig. 4c) but develops faster than the inner one, and completely covers it at the stage of the mature embryo sac development. As a result, the ovule becomes anatropous and bitegmic with a micropyle derived from the inner and outer integuments (Fig. 4e, f, Fig. 7).

At the stage of megasporocyte, the inner integument is about one-half of the length of the nucellus, and the outer integument is still shorter, while at the stage of the mature embryo sac, the outer integument grows over the inner integument and surrounds it (Figs. 4e, f and Fig. 7). During the fertilization stage, the outer integument extends, leaving the entrance of the micropyle open. Thus, the inner integument forms a thin micropyle with the closely spaced ends of the integument.

The degeneration of the inner integument starts after the fertilization and the first divisions of the zygote. The process begins with the thinning of the inner layer of the inner integument. The blades of the suspensor leave the embryonic sac through a micropyle into the space, which is formed during the growth of the seed coats and the subsequent cell elimination of the inner integument and the inner layer of the outer integument (the so-called micropylar cavity). The two cells of the postament are still visible at the four-celled embryo stage (Fig. 9e), and they will further simultaneously disappear with the elimination of the inner integument.

Our data reveals the variability in the embryogenesis of *D. nobile*, which might be seen in both morphological diversity and dynamics of the appearance and elimination of the embryonic structures. The fruits of *D. nobile* with the mature seeds are usually opened within 12 months after the pollination. Interestingly, there is a period of 120 to 180 days during the embryogenesis, when one fruit may contain the ovules of different developmental stages (starting from the quadrant stage to the well-developed multicellular embryos). The following 6 months are needed for the maturation of the lagging embryos. Finally, the suspensor is eliminated, and the

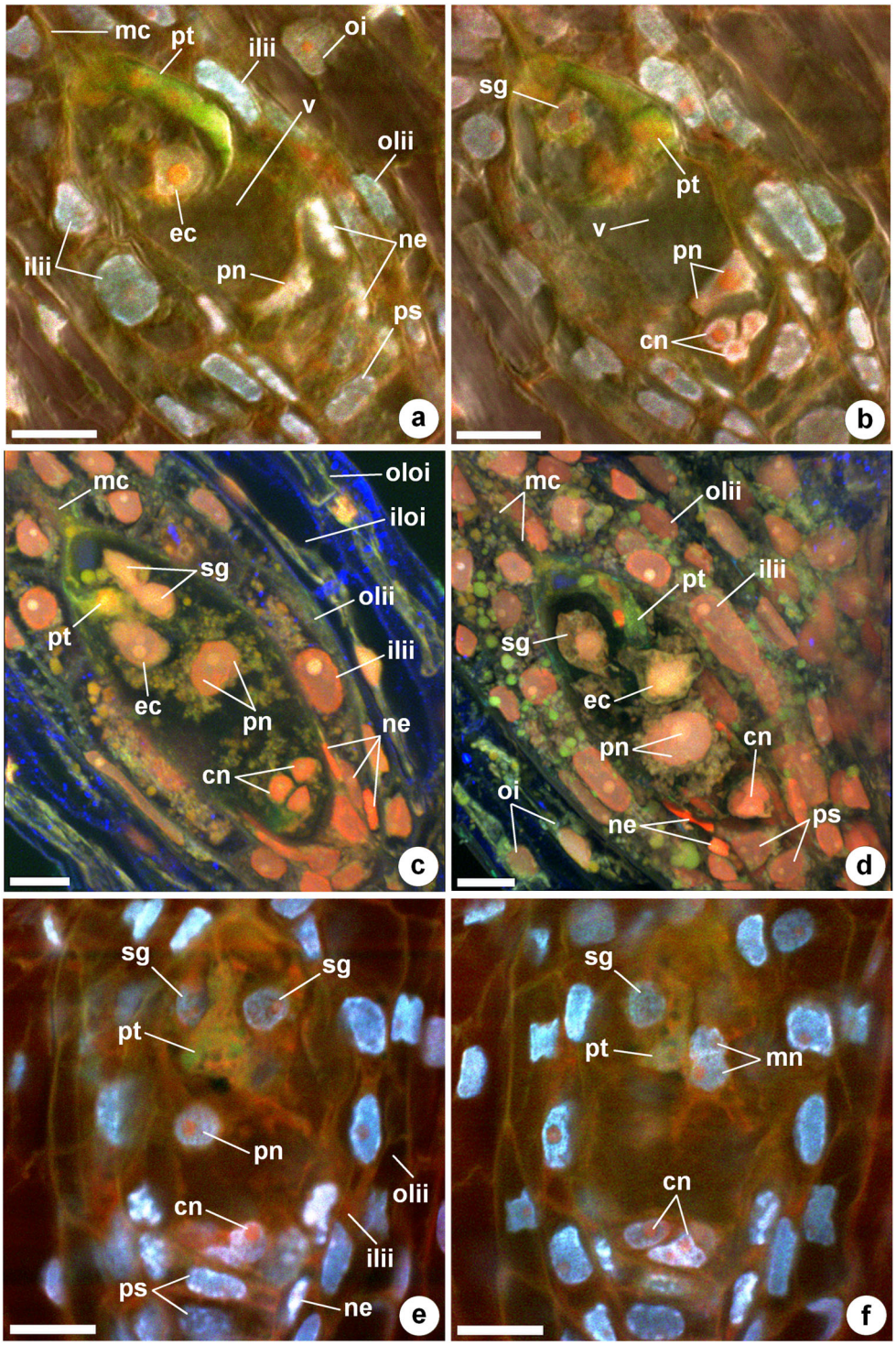

Fig. 8 Fertilization (90 days after pollination; ethidium bromide staining). **a**, **b**, **e**, **f** The paired optical sections of two different stacks. **a**–**d** The content of the pollen tube penetrates the embryonic sac in the micropylar zone through the micropyle, extending around the synergid cell, and finally approaches the egg cell. **e**, **f** Both synergids are visible, a pollen tube passes between them. **b**, **c**, **f** All 3 chalazal nuclei are visible in the chalazal part of the embryo sac; the attached polar nuclei are located in the center of the embryo sac (**c**) or its chalazal part (**b**, **d**). **f** The daughter nuclei formed after the division of one of the micropylar nuclei remained attached. In all images, the degenerating cells of the nucellar epidermis are visible in the chalazal part. The micropylar end is in the upper left corner. Scale bar: **a**–**f** 10 μm. *cn*, chalazal nuclei; *ec*, egg cell; *mc*, micropyle; *mn*, micropylar nuclei; *ne*, nucellar epidermis; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *olii*, outer layer of inner integument; *oi*, outer integument; *oloi*, outer layer of outer integument; *pn*, polar nuclei; *ps*, postament; *pt*, pollen tube content; *sg*, synergids; *v*, vacuole

integuments are degenerated, except the outer layer of the outer integument, which then transforms into the single-layered seed coat.

The shape of *Dendrobium* seeds is oblong, about 450–550 μm in length (Fig. 3c, e). The seed coat of *D. nobile* seeds is uniseriate and derived from the outer layer of the outer integument. The cells of the seed coat are elongated. The longitudinal anticlinal cell walls of the outer cells of the ovule outer integument get closer together after the protoplast degradation. Their walls form ridges covered with minute scattered verrucosities (Fig. 3d). There are no distinct gaps or perforations between the walls. When mature, the seed color is light yellow to brownish. The seed coat degradation is taking place during the germination process when the embryo swells and enlarges with the further conversion into the protocorm-like body (Fig. 3f).

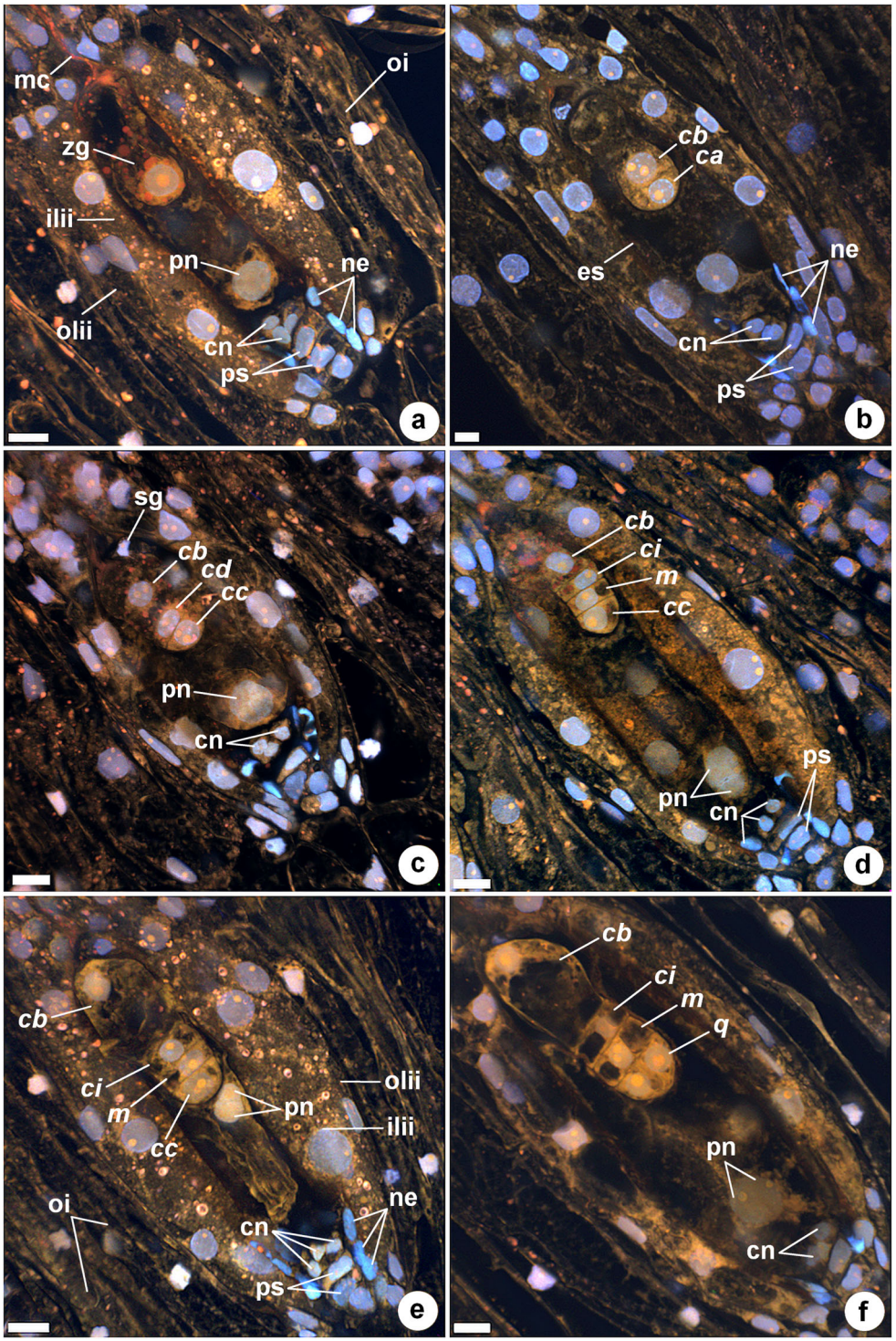

Fig. 9 Embryogenesis (100–120 days after pollination, autofluorescence). **a** A zygote, attached polar nuclei and three chalazal nuclei in the embryo sac. **b** A two-celled embryo. **c** A three-celled embryo; *ca* cell is divided into *cd* cell and *cc* cell. **d** A four-celled embryo, the attached different sized polar nuclei and chalazal nuclei. **e** A four-celled embryo, the attached different sized polar nuclei are located near the embryo. **f** A five-celled embryo and a central cell nucleus consisting of two or three attached nuclei. The micropylar end is in the upper left corner. Scale bar: **a**–**f** 10 μm. *ca*, *ca* cell; *cb*, *cb* cell; *cc*, *cc* cell; *cd*, *cd* cell; *ci*, *ci* cell; *cn*, chalazal nuclei; *es*, embryo sac; *ilii*, inner layer of inner integument; *m*, *m* cell; *mc*, micropyle; *ne*, nucellar epidermis; *oi*, outer integument; *olii*, outer layer of inner integument; *pn*, polar nuclei; *ps*, postament; *q*, quadrant layer of the embryo; *sg*, synergids; *zg*, zygote

Discussion

One of the distinctive features of the reproductive strategy of orchids is the formation of a large number of tiny seeds. Under natural conditions, they can only germinate with an appropriate mycorrhizal fungus. The range in length of the "dust seeds" of orchids is varying between 50 μm in *Anoectochilus imitans* Schltr. (Arditti and Ghani 2000) and 2860 μm in *Sobralia macrantha* Lindl. (Kolomeitseva et al. 2012). The minute size provides a relatively small energy investment and it is realized through the (i) formation of the small and undifferentiated embryo without cotyledon and embryo root, (ii) significant reduction of the endosperm, and (iii) the elimination of the ovule's maternal tissues, except the outer layer of the outer integument.

Table 1 The embryogenesis of *Dendrobium nobile* from the zygote to the octant stage embryo

Stages of embryogenesis	Formula
Fertilized ovule	Zygote
Two-celled embryo	*ca* + *cb*
Three-celled embryo	*cc* + *cd* + *cb*
Tetrad stage	*cc* + *m* + *ci* + *cb*
Quadrant stage	*q* + *m* + *ci* + *cb*
Octant stage	*l* + *l'* + *m* + *ci* + *cb*

Thus, the underdevelopment and elimination of some embryonic structures are a key point of the orchid embryogenesis.

As we see for the ovary of *Dendrobium nobile*, it is inferior, unilocular, tricarpellary, and syncarpous, which is consistent with the data obtained for *D. ovatum* (Gurudeva 2016). In the ovaries of the unpollinated *D. nobile* flowers, there is no or faintly differentiated ovules, consisting of few cells, which are located on the outgrowths of the parietal placentae. After the pollination, these placental outgrowths begin to branch and form numerous nucellar filaments. According to our data, the axial row of the ovule primordia in *D. nobile* is already expressed at the stage of the first divisions of the initials in the inner integument. It consists of 4 cells, excluding an archesporial cell, whereas two distal cells of the axial row are the postament cells, and its two basal cells are the central cells of the initials of the integuments. Ochora (2000), studying the reproductive biology of some Kenyan species of the orchids, reported that the number of cells in the axial rows of the ovules of the different orchid species is strictly predetermined. However, it was shown recently that there was a different number of axial cells among the species of the genus *Dendrobium*. *Dendrobium ovatum* possesses from 6 to 8 of them (Gurudeva 2016), whereas there are from 3 to 7 cells in an axial row of a hybrid *Dendrobium* sp. (Israel 1962). Such discrepancies may be explained by the undetermined definition of which cells should be considered axial row ones. In the case of *D. nobile*, we considered that apart from the archesporial cell, the axial row contains of only two cells of the postament and two cells of hypostase (Fig. 4c). Developed ovules of *D. nobile* are anatropous and bitegmic.

Previously, most orchid species were classified into the tenuinucellate type, based on the slender, single-layered, and short-lived nucellus (Maheshwari 1950). Other authors (Lysyakova and Klimenko 2009) reported the crassinucellate ovules in *Orchis pallens* L. with the developed lateral part of the massive, multilayered, and long-lived nucellus. Shamrov (1998, 2008) distinguished another type of the ovule morphogenesis, an intermediate medionucellate type of multilayered subvariation, with a long-existing lateral area of the nucellus, including the postament and the chalazal part of the nucellar epidermis.

The results of this study show that the biggest part of the nucellar epidermis of *Dendrobium nobile* disintegrates before the fertilization, the hypostase is formed under the two-celled postament, and the lateral region is absent. The basal part of the nucellus, represented by a 2–3-rowed epidermal layer and a two-celled postament, does not disappear until the degeneration of the embryo sac and the inner integument. Thus, the nucellus of *D. nobile* refers to the medionucellate type.

There is no vascular bundle in the orchid ovules (Clements 1999), so the role of the funiculus is to connect the placenta with the embryo sac (Shamrov 2008). In *D. nobile*, the funicular cells, which may differ by size or color (Fig. 4c), are attached to the corresponding layers of the outer integument in the dorsal part of the ovule. This

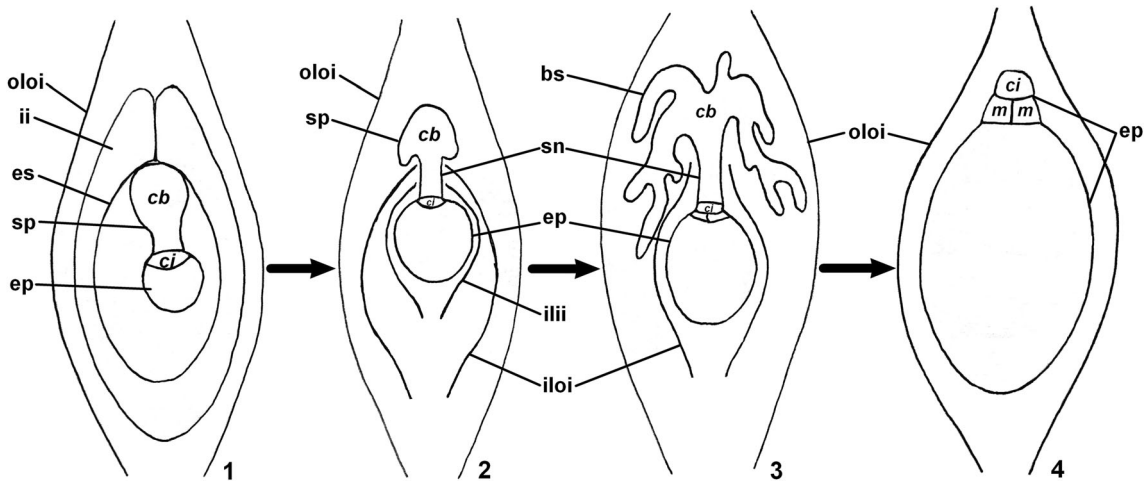

Fig. 10 The stages of suspensor development and degeneration in *Dendrobium nobile*. 1, unbranched unicellular (*cb*) suspensor within the embryo sac. 2, the exit of the suspensor outside the embryo sac and inner integument. 3, the development of suspensor blades in the micropylar space of the outer integument. 4, the embryo with eliminated suspensor; in the basal part, *ci* cell and 2 cells of layer *m* are preserved. *bs*, suspensor blades; *es*, embryo sac; *ep*, embryo proper; *ii*, inner integument; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument; *sn*, suspensor neck; *sp*, suspensor

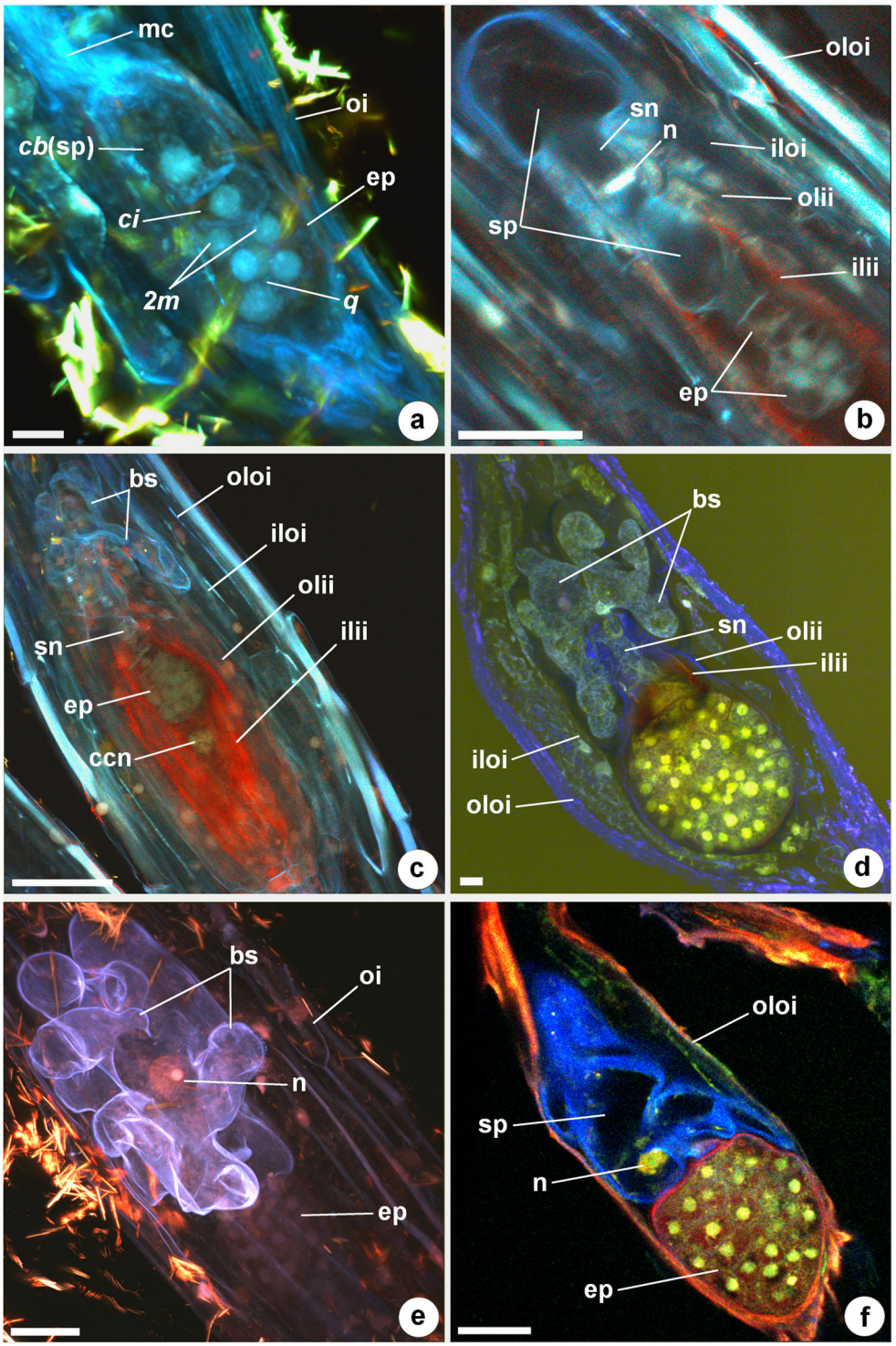

Fig. 11 A mature embryo of *Dendrobium nobile*. **a** An eight-celled embryo with an unbranched suspensor (calcofluor staining). **b** The transition of the suspensor cell into the micropylar cavity at the six-celled embryo stage (calcofluor staining). **c** A multicellular embryo with a branched suspensor and unfused polar nuclei, the outer layer of the inner integument is not yet degenerated (calcofluor staining). **d** A multicellular embryo with a well-developed branched suspensor, the inner layer of the outer integument is absent (fluorescein diacetate staining). **e** A unicellular branched suspensor (calcofluor staining). **f** A multicellular embryo with a unicellular branched suspensor (ethidium bromide staining). The micropylar end is in the upper left corner. Scale bars: **a**, **d** 10 μm; **e** 20 μm; **b**, **f** 30 μm; **c** 50 μm. *2m*, two-celled *m*-layer; *bs*, suspensor blades; *cb*, *cb* cell; *ci*, *ci* cell; *ep*, embryo proper; *es*, embryo sac; *ilii*, inner layer of inner integument; *iloi*, inner layer of outer integument; *mc*, micropyle; *n*, nucleus; *oi*, outer integument; *olii*, outer layer of inner integument; *oloi*, outer layer of outer integument; *pn*, polar nuclei, and sperm; *q*, *q*-layer; *sn*, suspensor neck; *sp*, suspensor

data confirms the results reported previously for another angiosperm, *Ornithogalum caudatum* Ait. (Liliaceae), in which the proximal portion of an outer integument is congenitally fused with the funiculus (Tilton and Lersten 1981). The acridine orange-stained unfixed ovules displayed the same color of the funicular cells and the cells of the inner integument of *Dendrobium nobile* (Fig. 4e). Thus, morphologically, the funiculus is attached to the outer integument, whereas histochemically, it is more similar to the inner integument. This might be explained by the fact the tissues are both involved in the transport of the nutrients to the embryo. On the stage of the globular embryo, the necessity for such transport is no longer required, and the cells of the inner integument die off. The outer layer of the

Table 2 The development and the elimination of the maternal structures in *Dendrobium nobile* ovules and seeds

The structure	The number of layers (longitudinal section)	The initiation stage	The elimination stage	Presence in the mature seed
Nucellus (epidermis)	1	The ovule attached to the placenta	Partially eliminated on the fertilization stage, and degenerated completely on the stage of the 7- to 8-celled embryo	Absent
Nucellus (postament)	2	The isolation of the archespore cell	The elimination of the inner integument	Absent
Hypostase	2	The isolation of the archespore cell	Fertilization	Absent
Inner integument	2	Megasporocyte	Octant stage	Absent
Outer integument	2, 3	Megasporocyte	The inner layer eliminates at the stage of the globular embryo	The dead outer layer only
Funiculus	3 × 3	Start of meiosis	Early globular embryo	Absent

outer integument serves its protective role during all stages of the embryo development, forming the seed coat.

In *D. nobile*, the meiosis II and cytokinesis occur only in the functional chalazal cell of the dyad, resulting in the megaspore triad formation. Such a scenario has been confirmed in many orchids (Swamy 1949; Govindappa and Karanth 1980; Attri et al. 2007; Gurudeva 2016). We also observed the formation of a bipolar and eight-nucleated embryo sac with three mitotic divisions within the chalazal functional megaspore (Fig. 6c–f). A further simplification with the reduction of mitosis in the second nucleus of the chalazal zone as well as the formation of a unipolar embryo sac was observed in *D. ovatum* (Gurudeva 2016).

The reduced embryo sacs with six (Poddubnaya-Arnoldi 1958) or five to six nuclei (Poddubnaya-Arnoldi 1959) were found in *D. nobile*. Gurudeva (2016) also reported six nuclei in the embryo sac of *D. ovatum*. All those five to six-nucleated embryo sacs possessed a single chalazal nucleus. In this study, a typical development, resulting in the formation of a mature eight-nucleated embryo sac of *D. nobile*, was noticed. Rarely, at the gametogenesis stage, there was an abnormal development with the undivided nuclei of the embryo sac observed. In orchids, the reduction in the number of nuclei also leads to a decrease in the number of antipodals. In the six-nucleated embryo sacs of *Calanthe* Veitchii (Poddubnaya-Arnoldi 1958) and *Gymnadenia conopsea* (L.) R.Br. (Shamrov and Nikiticheva 1992; Shamrov 2008), only one chalazal nucleus has been observed. In our experiments, in the normally developed eight-nucleated embryo sac of *Dendrobium nobile*, the trophic complex with three chalazal nuclei appeared to be quite stable. It was degraded only after the formation of a 6–8-cell embryo.

The presence of three antipodals or three chalazal nuclei is a key characteristic of the normal eight-nucleated embryo sac formation. In this study, the embryo sacs with three chalazal nuclei prevailed. In case when there were less than three antipodals, the conclusion about the initial number of nuclei in the embryo sac could not be made, since it was not evident, whether this was the final number of nuclei or they would divide.

At the moment, the cellular and nuclear structure of the antipodals in the embryo sacs of orchids is not keenly studied. According to our observations, the antipodals of *D. nobile* are only represented by nuclei without any cell walls (Figs. 8, 9). The same results were recently reported for some other orchids, such as *Epidendrum radicans* Pav. ex Lindl. (Gurudeva and Govindappa 2008), *Dendrobium ovatum* (Gurudeva 2016).

In his early works, Navaschin (1900) considered the absence of the endosperm in orchids. Poddubnaya-Arnoldi (1958, 1959) proposed several options for the possible sequence of events after the two attached but not fused polar nuclei meet the sperm nucleus. Later (Poddubnaya-Arnoldi 1976), she reported for *D. nobile* the existence of a primary endosperm, which was formed by the fusion of the polar nuclei with sperm; however, this single endosperm structure degraded very rapidly. In our work, we saw the central cell nucleus, which was also formed by the two joined but not fused polar nuclei (the most typical option) (Figs. 8b, c and 9d, e) or a single large central cell nucleus (Fig. 9a, c). Three joined but not fused nuclei with one belonging to the sperm nucleus were sometimes observed (Online Resource 2a, b).

In *D. nobile*, the morphological lability of the suspensor cell, globular at the beginning and finally elongated and brunched, was reported by Poddubnaya-Arnoldi (1959, 1976), where the suspensor blades tightly covered the embryo. However, in our study, all the suspensor blades were raised above the embryo proper due to the suspensor neck (a narrowed zone that connects the basal part of the suspensor

cell to the embryo proper), thus entering into the micropylar cavity of the outer integument. It is also known that when cultivated on the nutrient media, the suspensor of the fertilized ovules of *D. nobile* remained globular and unbranched (Poddubnaya-Arnoldi 1976).

As we showed, the one-cell suspensor of *D. nobile* passed in its development through the three stages: the stage of an unbranched suspensor inside the embryo sac, the stage of the branched suspensor within the integumental space, and the final stage of the cell death. Starting from the degeneration of the embryo sac (about 120–140 days after the pollination) up to the elimination of the inner layer of the outer integument (160–180 days after the pollination), it is more likely that a large and branched suspensor with amoeboid branches becomes the main nutrition source for an embryo by absorbing nutrients from the inner layer of the outer integument. The formation of the branched suspensor cell with long and winding blades was observed simultaneously with the degeneration of residual nucellus, endosperm, and the living cells of the inner integument.

There are different classifications of the orchid embryogenesis. Swamy (1949) classified it into three groups, according to the cell division types: group A (Asterad type), group B (Onagrad type), and group C (Cymbidium type). In group A, the initial suspensor cell (*ci*), a middle cell (*m*), and a terminal cell (*ca*) participate in the embryo formation. In group B, the *ci* cell gives rise to the suspensor, whereas the two other cells, *m* and *ca*, participate in the embryo formation. In group C, the irregular divisions of the two-cell proembryo are typical for both the basal (*cb*) and the terminal (*ca*) cells. Sriyot et al. (2015) reported the Onagrad type of embryogenesis in *Spathoglottis plicata* Blume, whose embryo was formed from the derivatives of the terminal (*ca*) and middle (*m*) cells. Clements (1999) characterized this type of embryogenesis as plesiomorphic (sharing some primitive traits). However, the *cb* cell of *S. plicata* divides, giving *ci* and *m* cells, whereas, in *D. nobile*, the *cb* cell does not divide during the embryogenesis at all. That is why, in our study, we used another classification that was reported by Souèges (1939). According to Souèges, the embryos with a non-divided *cb* cell correspond to the second cell generation. The *cb* cell does not change its literal designation, and the *ca* cell is considered a non-divided zygote. The *cb* cell did not divide during the whole process of the embryogenesis; thus, we could not classify the embryogeny of *D. nobile* either to Onagrad type, as it was described by Sriyot et al. (2015), or to the plesiomorphic Cypripedioid-type (Clements 1999). In general, the order of the first cell divisions in the embryo of *D. nobile*, up to its octant stage, corresponds to the Caryophyllad-type of the embryogenesis (Johansen 1950). The main differences are in the earlier finishing of the embryo formation and the failure of the stages for the development of the radicle and cotyledons, since they are absent in the family Orchidaceae.

Our results clearly show that the cell divisions of *D. nobile* embryo, starting from the zygote up to the octant stage, scarcely differ from those of the orchids with the developed one-celled suspensor. As an example, *Dienia ophrydis* (J.Köenig) Seidenf. and *Liparis parviflora* (Blume) Lindl. from the Malaxideae tribe might be considered (Kolomeitseva et al. 2017, 2019). The embryogenesis of these species is also comparable by the absence of the *ci* cell division. However, in these three species, the shape of the suspensor differs at the latter stages of the embryogenesis. Thus, in *Dienia ophrydis*, the suspensor is spherical and unbranched, with a neck, and in *Liparis parviflora*, the suspensor is lobed and sessile, without neck, whereas in *Dendrobium nobile*, the suspensor is lobed with a neck. In general, the consequence of the first cell divisions (up to the octant stage) corresponds to the Caryophyllad-type of the embryogenesis (Johansen 1950). The only exclusion is that there is no formation of the radicle and/or cotyledons.

The seed germination of some orchids is prevented by the cutinized layer resulting from the dying off of the inner integument. During the seed maturation of the terrestrial orchid *Cephalanthera falcata* Blume, the inner layer of the inner integument becomes cutinized, and a layer outside the inner integument becomes lignified (Yamazaki and Miyoshi 2006). Previously, Vasudevan and van Staden (2010) reported the presence of the similar cutinized layer in the inner integument of *Dendrobium nobile*, which was discontinuous at the suspensor region of the embryo sac. The gaps in the cuticular layer of the inner layer of the inner integument might prevent the accumulation of the phytoinhibitors and promote the water and nutrient uptake by the germinating seed. According to our observations, the fluorescent material that surrounds the embryonic sac probably marks the same morphological structure. However, we did not observe any perforations, except for the hole in the micropylar zone formed during the destruction of the inner integument. The suspensor blades break through the degenerating inner integument and spread into the space limited by the outer integument.

The results of our study are in agreement with those of Poddubnaya-Arnoldi (1958, 1959) and Vasudevan and van Staden (2010). The most important is that several structures of the female gametophyte and embryo of *D. nobile* have a temporary function and then they are eliminated. The embryonic development of *D. nobile* has several characteristic features, many of which are described in this paper for the first time. The *D. nobile* ovules are characterized as medionucellar with the remnant nucellus (consisting of two cells of the postament and 2 to 3 rows of cells of the nucellar epidermis). It remains in the chalazal section up to the elimination of the inner integument. The normally developed embryo sacs contain eight nuclei. At the latter stages, the presence of three chalazal nuclei is a marker of the eight-nucleated embryonic sac. The central cell nucleus, which is formed by two or three

attached but not fused nuclei as well as their fusion products, remains until the inner layer of the inner integument is degraded. During the embryogenesis, no division was noted in the suspensor cell (*cb*). This branched structure remains unicellular during the embryogenesis of *D. nobile*.

In conclusion, we described the unique features of the early embryo development in *D. nobile* orchid. This knowledge is of special importance for the fundamental understanding of the female gametophyte ontogeny and embryo organization. Our data show the prominent role of the underestimated transitory embryonic structures. All together, our results will contribute to the understanding of the reproductive biology of the orchids and further development and application of new conservation strategies.

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Authors' contributions G.L. Kolomeitseva, A.V. Babosha, and A.S. Ryabchenko are responsible for the conceptualization, microscopic observations, formal analysis, investigation, and methodology. E.A. Tsavkelova is responsible for the translation, revision, and editing of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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