J. Orchid Soc. India, 26 (1-2): 93-99, 2012

ISSN 0971-5371

ONTOGENY OF MICROSPORANGIUM AND DEVELOPMENT OF MALE GAMETOPHYTE IN *HABENARIA DIPHYLLA* DALZ.

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Abstract

The anther in *Habenaria diphylla* Dalz. is dithecous and tetrasporangiate. Its wall development conforms to the monocotyledonous type. Each archesporial cell develops into a block of sporogenous cells before organizing into massulae. The anther wall is 4-layered. The endothecial cells develop ring-like tangential thickening on the inner walls. Tapetal cells are binucleate and of dual origin. The microspore tetrads are tetrahedral, decussate, linear and T-shaped. Pollen sheds at 2-celled stage.

Introduction

THE GENUS *Habenaria* Willd. comprises nearly 600 species of terrestrial orchids. Seventy two species and one variety are known from India (Sathish Kumar and Manilal,1994); Karnataka accounts for 18 of these (Ananda Rao and Sridhar, 2007). Embryological data in the genus is rather meagre despite the involvement of severval workers (Abe, 1972; Brown, 1909; Leavitt, 1901; Mohana Rao and Rao, 1985; Mohana Rao and Sood, 1979; Sharma and Vij, 1987; Sood, 1985,1986; Swamy, 1946). Information on the embryology of *Habenaria diphylla* has remained elusive. This communication deals with the development of microsporangium and male gametophyte in this taxon.

Material and Methods

Habenaria diphylla Dalz. is a small terrestrial herb with an ellipsoid underground tuber. It bears one - two ovate-oblong coriaceous, acute or obtuse leaves that lie flat on the ground; a terminal raceme; greenish white sepals and petals; 3-lobed and white lip; long spur; stout column; two pollinia and; inferior ovary (Figs.1,2). The flower buds were collected, at different stages of development, from Manipal and Udupi (Karnataka, India) during August and October 2011. These were fixed in formalin-acetic-alcohol and stored in 70% ethanol following a thorough wash in running water. Conventional micro techniques were followed. The serial transverse sections at 10-12μm 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 a watch glass treated with 1N HCl and gently warmed over the flame. The treated anthers were macerated with Crystal Violet and mounted in glycerine. Drawings were made using Camera Lucida and Meopta microscope. Phtomicrographs were taken by using Olympus-CH20i microscope with built in analogue camera (CM OF 1.4 megepixel). Computer images were captured using Av-digitaliser having Grand VCD-2000 captured guard.

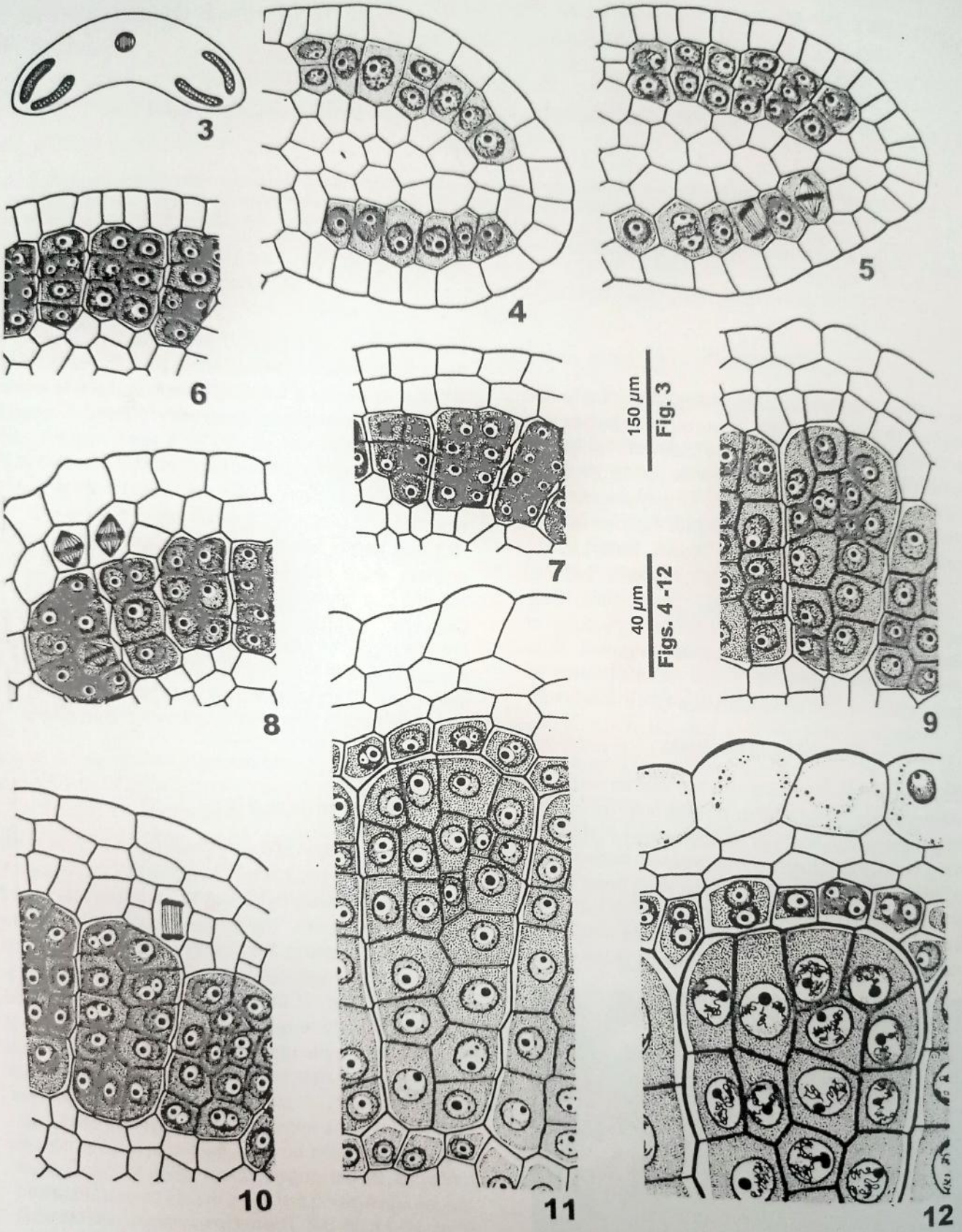
Figs. 1, 2. *Habenaria diphylla* : 1, Two flowering plants; 2, Flowers (close up).

Results

Microsporangium (Figs. 3-34)

A transverse section of a very young anther shows two lobes, each with two rows of hypodermal archesporial cells that are densely cytoplasmic and have prominent nuclei (Figs. 3, 4). The archesporial cells divide periclinally delimiting the primary parietal layer from the primary sporogenous layer (Figs. 4, 5). The density of the cytoplasm of the cells of the primary parietal layer is gradually reduced as the cells of the primary sporogenous layer undergo anticlinal and periclinal divisions giving rise to small blocks of sporogenous cells (Figs. 6, 7). A periclinal division of the parietal layer results in the formation of two layers of cells (Figs. 8, 9, 35). The outer layer below the epidermis directly functions as the endothecium while the inner layer divides again giving rise to the middle layer and tapetum (Figs. 10, 11, 36, 37). The microsporangial wall consists of four layers, namely the epidermis, the endothecium, a middle layer and the glandular tapetum. Its development, thus, conforms to 'monocotyledonous type' (Davis,1966). Meanwhile, cell of the connective

Received : November 15, 2011; Revised : August 17, 2012; Accepted : November 9, 2012



Figs.3-12. *Habenaria diphylla*, diagrammatic cross sections of developing microsporangium: 3, Showing four groups of archesporial cells; 4-6, Showing the origin of primary parietal and sporogenous cell layers; 7-9, Showing the development of two parietal layers; note groups of sporogenous cells; 10-11, Showing origin of the tapetum and middle layer from the inner parietal layer; 12, Showing binucleate tapetal cells and microspore mother cells in the developing massulae.

bordering the sporogenous tissue acquire dense cytoplasm and larger nuclei and align with the tapetal layer on the wallward side of the microsporangium and function as such (Figs.11, 37). The tapetal layer surrounding the sporogenous tissue of a microsporangium, therefore, is of dual origin.

During the later stages of development, the cells of the microsporangium wall divide anticlinally and extend laterally to accommodate the increase in volume of the sporogenous tissue from within. Starch grains accumulate in the epidermal cells. The uninucleate tapetal cells become binucleate by the time the spore mother cells are organized in the sporangium (Figs.12, 37). The tapetal layer breaks down along with the middle layer. The cells of the endothecium acquire ring-like heavy thickenings, one per each cell, tangentially disposed on the inner surface of the wall (Figs.13-15, 38-40).

Meanwhile cells of the primary sporogenous layer undergo periclinal and anticlinal divisions to produce a massive sporogenous tissue in which clear cut groups of cells can be easily discerned. Each group could be traced back to an archesporial cell. Therefore, there will be as many groups of sporogenous cells in the mass as there are archesporial cells present in the very young microsporangium (Figs.5-12). Ultimately the sporogenous cells in each of the groups give rise to the microspore mother cells (Fig.12). Each group of spore mother cells would later organize a pollen massula.

Microsporogenesis and Development of Male Gametophyte

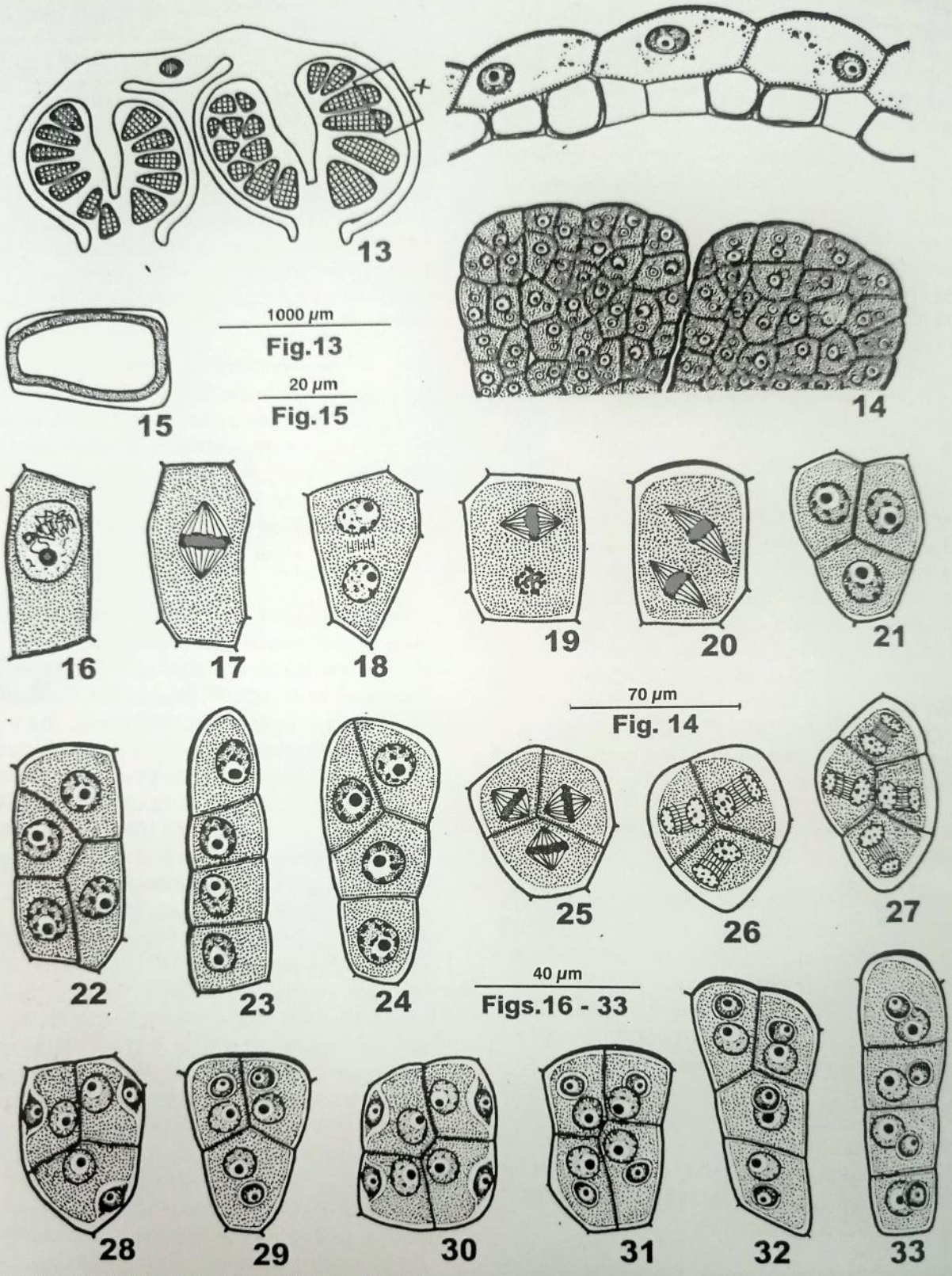
Meiotic divisions occur in the spore mother cells. Meiosis-I is not followed by a cell partition (Figs.16-18). The dyad nuclei resulting after meiosis-I pass through meiosis-II producing four haploid nuclei. Quadripartition of the mother cell now occurs and is of the simultaneous type. The microspore tetrads produced may be tetrahedral, rhomboidal, linear or T-shaped, depending the orientation of the spindles of the dividing dyad nuclei (Figs.19-24). Therefore, within a single massula all the above types of tetrads are recognized. Of all the tetrads rhomboidal, linear and T-shaped tetrads are located towards the periphery rather than at the centre of the massula.

During subsequent development, the microspores of a tetrad do not separate apart, so also the tetrads of a massula. The nuclei of microspores of tetrads then divide synchronously. However, this division is asynchronous or synchronous in microspores of adjacent tetrads of a massula. No vacuolation of spore cytoplasm is apparent prior to nuclear division. The dividing nuclei will have their spindles disposed in proximal-distal axis of the spore in the tetrad (Figs. 25-27). Of the two resulting cells within the microspore, the smaller generative cell is always located towards the distal end, adjoining the spore coat, while the larger tube cell lies at the proximal side (Figs. 28-30). The generative cell soon separates itself from the spore coat and enters into the cytoplasm of the tube cell (Figs. 29, 31-33). The pollen grain derived from a microspore remains two-celled. Meanwhile, deposition of a coat of sporopollenin occurs over each pollen massula and the massulae of a microsporangium appear distinct from one another.

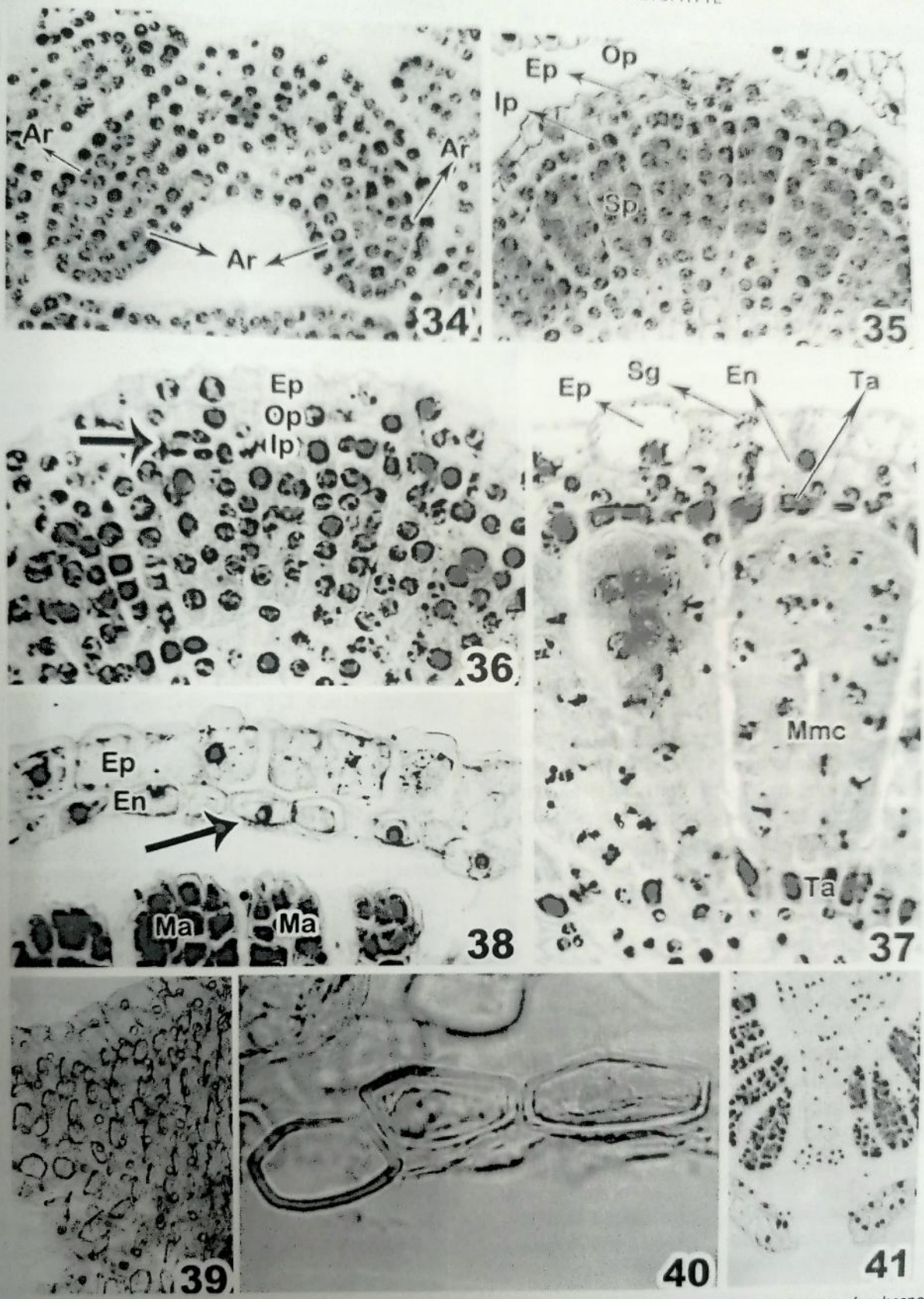
Cells of the separating layers between the adjacent microsporangia, especially below the region where the wall of the two microsporangia meet, break down freeing the two microsporangial walls and creating a stomium (Figs.13, 41) so as to facilitate the easy exit of the massulae of the adjacent microsporangia of the anther half through a common opening.

Discussion

The development of anther wall corresponds to the monocotyledonous type (Davis,1966). A similar mode of wall development has also been reported in *Habenaria intermedia, H. edgeworthii, H. elisabethe and H. galeandra* (Sood,1984,1985,1986). The anther wall comprises of epidermis, endothecium, middle layer and tapetum. Similar feature has been reported in most of the investigated orchid taxa (Mohana Rao and Sood,1987; Sharma and Vij,1984; Swamy,1949). The epidermis is single layered and shows the presence of starch grains, indicates that this layer is concerned with nutrition besides their usual function of protection. Nutritive role of epidermis has also been recorded in *Zeuxine longilabris* (Karanth, *et. al.*,1979) and *Epipogium roseum* (Govindappa and Karanth,1980). The endothecium is single layered. At maturity the cells acquire thickenings on the inner surface of their walls. A single ring-like and tangentially disposed thickening has been observed in each of the endothecial cells. The type of endothecial thickening corresponds to Type-II of Freudenstein (1991).Similar type of ring-like thickening has been reported in *Aa achalensis* (Cocucci,1964), in some species of *Habenaria* (Sharma and Vij,1987) and *Aphyllorchis montana* and *Dendrobium microbulbon* (Krishna Swamy, *et. al.*,2003). However, Untawale and Bhasin (1973) observed spiral thickenings in certain species of *Habenaria* and fibrillar or band like thickenings in many orchids(Bhanwra, *et.al.*, 2006; Prakash and Aow, 1973; Ravikant and Bhanwra, 2010; Sood, 1985, 1986, 1989; Sood and



Figs. 13-33. *Habenaria diphylla*, cross sections showing different stages of microsporogenesis and pollen development (diagrammatic): 13, Mature anther; 14, Persistant epidermis with starch grains, endothecial cells with ring-like thickenings and parts of massulae; 15,An endothecial cell to show single tangential heavy thickening in the wall; 16-18, Meiosis-I in the microspore mother cells; 19-20, Simultaneous divisions of dyad nucleus; 21, Three microspores in a tetrahedral tetrad; 22, Rhomboidal tetrad; 23, Linear tetrad; 24, T-shaped tetrad; 25-27, Divisions of microspores in the tetrad; note proximal and distal orientation of nuclear spindles of the dividing nuclei; 28-33, Migration of parietal disposed generative cell into the cytoplasm of the vegetative cell in a pollen tetrad.



Figs.34-41. *Habenaria diphylla*, photomicrograph showing different stages during microsporangium ontogeny: 34, Groups of archesporial cells. x 197; 35, Parietal cell layers and distinct groups of cells in sporogenous tissue. x 227; 36, Periclinal division in the inner parietal cell layer (arrow). x 241; 37, Wall layers showing epidermis with starch grains, binucleate tapetal cells and microspore mother cells x 250; 38, Mature microsporangium with persistent epidermis, endothecial cells with ring-like thickenings (arrow), and massulae. x 769; 39, Whole mount of endothecial layer x780; 40, Ring shaped thickenings on endothecial cells. x 1500; 41, Stomium and line of dehiscence. x 384. (Ar, Archesporial cell; En, Endothecium; Ep, Epidermis; Ip, Inner parietal layer; Ma, Massulae; Mmc, Microspore mother cell; Op, Outer parietal layer; Sg, Starch grains; Sp, Sporogenous tissue; Ta; Tapetum).

Mohana Rao,1986; Sood and Neelu Sham,1987; Swamy,1947, 1949; Swamynathan, 1967). The taxonomic significance of endothecial thickenings in Orchidaceae has been discussed by Freudenstein (1991). The middle layer and tapetum are single layered. Tapetum is glandular and dual in origin. Similar observations have been made in *Oreorchis foliosa* (Mohana Rao and Sood,1987) and in species of *Habenaria* (Sood,1986). Tapetal cells remain binucleate throughout and in conformity with several members of orchids such as *Paphiopedilum druryii* (Swamy,1949), *Spathoglottis plicata* (Prakash and Aow,1973), *Epipactis latifolia* (Sood,1997), and *E. veratrifolia* (Bhanwra, *et.al.*, 2006). Finally this layer breaks down leaving its remnants within the confines of the locule.

The archesporial cells after cutting off a parietal layer function as sporogenous tissue. The sporogenous cells belonging to a massula are derived from single archesporial cell. Similar condition has been reported in *Orchis maculata, Calanthe veratrifolia* and *Neottia ovata* (Guignard, 1882), *Himantoglossum hircinum* (Heusser,1915) and in species of *Habenaria* and *Peristylus* (Swamy, 1946, 1949). The sporogenous cells enlarge and become microspore mother cell. They undergo usual meiotic divisions and results in tetrahedral, decussate, linear and T-shaped tetrads. Quadripartition of microspore mother cell is simultaneous in conformity with other investigated orchid taxa (Mohana Rao and Sood,1987; Prakash and Aow, 1973; Sood and Mohana Rao,1988; Swamy, 1941,1946,1947,1949). The nuclear division within the microspore tetrad is synchronous and asymmetrical in conformity with the earlier records (Hagerup,1939; Mohana Rao and Sood,1986; Swamy,1949). The pollen grains are two celled when massulae are ready for pollination. Similar observations has been made in *Malaxis saprophyta* (Sood,1992) and *Cypripedium cordigerum* (Sood and Mohana Rao,1988). At the time of anther dehiscence a well developed stomium is formed at wall cells at the junction of the two adjacent microsporangia disorganize leading to the formation of vertical slit in each of the two anther lobes which facilitates to carry the massula.

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