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DEVELOPMENT OF MALE AND FEMALE GAMETOPHYTES IN *HABENARIA OVALIFOLIA* WIGHT (ORCHIDACEAE)

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Abstract

The anther in *Habenaria ovalifolia* Wight was dithecous and tetrasporangiate. Its wall development confirmed to the monocotyledonous type. Each archesporial cell developed into a block of sporogenous cells and finally organized into pollen massulae. The anther wall was 4-5 layered. The endothecial cells developed ring-like tangential thickening on their inner walls. Tapetal cells were uninucleate and showed dual origin. The microspore tetrads were linear, tetrahedral, decussate and isobilateral. The pollens were shed at 2-celled stage. The ovules were anatropous, bitegmic and tenuinucellate. The inner integument alone formed the micropyle. The development of embryo sac was monosporic and G-1a type. The mature embryo sac contained an egg apparatus, secondary nucleus and three antipodal cells. Double fertilization occurred normally.

Introduction

THE ORCHIDACEAE, one of the largest families of angiosperms is the most evolved amongst the monocotyledons. The orchid embryology is interesting, as these plants exhibit great diversity in the development of male and female gametophyte. The first embryological study in the family was made by Muller in 1847. Since then several investigations have been carried out (Abe, 1972a,b; Schnarf, 1931; Swamy, 1949a,b; Wirth and Withner, 1959). Some of the recent works in the area include those of Bhanwra *et al*. (2006), Fredrikson (1991), Govindappa and Karanth (1980), Gurudeva (2009, 2010, 2011a,b, 2012, 2014), Gurudeva and Govindappa (2008), Krishna Swamy *et al*. (2003, 2005), Kant and Bhanwra (2010), Kant and Hossain (2010), Kant *et al.* (2013), Sood (1984, 1985, 1987, 1989, 1992), Sood and Mohana Rao (1986, 1988), Vij *et al*. (1982).

The genus *Habenaria* Willd. (Sub-tribe: Orchidinae; tribe: Orchideae of Dressler and Dodson, 1960) is a large pantropical genus of about 600 species of terrestrial orchids characterized by an underground tuberous herb. Seventy two species and one variety are known from India (Sathish Kumar and Manilal, 1994) and Karnataka accounts for 18 species (Ananda Rao and Sridhar, 2007). The embryology of several species of *Habenaria* have been studied by Abe (1972a), Brown (1909), Leavitt (1901), Gurudeva (2012), Sharma and Vij (1987), Mohana Rao and Rao (1984), Mohana Rao and Sood (1979a), Sood (1986) and Swamy (1946). Perusal of literature indicates that the embryology of *H. ovalifolia*, has remained elusive, hence, an attempt was presently made to study the development of male and female gametophyte in this species.

Materials and Methods

Habenaria ovalifolia Wight is a terrestrial herb with ellipsoidal underground tubers. There are about 4-6 oblong or obovate, acute, entire leaves cluster below the middle of the stem (Fig. 1). The *inflorescence* is a many flowered raceme. The *flowers* are green, bracteate and pedicellate (Fig. 2). The *sepals* and *petals* are entire. The *lip* is 3-lobed. The *mid lobe* is arched upward towards the dorsal sepal. The *spur* is pale green, longer than the ovary either curved or straight (Figs. 3,4). *Ovary* is inferior.

The flower buds and post-pollinated and mature ovaries were collected near Manjrabad fort and Donigal, near Sakleshpur town, Hassan district, Karnataka, India, during the months of August and December 2011. These were fixed in formalin-acetic acid-alcohol, and stored in 70% ethanol followed by thorough wash in running water and conventional microtechnique. The serial transverse and longitudinal sections at 10-12 μm were stained with Heidenhain's iron-alum and haematoxylin. Erythrosin in clove oil was used as counter stain. Mature anthers were selected and placed in watch glass, treated with 1N HCl and gently warmed over flame. The treated anthers were macerated with crystal violet and mounted in glycerin. Drawings were made using camera lucida and Meopta microscope.

Results

Microsporangium

The very young anther in transaction showed two lobes

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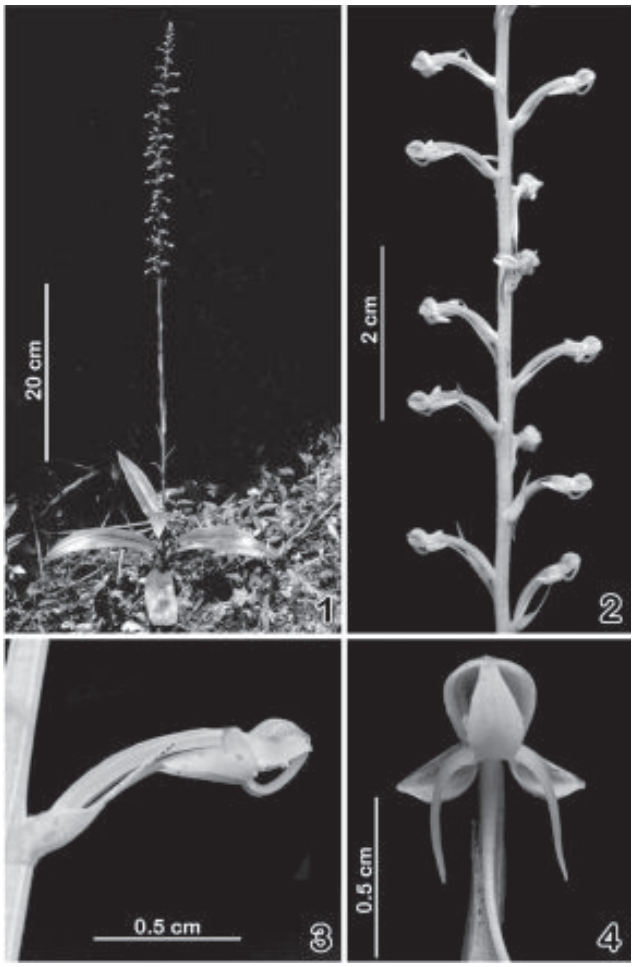


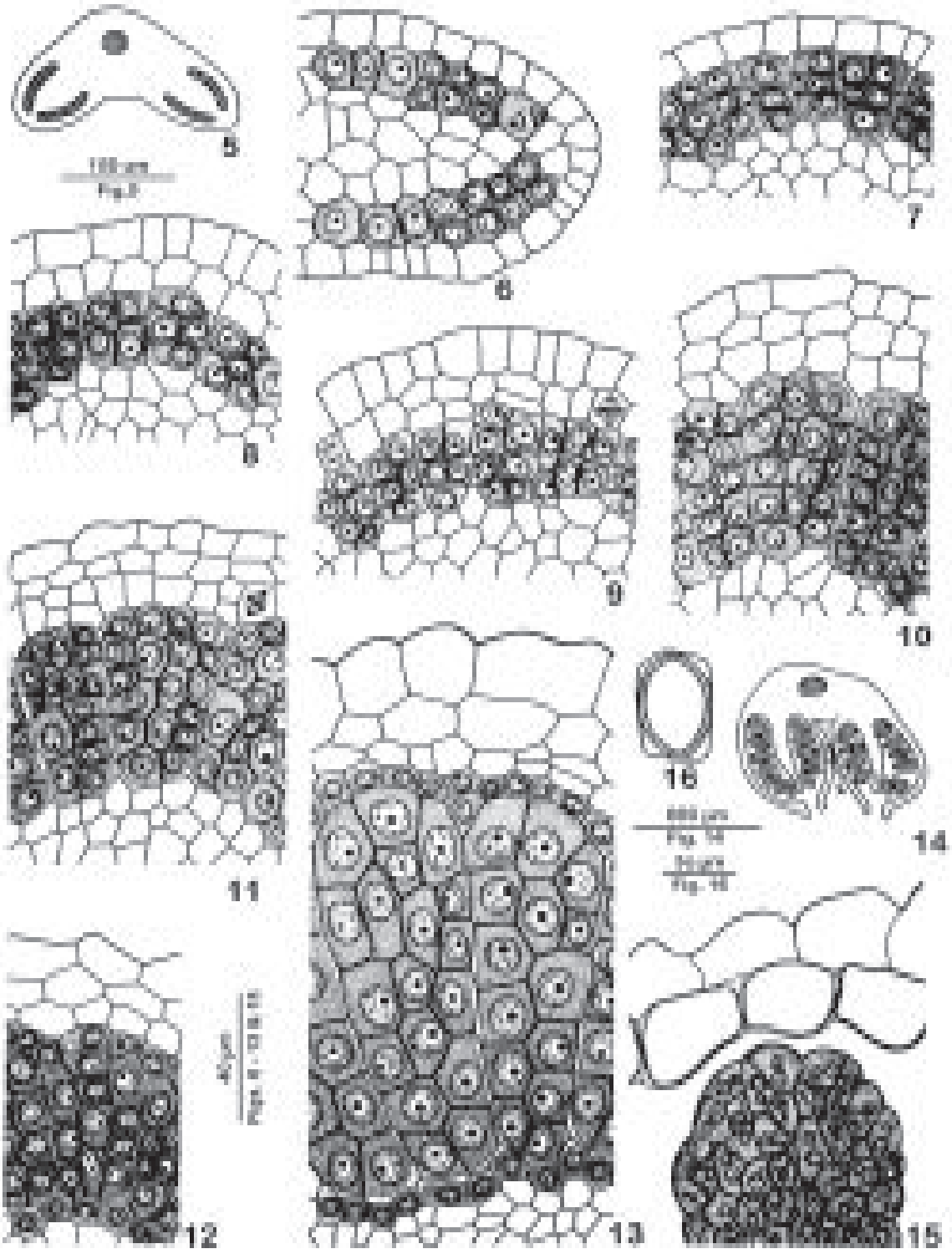
Figs. 1-4. *Habenaria ovalifolia* : 1, Habit of flowering plant; note the cluster of leaves below middle of the stem; 2, Close-up of inflorescence; note the bracteates and pedicellate flowers; 3, Lateral view of the flower; 4, Ventral view of the flower.

(Fig. 5). Each lobe had two rows of hypodermal archesporial cells. Each row represented the site of a future microsporangium. The archesporial cells underwent periclinal divisions to produce the primary parietal and primary sporogenous layers (Fig. 6). The cells of the primary sporogenous layer divided anticlinally to form 2-3 layers of sporogenous cells (Fig. 7). This was followed by similar divisions in the sporogenous cells also (Fig. 8). The density of the cytoplasm in the cells of the primary parietal cells decreased and as a consequence they began to appear more translucent under the microscope. A periclinal division of these cells resulted in two layers (Fig. 9); the outer layer functioned as the endothecium while the inner divided again in a similar manner to produce the glandular tapetum and the middle layer (Figs. 10-11). The ontogeny of the microsporangium wall, therefore, was of the monocotyledonous type (Davis, 1966). The tapetal cells on the sides of the wall acquired dense cytoplasm, the cells on the connective side and those bordering the sporogenous cells also became the tapetum in alignment with the layer towards the side of the wall. The tapetum ensheathing the sporogenous tissue, therefore, was of dual origin (Fig. 12). Occasionally some of the cells of the tapetum as well as the middle layers divided at places forming two layers of cells (Fig. 13). The tapetal cells remained uninucleate throughout their existence. During later stages of development, the glandular tapetum provided nourishment to the spore mother cells and the pollen grains and got absorbed along with the middle layers. The endothecial cells enlarged in size and each of them developed a ring-like tangential thickening on its inner wall. The epidermal cells extended laterally but remained thin-walled at the maturity of the microsporangium (Figs. 14-16). Concomitant with the ontogeny of the microsporangium wall, marked changes occurred in the sporogenous region. Blocks of sporogenous cells became distinct within the mass. Each block could be traced back to a single archesporial cell (Figs. 7-12). Each one of these cell blocks later organized into a pollen massula.

Microsporogenesis and Development of Male Gametophyte

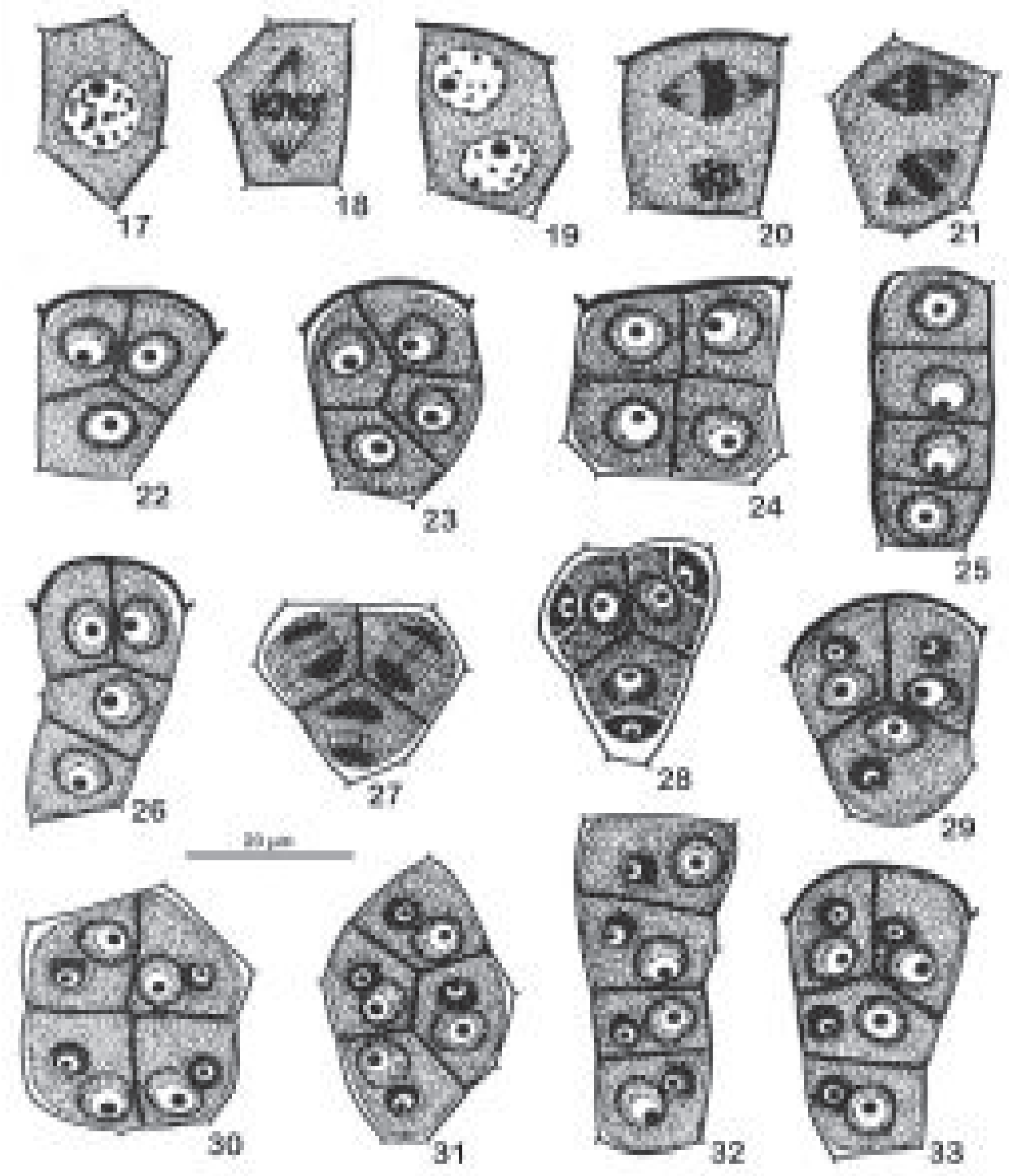
After a series of mitotic divisions, the sporogenous cells of a block differentiated into microspore mother cells (Fig. 13). These cells became spherical but remained *in situ* and underwent meiotic divisions. No cell wall was laid down after meiosis-I (Figs.17-19). The two resulting dyad nuclei then divided synchronously. The orientation of the spindles of these nuclei varied in different spore mother cells of a massula (Figs. 20,21). Meiosis-II was followed by laying down of cell walls. The simultaneous quadri-partition of the mother cells lead to the organization of tetrahedral, rhomboidal, isobilateral, linear and T-shaped tetrads of microspores (Figs. 22-26). These tetrads remained packed within the young massula. Within a massula, rhomboidal, isobilateral, linear and T-shaped tetrads were more towards the periphery than at the centre.

Soon the nuclei of microspores of a massula in a sporangium divided. It was observed that the spindles of the dividing nuclei of the tetrads were always disposed in a proximal-distal axis of the spore in the tetrad. Of the two resulting daughter cells, the smaller one was located at the distal end of the young pollen grain (Figs. 27, 28). Later, the generative cell separated itself from the microspore wall and entered into the cytoplasm of the large tube cell (Figs. 29-33). The pollen grain remained two celled when the massulae of the sporangium were ready for release. By this time, a conspicuous sporopollenin wall was laid down around



Figs. 5-16. Onotogeny of microsporangium in *Habenaria ovalifolia*: 5, Diagramatic cross section of very young anther showing four sites of archesporial cells; 6, Anther lobe showing periclinal division in archesporial cells; 7-8, Portion of anther lobe showing anticlinal division in primary parietal and sporogenous layers; 9-10, Part of microsporangis showing origin of two parietal layers; note blocks of sporogenous cells; 11-12, Part of microsporangium showing origin of middle layer and tapetum; 13, Part of microsporangium at microspore mother cell stage; note two rows of cells in middle layer and tapetum; 14, Diagrammatic cross section of mature anther; 15, Part marked 'x' in fig. 14 enlarged; note ring like thickenings in the endothecial cells and part of massulae; 16, An endothecial cell showing single tangential heavy thickening in the wall.

each massula. As the massulae were ready for release, a stomium was organized in the anther wall between the two adjacent sporangia of an anther half. The group of thin-walled cells located in the sub-epidermal region at the junction of the two microsporangial walls broke down. Aided by the heavy rings of thickenings, a line of opening in the anther half was created (Figs. 14-16), facilitating the insect visitor to collect and



Figs. 17-33. *Habenaria ovalifolia* -microsporogenesis and pollen development: 17-19, Meiosis – I in microspore mother cells; 20-21, Simultaneous division of dyad nucleus; 22, Tetrahedral tetrad; 23, Rhomboidal tetrad; 24, Isobilateral tetrad; 25, Linear tetrad; 26, T-shaped tetrad; 27, Synchronous divisions of micropores in the tetrads; note the proximal and distal orientation of nuclear spindles of the dividing nucleus; 28-33, Migration of parietal disposed generative cell into the cytoplasm of vegetative cell in the pollen tetrad.

transport the pollen massulae from one flower to the other.

Megasporangium

The ovary was tricarpellary, syncarpous, and inferior. After pollination, many protuberances developed from the three bilobed parietal placental ridges. The protuberances branched once or twice and the final branches ended up in ovular primordia. It composed of an axial row of 9-10 cells surrounded by an epidermis. A single archesporial cell situated terminally in the axial row (Fig. 34) did not cut off a parietal cell, but enlarged to function directly as a megaspore mother cell (Figs. 35,36). The two integumentary primordia became initiated by this time in the developing ovule (Figs. 34,35). The inner integument grew rapidly until it surrounded the nucellus. The outer, which arised a little later ultimately outgrew the inner by the time, the ovule reached the 2-nucleate embryo sac stage (Fig. 45) when the tenuinucellate ovule attains the anatropous status. The micropyle was formed only by the inner integument. Both the integuments were two cell-layered in thickness.

Megasporogenesis and Development of Female Gametophyte

The megaspore mother cell underwent meiosis-I to form two dyad cells. The micropylar dyad cell was smaller than the chalazal one (Figs. 37-40). The chalazal dyad cell underwent meiosis-II to give rise to two megaspores (Figs. 39,40). Therefore, a triad was organized (Fig. 40). Sometimes the micropylar dyad cell also underwent the second division of meiosis, the plane of which may be oblique or transverse. The resulting megaspores were arranged obliquely or one above the other (Figs. 41,42). The chalazal megaspore was functional and its nucleus underwent a free nuclear division. The two daughter nuclei produced were pushed apart to the opposite poles as a central vacuole appeared between them in the cytoplasm (Figs. 43-45). These two nuclei divided simultaneously twice in succession forming eight nuclei, disposed as micropylar and a chalazal quartets (Figs. 46-49). Three of the micropylar quartet of nuclei organized into the egg apparatus and three of the chalazal ones to the antipodal cells and the remaining two, one from each quartet, fused together to form the secondary nucleus prior to fertilization. The mature embryo sac remained in direct contact with inner integument. Its micropylar part is larger in size and it lodged the egg apparatus. The secondary nucleus was near the egg apparatus. The three antipodal cells were smaller in size (Fig. 50).

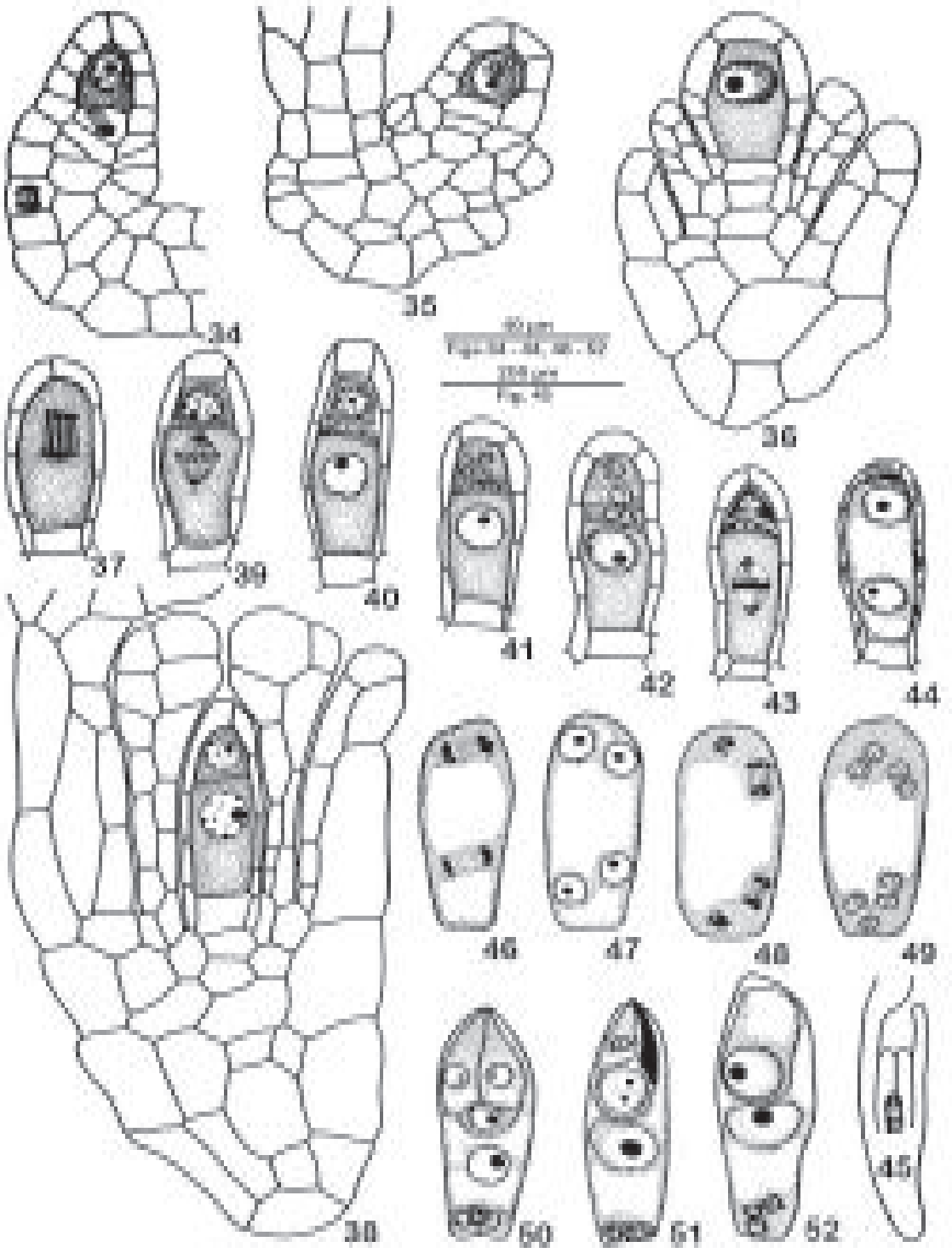
Fertilization

Double fertilization occurred. A pollen tube entered the embryo sac at the micropylar part with the degeneration of the synergids and discharged the two male gametes. One of them fused with the egg and gave rise to the zygote. The other united with secondary nucleus so as to form the primary endosperm nucleus (Figs. 51,52).

Discussion

Male Gametophyte

The anther wall development in *Habenaria ovalifolia* corresponds to the monocotyledonous type (Davis, 1966). A similar method of wall development was recorded earlier in *Aphyllorchis montana, Dendrobium microbulbon,* and *Sirhookera latifolia* (Krishna Swamy *et al.,* 2003), *Goodyera repens* (Sharma and Vij, 1984; Sood, 1988), *Habenaria edgeworthii, H. elisabethae, H. galeandra,* and *H. intermedia* (Sood, 1984, 1985, 1986), *Malaxis saprophytica* and *Neottia listeroides* (Sood, 1992), *Satyrium nepalense* (Mohana Rao and Sood, 1979b), and *Zeuxine strateumatica* (Vij *et al.,* 1982). It was very likely that in others where the type of organization was not assigned could also be a similar pattern. The anther wall comprised of epidermis, endothecium, middle layers and tapetum, similar feature was reported in most of the investigated taxa (Cocucci, 1964; Sharma and Vij, 1984; Mohana Rao and Sood, 1987; Sood, 1986; Swamy, 1949a). Occasional increase of cell layers in any of the zone barring the epidermis could have occurred at places as has been recorded in *H. ovalifolia*. Such a feature was also recorded in several species of *Epipactis* (Vij and Sharma, 1987) and *Habenaria* (Swamy, 1946). The epidermis was always single layered. Its cells were larger in size and tangentially extended. It is basically protective in function and remains persistent even at anthesis. A similar observation had been made in *Aa achalensis* (Cocucci, 1964) and *H. densa* (Mohana Rao and Sood, 1979a). Endothecium was single layered. At maturity the cells acquired thickenings on the inner surface of their walls. A single ring-like and tangentially disposed thickening was observed in each of the endothecial cells. The type of endothecial thickening corresponded to Type-II of Freudenstein (1991). Similar type of ring-like thickening was reported in *Aa achalensis* (Cocucci, 1964), species of *Habenaria* (Sharma and Vij, 1987) and in *Habenaria diphylla* (Gurudeva, 2012). However, Untawale and Bhasin (1973) reported spiral thickenings in certain species of *Habenaria* and fibrillar, band like,'U' or 'V' shaped thickenings in many orchids (Bhanwra *et. al.*, 2006; Prakash and Aow, 1973; Kant and Bhanwra, 2010; Sood, 1985, 1986, 1989; Sood and Mohana Rao, 1986; Sood and Sham, 1987). The taxonomic



Figs. 34-52. Ontogeny and organization of female gametophyte in *Habenaria ovalifolia:* 34, L.S. young ovular primordium showing archesporial cell; 35-36, L.S. of young ovule with megaspore mother cell; 37, Megaspore mother cell at meiosis – I; 38, L.S. of ovule with unequal dyad cells; 39, Lower dyad cell at meiosis – II; 40, A triad; 41, Tetrad of megaspores; note obliquely arranged megaspores derived from micropylar dyad cell; 42, Linear tetrad of megaspores; 43, Nuclear division in functional megaspore; 44, Two nucleate embryo sac; 45, L.S. of ovule outline at 2-nucleate embryo sac stage; note longer outer integument; 46, Shows simultaneous division of nuclei in the 2-nucleate embryo sac; 47, Four nucleate embryo sac; 48, Shows synchronous division of nuclei in a 4-nucleate embryo sac; 49, Eight nucleate embryo sac; note quartet of nuclei at each pole; 50, Organised embryo sac; 51-52, Show stages of double fertilization.

significance of endothelial thickenings in orchidaceae was discussed by Freudenstein (1991). The middle layer was single layered, however, at certain places became bilayered because of periclinal division of certain cells, a feature also recorded in *Spathoglottis plicata* (Prakash and Aow, 1973) and *Vanilla planifolia, Zeuxine sulcata* (Swamy, 1946a, 1947). Tapetum was glandular and dual in origin. Similar observations were made in *Oreorchis foliosa* (Mohana Rao and Sood, 1987) and in species of *Habenaria* (Gurudeva, 2012; Sood, 1986). Tapetum was single layered however; it became two layered at certain places, a feature also recorded for *Eulophia epidendraea, Vanilla planifolia* (Swamy, 1943b, 1947) and *Zeuxine strateumatica* (Vij *et al.*, 1982). Tapetal cells remained uninucleate throughout and it was in conformity with previously investigated orchids (Sood, 1985; Sood and Mohana Rao, 1986; Sood and Sham, 1987). However, binucleate tapetal cells were reported in *Habenaria diphylla* (Gurudeva, 2012) and *Spathoglottis plicata* (Prakash and Aow, 1973). Finally the tapetal layer broke down leaving its remnants within the confines of the locule.

The archesporial cell after producing a parietal layer functioned together as sporogenous tissue. The sporogenous cells belonging to a massula were derived from a single archesporial cell. Similar condition was reported in *Calanthe veratrifolia, Neottia ovata* and *Orchis maculata* (Guignard, 1882), in several species of *Habenaria* and *Peristylus* (Swamy, 1946b, 1949a) and *Himantoglossum hircinum* (Heusser, 1915). The sporogenous cells enlarged and became microspore mother cells. They underwent usual meiotic divisions and resulted in different types of microspore tetrads. Quadripartition of microspore mother cell was simultaneous in most of the taxa investigated so far (Bhanwra, 2010; Bhanwra *et al.*, 2006; Mohana Rao and Sood, 1987; Prakash and Aow, 1973; Sood and Sham, 1987; Swamy, 1941, 1946b, 1947, 1949a) including the present study. After meiotic divisions, the microspores were held together in tetrads. The nuclear division within the microspore tetrad was synchronous. A similar feature was reported by Yeung and Blackman (1983). The nuclear division was asymmetrical in conformity with the earlier records (Hagerup, 1938; Mohana Rao and Sood, 1986; Swamy, 1949a). The small newly formed generative cell was initially adpressed to the wall of the microspore. Later, it separated itself from the microspore wall and entered into the cytoplasm of the tube cell as reported in *Rhynchostylis retusa* (Sood and Sham, 1987), species of *Habenaria* (Gurudeva, 2012; Sood, 1986; Swamy, 1946b). The pollen grains are 2-celled when the massulae are ready for pollination. Similar observations have been made in *Cypripedium cordigerum* (Sood and Mohana Rao, 1988), and *Malaxis saprophyta* (Sood, 1992). At anther dehiscence, a well developed stomium formed at wall cells at the junction of the two adjacent microsporangia disorganized leading to the formation of a vertical slit in each of the two anther lobes which facilitated the pollinators to carry the massula.

Female Gametophyte

The gynoecium was tricarpellary, syncarpous and inferior and the ovary was unilocular. Numerous anatropous ovules developed on the divided parietal placentae. The initiation of ovule on the placenta was triggered after pollination. This observation was in conformity with earlier records on this aspect (Brown, 1833; Niimoto and Sagawa, 1961; Vij and Sharma, 1986; Yeung and Law, 1989). Finger-like ovular primordia consisted of an axial row of cells ensheathed by a layer of epidermis is a constant feature. This was also true with the orchids studied (Abe, 1972; Gurudeva and Govindappa, 2008, 2009, 2014; Krishna Swamy *et al.*, 2003; Lee and Yeung, 2012; Kant and Bhanwra, 2010; Sood, 1989; Sood and Mohana Rao, 1988; Swamy, 1949a).

The integumentary initials took their origin from nucellar epidermal cells. The inner integument was always the first to be differentiated, the first to become active and first to degenerate unlike in *Vanilla planifolia* where it was intact at embryo stage (Swamy, 1947). The micropyle was organized by the inner integument alone. This was in complete agreement with earlier findings (Abe, 1972b; Govindappa and Karanth, 1980; Gurudeva and Govindappa, 2008, 2009, 2014; Mohana Rao and Sood, 1986, 1987; Sood, 1986b; Sood and Mohana Rao, 1986; Swamy, 1949a).

The archesporial cell enlarged in size and directly functioned as the megaspore mother cell. It underwent meiosis-I giving rise to two superposed dyad cells. The small upper dyad cell degenerated and the lower one passed through meiosis-II giving rise to a smaller upper non-functional and a lower functional megaspore. This led to the organization of a triad which was a common feature. Similar triad was reported in majority of orchids (Abe, 1972a; Attri *et al.*, 2007; Govindappa and Karanth, 1980a; Gurudeva and Govindappa, 2008, 2009, 2014; Mohana Rao and Sood, 1986, 1987; Sood, 1986; Swamy, 1949a; Ward, 1880). However, sometimes the micropylar dyad cell did not degenerate and participated in meiosis-II in which case the resulting tetrad would have either been T-shaped or linear tetrad. Co-existance of tetrad and triads was also recorded in *Bletilla striata* and *Habenaria radiata* (Abe, 1972a)

and *H. densa* (Mohana Rao and Sood, 1979a). The formation of a triad was considered to be an advanced character compared to the development of a linear tetrad of megaspore. According to Abe (1972b), triad formation was more common in orchids.

The nucleus of the chalazal megaspore divided successively and developed into 8-nucleate embryo sac. The mature embryo sac consisted of egg apparatus with two synergids and an egg, a micropylar and chalazal polar nuclei and three antipodal cells. Similar type of embryo sac organization was recorded in *Aerides maculosum* (Gurudeva, 2009); *Habenaria edgeworthii, H. elisabethae,* and *H. galeandra* (Sood, 1986); *H. clavigera, H. intermedia, H. latilabris,* and *H. pectinata* (Sharma and Vij, 1987); *Liparis paradoxa* (Sood, 1989); *Microstylis cylindrostachya* (Sood, 1985); *M. wallichii* (Sood and Mohana Rao, 1986); and *Oreorchis foliosa* (Mohana Rao and Sood, 1987). The embryo sac contained three antipodal cells and as recorded in many species of orchids (Abe, 1972a, 1977; Brown, 1909; Sood, 1986) is a basic feature. The next step in simplification was the elimination of walls of antipodal cells as observed in several species of *Epidendrum* (Gurudeva and Govindappa, 2008) and *Habenaria* (Swamy, 1946b). On the other hand, number of antipodal cells was reduced from three to one in *Goodyera procera* (Gurudeva, 2014). Further, with the elimination of wall of antipodal cell and only one antipodal nucleus arrived at, as in *Trias stocksii* (Gurudeva, 2010) and *Zeuxine gracilis* (Gurudeva, 2011a). The process of simplification appeared to extend to the number of antipodal nuclei from three to two, one and none, a feature which was recorded for a few species of orchidaceae (Arekal and Karanth, 1981; Savina, 1978; Swamy, 1949a). It was obvious, therefore, that functionally the chalazal end of the embryo sac was not as important as the micropylar end. The development of the female gametophyte conformed to G 1a type of Abe (1972b).

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