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Original Scientific Paper

The formation of integuments, megasporogenesis and megagametogenesis in *Dendrobium catenatum*, with special discussions on embryo sac types and section techniques

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ABSTRACT:
The formation of integuments, megasporogenesis and megagametogenesis in *Dendrobium catenatum*, an economically important orchid, are observed. After pollination, mitotic cell divisions of the placental epidermis result in the formation of a branching system of outgrowths. The tip of each branch consists of an archesporial cell derived from the differentiation of the terminal subepidermal nucellar cell. It differentiates directly into a megasporocyte. The first division of the meiosis of the megasporocyte produces a dyad approximately equal in size, in which the micropylar cell promptly degenerates. The second meiotic division of the remaining dyad cell results in the formation of two megaspores of unequal size. The larger chalazal cell becomes functional and eventually develops into a mature megagametophyte. The development of the megagametophyte conforms to the Monosporic *Polygonum* type. The final arrangement of the mature embryo sac conforms to a seven-celled/eight-nucleate structure. The mature ovule is bitegmic, tenuinucellate and has an anatropous orientation. In the present study, we also discuss the differences between three main types of embryo sac development and the improvement of section techniques.

Keywords:
Iron-skin dendrobe, embryo sac, Monosporic *Polygonum* type, Orchidaceae, Technovit section technique

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INTRODUCTION

Meiotic division allows for the random assortment of parental genes, whereas the union of parental genes upon a successful fertilization event enables the offspring to better adapt to the changing environment (Lee & Yeung 2012). In angiosperms (flowering plants), meiosis of the megasporocyte (the megaspore mother cell) and subsequent mitoses (megagametogenesis) give rise to a female gametophyte (megagametophyte, embryo sac) in which double fertilization, a phenomenon which characterizes angiosperms (Ao 2013), occurs. These two successive cell division events are critical for ensuring successful sexual reproduction (Ao *et al.* 2016).

Meiosis of the megasporocyte usually produces four independent megaspores. However, this is not always the case. For example, in the embryo sac development of *Fritillaria ussuriensis* Maxim (Li & Shen 1990) and *Rudbeckia bicolor* Nutt. (Musiał *et al.* 2012), meiosis of the megasporocyte yields four megaspore nuclei which co-exist in the coenocyte and all participate in the constitution of the embryo sac [see Maheshwari (1937) and Hu (2005) for a review]. According to the number of megaspore nuclei which participate in the constitution of the embryo sac, three main types of embryo sacs are recognized, i.e. the Monosporic *Polygonum* type, the Bisporic *Allium* type and the Tetrasporic *Fritillaria* type.

In more than 70% of flowering plants, meiosis of the megasporocyte results in four megaspores viz. a tetrad of megaspores, in which only the chalazal-most or the micropylar-most megaspore is functional and develops into a female gametophyte, while the other megaspores degener-

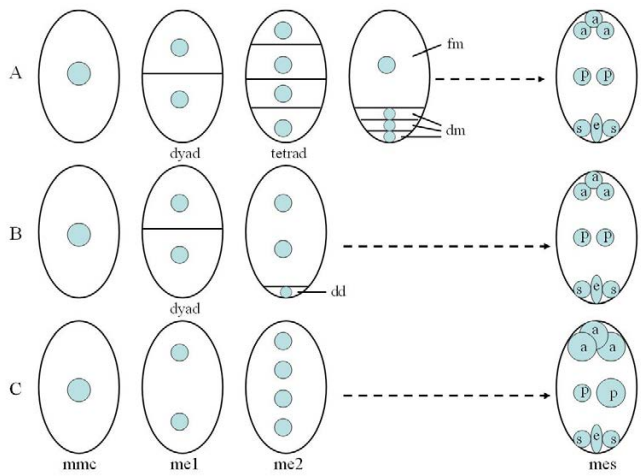

Fig. 1. A comparison of the Monosporic type, the Bisporic type and the Tetrasporic type embryo sacs. For all figures, the micropylar pole is at the bottom and the chalazal pole is at the top. A. the Monosporic *Polygonum*-type embryo sacs. B. the Bisporic type embryo sacs. C. the Tetrasporic type embryo sacs. Abbreviations: a, antipodal cells; dd, degenerating dyad cell; dm, degenerating megaspore; e, egg cell; fm, functional megaspore; mes, mature embryo sacs; me1, meiosis I; me2, meiosis II; mmc, megaspore mother cell. p, polar nucleus, s, synergid.

ate. Such patterns of embryo sac development are termed the Monosporic *Polygonum* type (Fig. 1A) and the Monosporic *Oenothera* type (Maheshwari 1937; Hu 2005), respectively. Meiosis of the megasporocyte is here termed megasporogenesis. In the Bisporic type embryo sacs, through meiosis I the megasporocyte produces a dyad in which the micropylar cell degenerates, while the chalazal cell becomes functional. No new cell wall forms after the functional dyad cell divides (i.e. meiosis II) and the two daughter nuclei (megaspore nuclei) co-exist in the coenocyte and both participate in the constitution of the embryo sac (Fig. 1B). In the Tetrasporic type embryo sacs, the megasporocyte gives rise to four daughter nuclei through meiosis, which co-exist in the coenocyte and all of them participate in the constitution of the embryo sac (Fig. 1C).

The iron-skin dendrobe (formerly *Dendrobium officinale* Kimura et Migo, now corrected as *Dendrobium catenatum* Lindl. by Liu *et al.* 2011), a perennial herb in Orchidaceae, is listed as No. 1 in the Chinese traditional Nine Fairy Herbs (Chen *et al.* 2018). In recent years, *D. catenatum* has drawn widespread concern due to its tremendous medicinal and economical value. It blooms and bears fruit from April to August every year (Fig. 2). The trimerous flower of *D. catenatum* is composed of three sepals (a mid-sepal and double lateral sepals), and three petals which have differentiated into two common petals and a labellum (Fig. 3A). The ovary is inferior (Fig. 3B-E) (Chen *et al*. 2018).

Orchid flowers are typically insect-pollinated and have formed a symbiotic relationship with their pollinators during the long period of coevolution in their original locations. In spite of its wild populations, which are now becoming increasingly scarce, and are under threat of extinction (Chen *et al.* 2018), in the culture status plants of *D. catenatum* lack pollinating insects and pollination is greatly limited. Hand-pollination is thus indispensable for the harvest of a fruit set. However, in the stereo-cultivated status (Fig. 2), the pollinia in the upper flowers are frequently blown down by the wind. Sometimes the dropping pollinia adhere to the gynandrium of the flower under them. This sort of spontaneous pollination can also result in a large number of fruits (capsules) that are readily available at the door, where the wind is relatively strong. Such a rich material system provides a good foundation for embryological studies in Orchidaceae.

Fig. 2. Stereo-cultivated plants of *D. catenatum*. In the stereo-cultivated status, the pollinia in the upper flowers are frequently blown down by the wind. Sometimes the dropping pollinia adhere to the gynandrium of the flower under them. The white dotted lines indicate the potential pollination routes. Scale bar = 10 cm.

The present study aims to document the key anatomical events during the course of the formation of integuments, megasporogenesis and megagametogenesis in *D. catenatum* by means of Technovit-embedded sections and to provide high-resolution, light microscopic photos of the various developmental stages of ovules. We will determine the type of embryo sac development in *D. catenatum* and evaluate the merits of the Technovit section technique.

MATERIALS AND METHODS

Plant material. *Dendrobium catenatum* is grown in greenhouses at Zhejiang Juyoupin Biological Technology Co., Ltd (Dajing, Wenzhou, Zhejiang, China). The flowers were naturally pollinated by wind in the stereo-cultivated status (Fig. 2). In the pre-experimental phase, a number of ovaries were hand-pollinated and their width was measured 10-18 days after pollination (DAP). Naturally pollinated ovaries or young fruits at different developmental stages were collected on July 10, 2018. A total of 30 ovaries at 10-18 DAP (Fig. 3) were used for this study. DAP was assessed by comparing their widths to the widths of hand-pollinated ovaries.

Light microscopy. Ovaries or young fruits at different developmental stages were fixed and stored in FAA (5 mL formalin: 6 mL acetic acid: 89 mL 50% alcohol). Prior to the experiments, the ovaries (young fruits) were washed in 50% alcohol three times and dissected to obtain placentae. The placentae bearing numerous ovules were dehydrated in an alcohol series, and embedded in Technovit 7100 (Kulzer, Wertheim, Germany). A total of 30 placentae, each representing an ovary at different developmental stages, were selected and five sections for each placentae of 4 μm thickness were made using a knife on a KD-1508 rotary microtome (Kedi Instrumental Equipment Co., Ltd., Jinhua, Zhejiang, China). The sections were stained with Heidenhain's haematoxylin and safranin, and were viewed; the images were captured digitally with a CCD camera (Olympus DP71, Japan) attached to a light microscope (Olympus BX51, Japan). All of the micrographs were processed to make plates using Adobe Photoshop CS software (Adobe Systems, San Jose, California, USA) and image adjustments were single applications to the entire image to amend brightness and contrast. The permanent sections were deposited in the Plant Morphology Research Room (10A-504) in the South Campus of Wenzhou University.

RESULTS

Formation of integuments. After successful pollination, the ovary starts to enlarge, turning into a fruit (capsule). In the meantime, the ovular tissue proliferates and ovule development and seed formation proceed within the ovary. Fig. 3 shows the ovaries at 10, 12, 14, 18 DAP (B, C, D and E, respectively).

The integument tissue was initiated and formed before the megasporocyte underwent meiosis (Fig. 4A-D). During meiosis of the megasporocyte and megagametogenesis, the integument continued to extend toward the tip of the nucellar filament. The integuments gradually enclosed the developing embryo sac, leaving a micropyle, thus creating an anatropous orientation.

The anticlinal divisions of the placental epidermis are primarily responsible for the increase in the surface area of the placental ridges, while the periclinal divisions in the subepidermal layer initiate branching (Yeung & Law 1989). The tip of each branch will become an ovule primordium. As the terminal subepidermal nucellar cell begins to differentiate into the archesporial cell, the filamentous ovule primordium starts to bend at the terminus of each branch (Fig. 4A). In the meantime, the archesporial cell enlarges and differentiates directly into the megasporocyte (Fig. 4B, C). The inner integument appears prior to the outer integument (Fig. 4B). The outer integument appears later (Fig. 4C). At the stage when the megasporocyte enters meiosis, the inner integument is about half of the length of the nucellus, and the outer integument is even shorter (Fig. 4D). However, the outer integument develops

Table 1. Variation in the number of nuclei in the mature embryo sacs in the genus *Dendrobium*

Species	Number of nuclei in the mature embryo sacs	Reference
D. nobile Lindl.	5, 6	Poddubnaya-Arnoldi 1958, 1959
	8	Kolomeitseva *et al.* 2021
D. macrostachyum Lindl.	6, 8	Rajan 1971
D. ovatum (L.) Kraenzl.	6	Gurudeva 2016
D. aqueum Lindl.	8	Govindappa & Karanth 1980
Dendrobium moniliforme (L.) Sw. (syn. *D. catenatum* Lindl.)	8	Present study

Table 2. A comparison of three kinds of section techniques

Section techniques	Pretreatment		Penetration and embedment	Temperature conditions	Knives
	Dehydration	Transition			
Paraffin section	Alcohol	Xylene	Paraffin	55 -60°C	Ordinary section knives or disposable razors
Resin section	Acetone	Propylene oxide	Resin (Epon 812, Spurr, etc.)	Room temperature	A diamond knife or glass knives
Technovit section	Alcohol	---	Technovit	Room temperature	Ordinary section knives or disposable razors

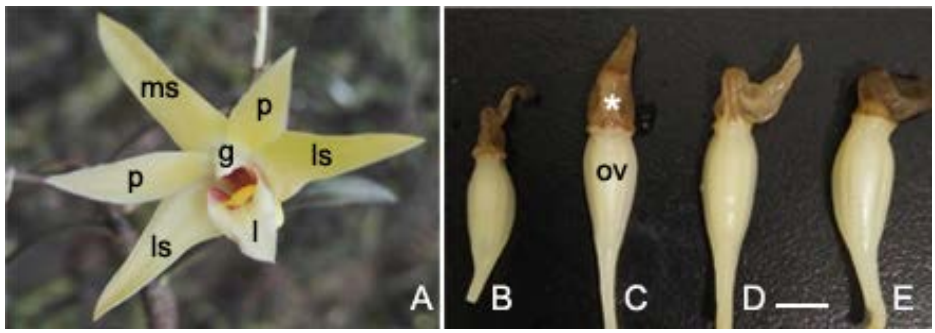

Fig. 3. Flower at anthesis showing mid-sepal, lateral sepals, petals, labellum and gynandrium (A) and ovaries at 10, 12, 14, and 18 DAP (B, C, D and E, respectively). Note the withered sepals and petals (e.g. asterisk in C) and the inferior ovary. Scale bar = 5 mm. Abbreviations: g, gynandrium; l, labellum; ls, lateral sepals; ms, mid-sepal; ov, ovary; p, petals. Scale bar = 5 mm.

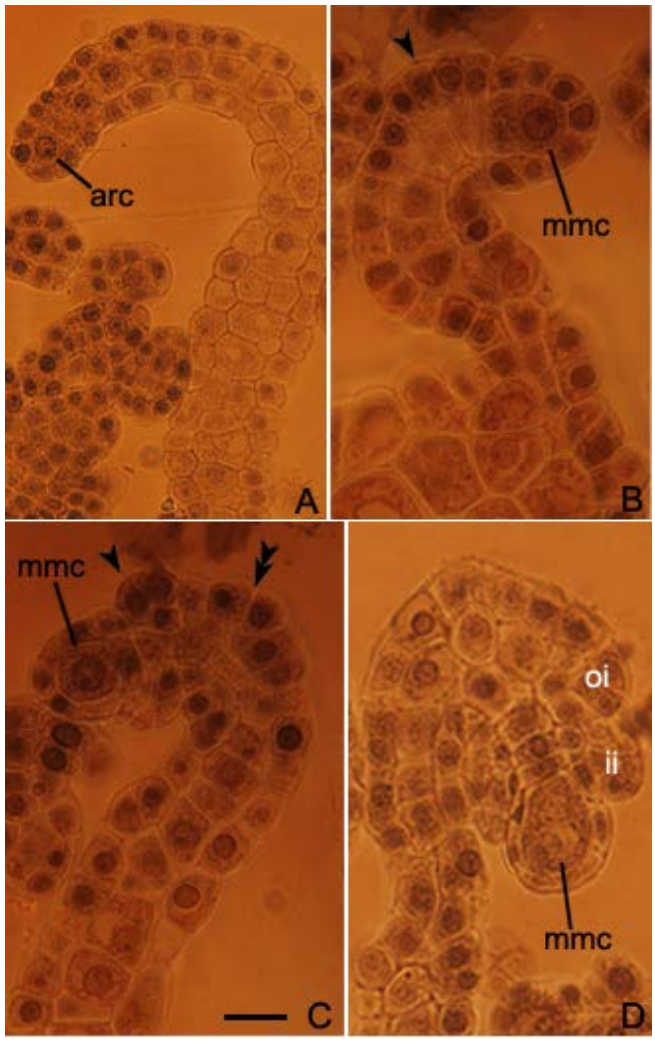

Fig. 4. Formation of the inner and outer integuments in *D. catenatum*. Scale bar = 20 μm and shared. A. The subepidermal cell of the nucellar filament differentiates directly into an archesporial cell. The ovule primordium begins to curve back. B. The ovule primordium continues to curve back. The inner integument (arrowhead) initiates and the archesporial cell acts directly as a megasporocyte. C. The ovule primordium continues to curve back. The inner integument (arrowhead) appears and the outer integument (double arrowheads) initiates. D. The early ovule continues to curve back, creating an anatropous orientation and the megasporocyte has entered meiosis. Both the inner and the outer integuments have appeared. Abbreviations: ii, inner integument; mmc, megaspore mother cell (megasporocyte); oi, outer integument; arc, archesporial cell.

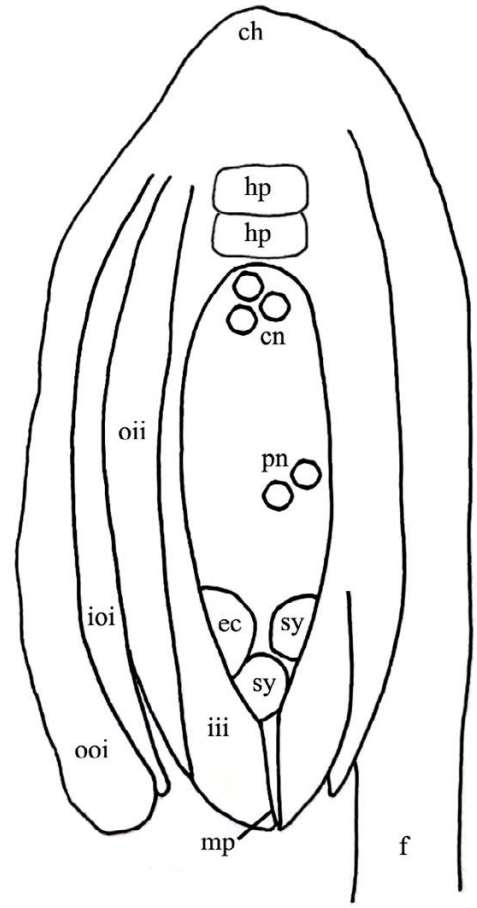

Fig. 5. The schematic organization of an ovule in *D. catenatum*. Abbreviations: ch, chalaza; cn, chalazal nuclei; ec, egg cell; f, funiculus; hp, hypostase; iii, inner layer of inner integument; ioi, inner layer of outer integument; oii, outer layer of inner integument; ooi, outer layer of outer integument; mp, micropyle; sy, synergid; pn, polar nuclei.

faster than the inner integument, and completely covers it at the mature embryo sac stage (Fig. 5). As a result, the ovule becomes bitegmic with a micropyle derived from the inner and outer integuments (Fig. 5).

The archesporial cell acts directly as a megasporocyte, and the megasporocyte, functional megaspore and mature embryo sac occur under one epidermal layer (tenuinucellate).

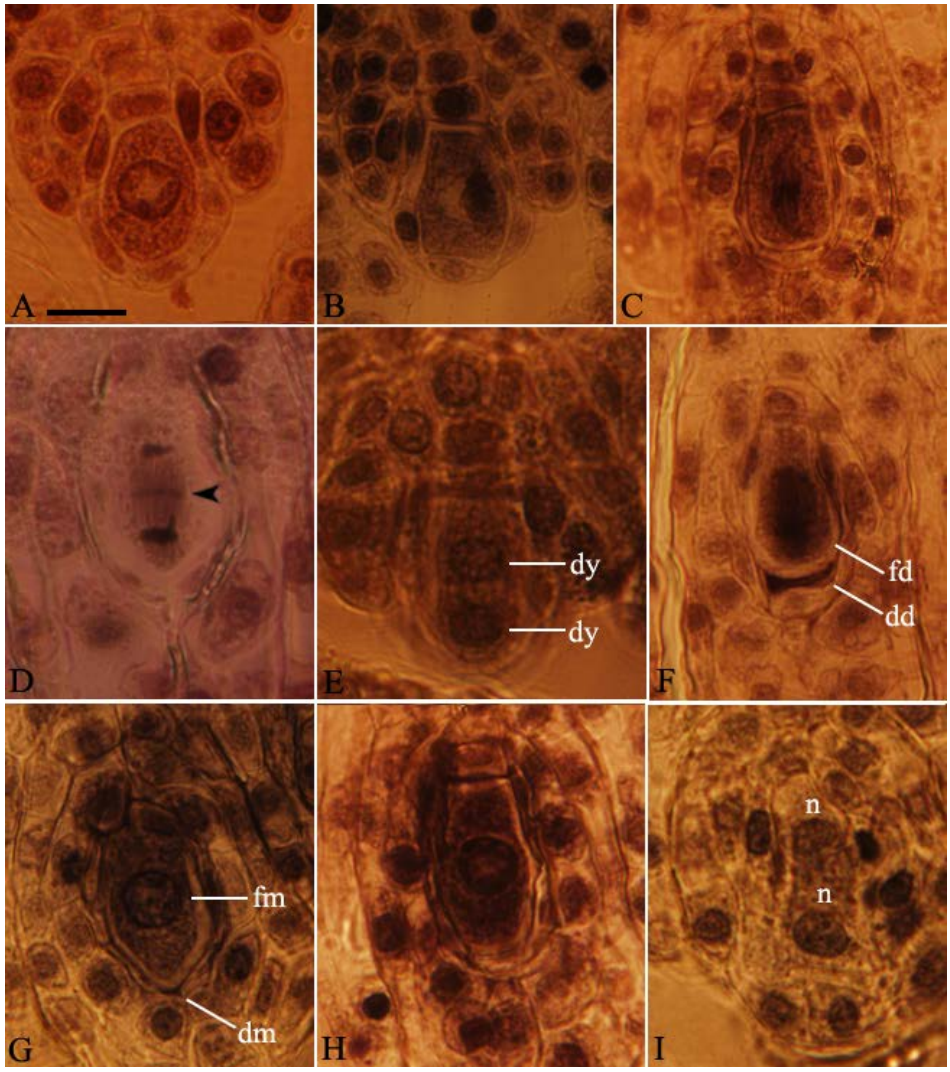

Fig. 6. Megasporogenesis and megagametogenesis in *D. catenatum*. For all figures, the micropylar pole is at the bottom and the chalazal pole is at the top. Scale bar = 15 μm and shared. (A) Megasporocyte. (B-D) Meiosis of the megasporocyte at prophase (B) metaphase (C), and telophase [D, note cell plate formation (arrowhead)]. (E) A dyad of cells which are approximately equal in size. (F) The complete degeneration of the micropylar dyad. The chalazal dyad has entered the second meiotic cell division. (G) The functional chalazal megaspore and the remains of a degenerating nonfunctional micropylar megaspore. (H) Mononucleate embryo sac. (I) Binucleate embryo sac. Abbreviations: dd, degenerated dyad; dm, degenerated megaspore; dy, dyad; fd, functional dyad; fm, functional megaspore; n, nucleus.

Megasporogenesis and megagametogenesis. The megasporocyte (Figs. 4B-C, 6A) is significantly larger than the archesporial cell (Fig. 4A) because of cell enlargement and remains highly cytoplasmic throughout the meiotic division cycle. The first meiotic cell division via prophase (Fig. 6B), metaphase (Fig. 6C), anaphase and telophase (Fig. 6D) creates a dyad of cells that are approximately equal in size (Fig. 6E). A marked cell plate forms during telophase (Fig. 6D), which decollates the cytoplasm almost evenly. When the micropylar dyad cell degenerates, the second meiotic cell division of the chalazal dyad cell then begins (Fig. 6F). This cell divides in an unequal way, forming two cells, viz. megaspores: of which the smaller one promptly degenerates. The remains of the degenerating megaspore could be detected (Fig. 6G). The remaining megaspore located at the chalazal end is functional and continues to develop into a mononucleate embryo sac (Fig. 6H), binucleate embryo sac (Fig. 6I) and a tetranucleate embryo sac, until a mature megagametophyte (Fig. 5) occurs.

The final development of the mature embryo sac conforms to a seven-celled/ eight-nucleate structure, i.e. an egg cell, two synergids, a central cell involving two polar nuclei, and three antipodal cells, each involving a chalazal nucleus (Fig. 5). During megagametogenesis, only one chalazal megaspore undergoes subsequent mitoses and takes part in the constitution of the embryo sac. Thus the embryo sac development of *D. catenatum* follows the Monosporic *Polygonum* type.

DISCUSSION

Orchids are unique among flowering plants in that ovule development is initiated after successful pollination (Yeung & Law 1989). In the overwhelming majority of angiosperms, the female gametophyte, an egg-producing structure, has matured and the egg cell is in the "ready for fertilization" status before pollination and pollination is simply the prelude to fertilization (Chen *et al.* 2018).

In *D. catenatum*, like in most orchids, pollination is the stimulator that triggers ovule development (Yeung & Law 1997; Chen *et al.* 2018), and the initiation of the archesporial cell and the subsequent megasporogenesis and megagametogenesis occur only after pollination (Chen *et al.* 2018). Such an unusual pattern of ovule development determines to some extent a distinctive type of embryo sac and variability in the constitution of mature embryo sacs.

Number of nuclei in the mature embryo sacs in the genus *Dendrobium*. The development of the female gametophyte in the genus *Dendrobium* has been previously described (Pastrana & Santos 1931; Swamy 1949; Poddubnaya-Arnoldi 1958, 1959; Rajan 1971; Chardard 1978; Govindappa & Karanth 1980; Vasudevan & van Staden 2010; Gurudeva 2016; Kolomeitseva *et al.* 2021). The number of nuclei in the mature embryo sacs varies in this genus (Table 1). This study represents a contribution to the knowledge about the number of nuclei in the mature embryo sacs in this genus and adds to studies in the literature that *D. catenatum* has a mature embryo sac with eight nuclei and seven cells.

Identification of the type of embryo sac in *D. catenatum*. While the majority of orchids have a Monosporic pattern of embryo sac development (Vij & Sharma 1986; Yeung & Law 1989), a few studies indicate that the *Cypripedium* and *Paphiopedilum* species follow a Bisporic pattern (Duncan & Curtis 1942; Carlson 1945; Lee & Yeung 2012). The occurrence of the dyad stage in both Monosporic-type and Bisporic-type embryo sac development obscures their differences (Fig. 1A, B). However, in the Bisporic-type embryo sac development, the megasporocyte produces a dyad through meiosis I, of which the micropylar cell degenerates, while the chalazal cell undergoes the second meiotic division (meiosis II), resulting in two daughter nuclei which co-exist in one cell (coenocyte). The two newly-formed daughter nuclei both participate in the formation of the embryo sac (Fig. 1B). By contrast, in the embryo sac development of the Monosporic type, the first meiotic division produces a dyad and both dyad cells undergo the second meiotic division, giving rise to a tetrad, of which three megaspores degenerate. Only one megaspore (the nucleus) participates in the formation of the embryo sac (Fig. 1A).

In *D. catenatum*, the first meiotic division of the megasporocyte produces an equal dyad (Fig. 6E). However, its subsequent second meiotic division differs from that of the typical embryo sac development of the Monosporic type, in that only the chalazal dyad cell undergoes the second division, while the micropylar dyad cell becomes highly compressed (Fig. 6F). Although this process appears to be similar to that of the Bisporic-type embryo sac development, the second meiotic division of the remaining dyad cell does not produce two free nuclei which co-exist in the coenocyte. Instead, it yields two megaspores of unequal size: a larger chalazal megaspore and a smaller micropylar megaspore. The chalazal megaspore continues to enlarge and serves as the functional megaspore, while the micropylar megaspore promptly degenerates (Fig. 6G). Thus, only one megaspore (the nucleus) participates in the constitution of the embryo sac. This still conforms to the Monosporic embryo sac development.

In *D. catenatum*, the non-functional dyad cell and megaspore degenerate in two steps. By contrast, three non-functional micropylar megaspores degenerate simultaneously in *Zephyranthes candida* with a typical Monosporic *Polygonum*-type embryo sac development (Ao *et al.* 2016; Fig. 1A). The relationship between the distinctive pattern of megasporogenesis and the unique mode of ovule development in *D. catenatum*, remains unclear.

A comparison of section techniques. The conventional paraffin section technique is convenient and less time-consuming and is thus widely adopted. However, this technique has many shortcomings. For example, the materials usually transition and are made transparent in xylene (dimethylbenzene) and are then penetrated by paraffin in an incubator at 55-60°C. This process harms the materials to a great extent, and as a result affects the quality of sections. In addition, exposure to xylene may cause harmful effects on the human nervous system. By comparison, when using resin (e.g. Epon 812 and Spurr) – the embedded materials are dehydrated with acetone and transition in propylene oxide during the pretreatment stage, and are thus free of the health hazards of xylene (Table 2). Besides, penetration by resins proceeds at room temperature and is rarely harmful to the materials. Because of these merits, resin sections can even be used for Transmission Electron Microscope (TEM) observations. However, resin-embedded materials need to be sectioned by an extremely expensive diamond knife. Although a glass knife can be used to cut the materials instead, such knives are not durable and need to be replaced too frequently.

Technovit sections are similar to resin sections in terms of both procedures and qualities [Figs. 4-6, compared with Figures in Ao (2019) and Ao *et al.* (2016), for example], but they can be made using an ordinary rotary microtome (e.g. the KD -1508 rotary microtome in the present study) attached with an ordinary section knife or a disposable razor which is almost as durable as in the case of paraffin section. Thus, this technique is particularly suitable for poorly-equipped laboratories and should be widely used in the future. In our experience, the numerous tiny ovules in the orchid capsules were sensitive and vulnerable to paraffin in an incubator at temperatures above 50°C and we believe that Nawaschin's (1900) failure to observe the second fertilization in Orchidaceae was due to the paraffin -embedded materials (Chen *et al.* 2018). The finding of the second fertilization (Chen *et al.* 2018) and the determination of the Monosporic *Polygonum*-type embryo sac in *D. catenatum* in the present study demonstrated that

Technovit is an ideal embedding medium for studies of orchid embryology.

CONCLUSION

We identified that the embryo sac development in *D. catenatum* followed the Monosporic *Polygonum*-type, and we concluded that Technovit-embedded sections are more practical than those using paraffin or resin as embedding media.

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REZIME

Botanica SERBICA

Formiranje integumenta, megasporogeneza i megagametogeneza kod *Dendrobium catenatum*, sa posebnim osvrtom na embrionalne kesice i tehnike sečenja

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Istraživani su formiranje integumenta, megasporogeneza i megagametogeneza kod *Dendrobium catenatum*, ekonomski važne orhideje. Nakon oprašivanja, mitotička deoba ćelija placentalnog epidermisa rezultirala je formiranjem razgranatog sistema izdanaka. Vrh svake grane uključuje arhesporijalnu ćeliju nastalu diferencijacijom terminalne subepidermalne ćelije nucelusa. Ona diferencira direktno u megasporocitu. Prva deoba mejoze megasporocita stvara dijadu približno jednake veličine, u kojoj se mikropilarna ćelija odmah degeneriše. Druga mejotička deoba preostalih ćelija dijade rezultira stvaranjem dve megaspore nejednake veličine. Veća, halazalna ćelija postaje funkcionalna i na kraju se razvija u zreli megagametofit. Razvoj megagametofita odgovara monosporičnom *Polygonum* tipu. Konačni raspored zrele embrionalne kesice odgovara sedmoćelijskoj / osmojedarnoj strukturi. Zreli semeni zametak je bitegmičan, tenuinucelatan i ima anatropnu orijentaciju. U ovoj studiji diskutuje se i o razlikama tri glavna tipa razvoja embrionalnih kesica i poboljšavanju tehnika sečenja.

Ključne reči: gvozdeno-kožna dendrobija, embrionalna kesica, monosporični *Polygonum* tip, Orchidaceae, Technovit tehnika sečenja