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Microsporogenesis in *Melilotus indicus* (L.) All

Nidhi Gautam* and V.K. Gautam

Department of Botany, Daulat Ram College, Deen Dayal Upadhyaya College, University of Delhi, Delhi-7, India

*Corresponding author: nidhigautam2004@gmail.com

ABSTRACT

The anther development in *Melilotus indicus*, an annual fodder legume follows the dicotyledonous type. The mature anther is tetrasporangiate and its wall is comprised of the outermost epidermis followed by endothecium, single middle layer and the innermost secretory tapetum. *Melilotus indicus* bears 10 stamens of around 1.5 to 2 mm long that are arranged in a 9+1diadelphous manner. These tiny dorsifixed stamens produce a small number of microspores in each microsporangium. At the sporogenous stage of microsporogenesis, tapetal cells become irregular, full of proteinaceous material and during late ontogeny a transverse septum is observed in some of the microsporangia. The septum is of tapetal origin that seems to be an adaptation for better nourishment of developing microspores. The mature pollen grain of *Melilotus indica* is oblong and tricolporate. The exine is reticulate, well differentiated into ektexine and endexine. The ektexine is well distinguished in a tectum, bacula, and foot layer.

Keywords: *Melilotus indicus,*forage legume, winter weed, microsporogenesis, tapetal septae, *in situ* pollen germination

Melilotus indicus (L.) All.(syn. *M.parviflora* Desf.), a small, herbaceous wild fodder legume belonging to tribe Trifolieae (Leguminosae), used traditionally as an anti-inflammatory agent. ethanolic extract of *M.indicus* is reported to have antioxidant, antibacterial and antifungal properties (Khan *et al.* 2018) that appears as a common winter weed in the month of November in Delhi. The plant is rich in coumarin, an anticoagulant Its reproductive phase starts in February and continues till March end. It branches profusely and attains a height of 50-60 cm. at maturity. Leaves are trifoliate with spiral phyllotaxy. Flowers develop in axillary racemes and each inflorescence bears 20-30 small, yellow, papilionaceous flowers. Almost all flowers develop into tiny one-seeded pods. The present investigations on *Melilotus indicus* were taken to correlate the structural changes in developing anthers and microspores at light microscopic and EM levels with *in situ* localization of major metabolites like insoluble polysaccharides and total proteins that would help to understand the biology of the pollen grain development in *Melilotus indicus* L. (All). The presence of tapetal septae in anthers and in situ germination of pollen grains are interesting features reported in the present work.

MATERIALS AND METHODS

Young buds of *Melilotus indica* L. All. at progressive developmental stages were collected from the botanical garden, University of Delhi, Delhi. The material was fixed in 10% aqueous acrolein for 24 hr. Usual methods of dehydration and infiltration were followed and the embedding of material was done in glycol methacrylate (Feder & O' Brien, 1968). Two-micron thick sections were cut on an AO Spenser rotary microtome using glass knives. Insoluble polysaccharides and total proteins were localized with Periodic acid Schiff's reagent (Jensen, 1962) and Coomassie brilliant blue (Fisher, 1968) respectively for histochemical studies. For electron microscopy, material was fixed in 5% glutaraldehyde in 0.2M phosphate buffer (pH 6.8) for 6 hr at 4^{o}C. The fixed material was washed with a cold buffer and postfixed in 2% OsO_4 for 2 hr., dehydrated in a graded ascending acetone series. For TEM the material was infiltrated and embedded in epon-araldite resin mixture (Mollenhauer, 1964). Sections cut on a Sorvall Ultramicrotome, were picked up on 200 mesh copper grids and stained with uranyl acetate and lead citrate. Stained grids were observed under Philips Electron Microscope 300 that was operated at 80 kV. The material was then sputter coated with silver for SEM studies and was observed under a Philips 501B microscope.

Observations

The anthers are tetrasporangiate in *Melilotus indicus*. The young anther shows a few hypodermal, archesporial cells that undergo periclinal division to give rise to outer primary parietal and the inner primary sporogenous layers. The primary parietal layer redivides by periclinal divisions to form secondary parietal layers. The development of the anther wall is of Dicotyledonous type where the outer secondary parietal layer divides again to produce endothecium and middle layer while the inner layer directly functions as the tapetum. The mature anther wall consists of an outermost epidermis, an endothecium, a middle layer and the innermost tapetum (Fig. 1, A, B Fig. 3 A). The tapetum is of secretary type. A few polysaccharide grains are seen in wall layers at this stage. With the onset of meiosis, the microspore mother cells are besieged by a callose layer. The tapetal cells increase in size, possess large polyploid nuclei and are rich in proteins (Fig. 3, A,B). Some of the central tapetal cells protrude into the locule forming a septum that divides microsporangium into two equal chambers at this stage (Fig. 3, B). The MMCs divide meiotically. The cytokinesis is of simultaneous type and gives rise to tetrahedral tetrads (Fig. 1, C Fig. 3 A). The tapetal cells stain intensely with PAS and are full of proteins at this stage. These microspores are released from the callose layer, enlarge, assume spherical shape and stain deeply for proteins (Fig. 2D). The uninucleate microspores also contain a few PAS positive grains (Fig. 1,D Fig. 3 C,D). Numerous tiny vacuoles appear in the microspore that fuse together to form a large vacuole pushing cytoplasm to the periphery (Fig. 1 D, Fig. 3C). The microspore wall differentiates into a thin intine and a thick exine layer.

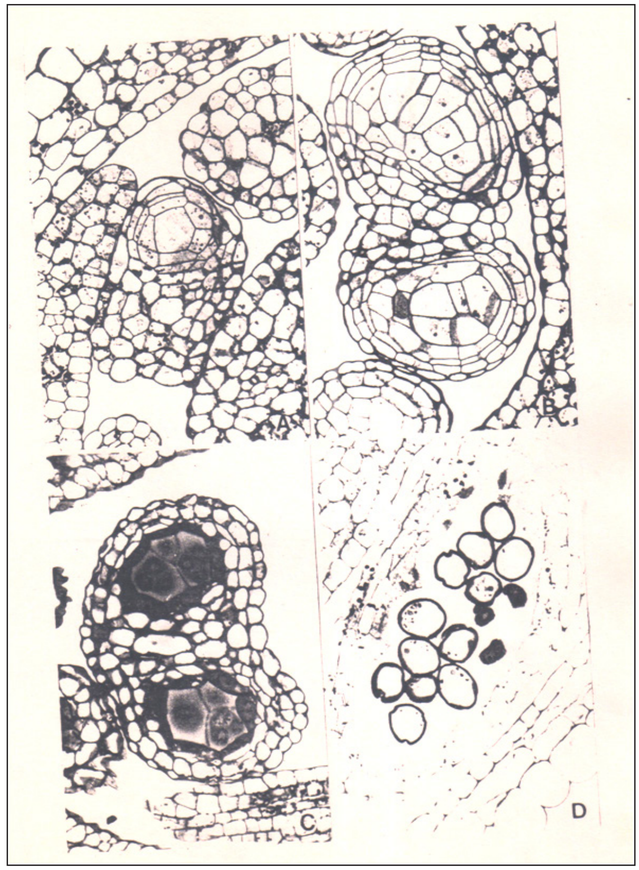

Fig. 1: **(A)** Section of young anther at archesporial. **(B)** Sporogenous cell stage showing various wall layers. **(C)** Microsporangium with microspore tetrads and PAS rich tapetum. **(D)** Uninucleate microspore stage (LM × 480).

The mature pollen grains of *Melilotus indicus* are oblong in shape and tricolporate with reticulate surface sculpturing (Fig. 4,B,C). The exine is distinguished into sexine and nexine. The sexine is differentiated into tectum and short, thick, closely placed columellae. The nexine is composed of massive nexine 1 and nexine 2. The nexine layer represents half of the total exine thickness (Fig. 4,D). The small,PAS positive exine connections, composed of tectal layers of adjacent pollen grains are also observed during the present study (Fig.

1,D). The endothecium shows well-developed PAS positive fibrous thickenings on their radial and inner tangential walls (Fig. 3, E). In *M. indicus* the mature anther dehisces by a longitudinal slit releasing two-celled pollen grains. *In situ* germination of pollen grains is another interesting feature observed in some of the dehisced anthers during the present investigations (Fig. 4, A).

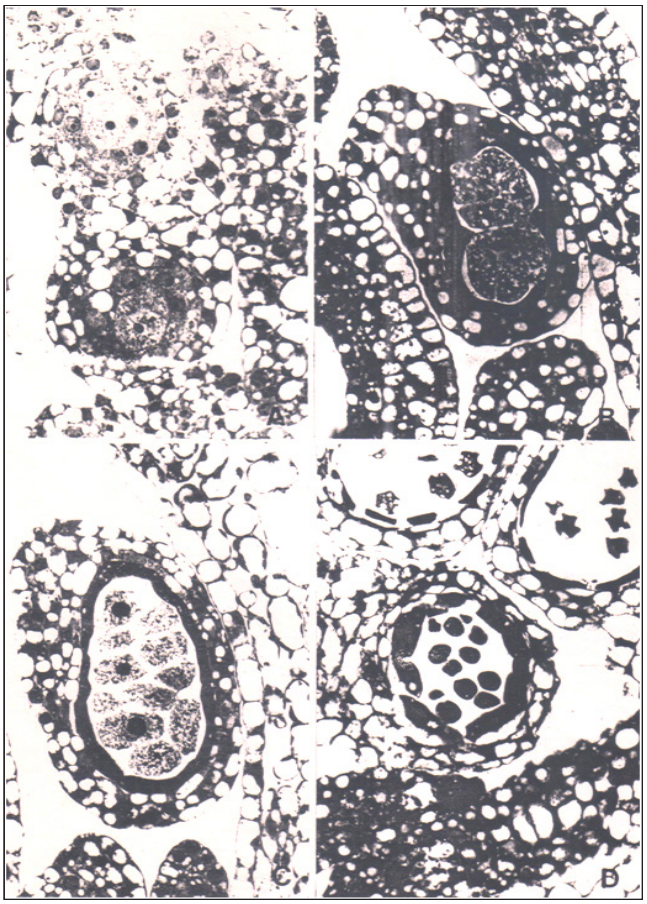

Fig. 2: (A) Section of young anther at archesporial tetrad stage. **(B)** Sporogenous cell stage showing protein rich tapetum. **(C)** MMC stage. **(D)** Late tetrad stage, note protein rich microspores and tapetum (LM × 480)

RESULTS AND DISCUSSION

The course of anther development in *Melilotus indicus* follows the Dicotyledonous type (Davis, 1966) of pattern. The presence of a single middle layer is an advanced feature as the primitive Angiosperms show a relatively higher number of middle layers (Bhandari, 1984). In angiosperms the glandular type of tapetum is considered as the primitive type whereas the amoeboid (plasmodial) type as the advanced type (Johri, 1992).

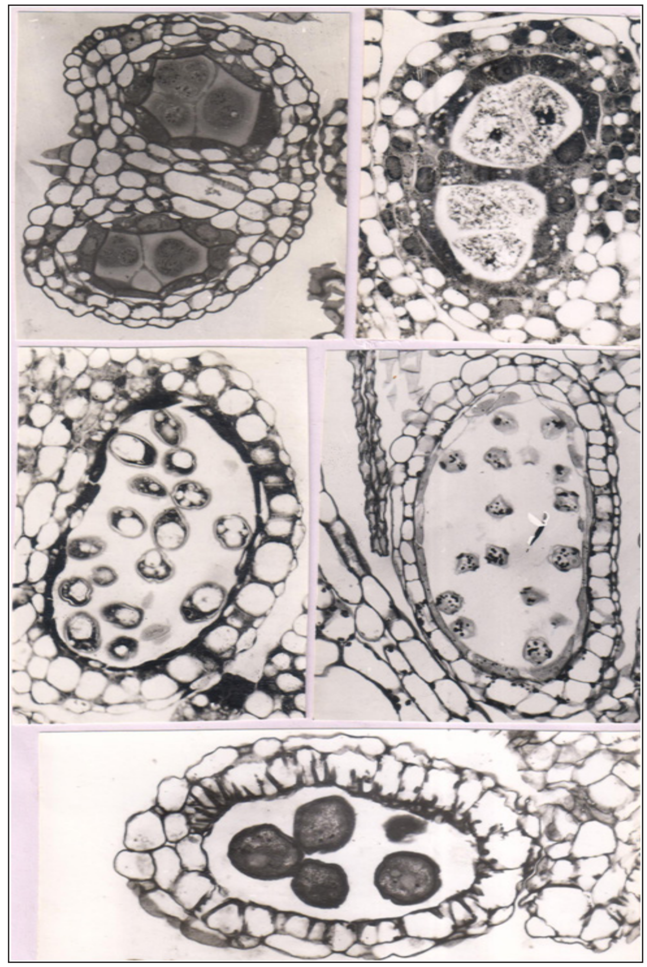

Fig. 3: (A) Section of anther at tetrad stage. **(B)** MMC stage showing protein rich tapetal septum. **(C-D)** Uninucleate microspore stage. **(E)** Bicelled stage of pollen grains. Also note PAS positive endothelial thickenings (LM × 480)

In *Melilotus indicus* (present study) though the tapetum is of secretory type but the formation of tapetal septae represent a special feature. The partitioning of sporogenous tissue due to the presence of plates of sterile tissue has been reported in a few plants (Eames, 1961; Davis, 1966; Johri & Bhatnagar, 1971; Albertsen & Palmer, 1979). In *Nymphaea cologata* (Rowley *et al.* 1992) tapetal cells protrude into anther locules at three distinct intervals as bridges, partitions or as invasive cells between or around microspore tetrads. In *Melilotus indicus* (present study) the septae are derived from the tapetal layer for the better nourishment of the developing microspores.

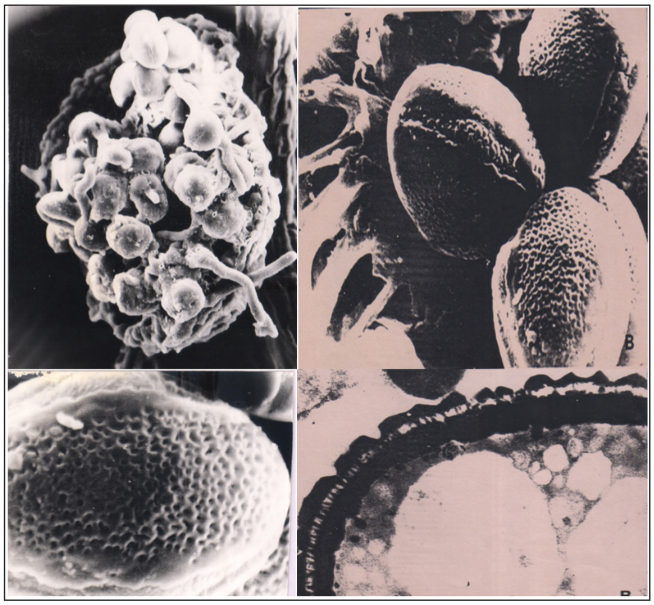

Fig. 4: (A) Scanning electron micrograph of anther with germinating pollen grains (×1280). (**B**) SEM of the dehisced anther locule with mature pollen grains and endothelial thickenings (×5000). **(C)** Mature pollen to show reticulate exine (×10,000). **(D)** TEM of pollen, note various layers of exine (×11,600)

The mature pollen grains of *Melilotus indicus* are oblong in shape and tricolporate with reticulate surface sculpturing. The pollen morphology of 28 species belonging to three genera (*Melilotus, Trifolium and Trigonella*) of subfamily Papilionoideae in Egypt were studied by Gazar (2003) and established three types of pollen in this group. Lashin (2006) reported pollen with tectate perforate, reticulate exine and distinct pores in *Melilotus* spp. Small exine connections are observed between the fertile pollen grains in *M. indicus*. The plant which is characterized by minute flowers with small stigma, two ovules inside the ovary and low pollen - ovule ratio, the existence of small exinal connections enhance the pollination efficiency (Gautam *et al.* 1991).

In angiosperms, pollen grains carrying either male gametes or their progenitor cell (generative cell) get deposited on the stigma through biotic or abiotic agents. These grains then germinate on the stigmatic surface as most of the basic requirements of pollen germination are met by the stigma. A complex and elaborate mechanism of pollen-pistil interaction regulates the post-pollination event in higher plants (Shivanna & Johri, 1985; Cresti, *et al.* 1992; Russel & Dumas, 1992). In a few systems, however, pollen grains do not seem to require any specific substances from the stigma for germination. In *Crotalaria retusa,* about 20-30 % of the pollen germinates when they are exposed to high humidity (Malti & Shivanna, 1984).

In most of the plants the amount of nutrients present in the pollen are insufficient to support pollen tube growth until they reach the ovule. An unusual observation has been reported in some of the members of Malpighiaceae (Anderson, 1980) and Callitricaceae (Philbrick & Anderson, 1992) where both chasmogamous as well as cleistogamous flowers are produced. In Malpighiaceae, pollen grains of cleistogamous flowers germinate inside the indehiscent anther; pollen tubes grow down the anther filament into the receptacle and then turn upward growing into the ovary and enter the micropyle (Anderson, 1980). In the case of Callitricaceae the phenomenon of "internal geitonogamy" has been reported (Philbrick & Anderson, 1992). In *Melilotus indicus* (present study) *in situ* germination of pollen grains in some of the dehisced anther is very interesting. The significance of this phenomenon will only be understood when the reproductive biology of this taxon is studied in more details.

Melilotus indicus is one of the unexploited fodder crops that is able to tolerate environmental stresses and can be grown on moderately saline soils (Maranon, 1989; Al Sherif, 2009)where other conventional forage crops cannot be cultivated. It also has a number of ethnomedicinal uses (Ahmed *et al.* 2014; Khan *et al.* 2018). *Melilotus indicus* is used in traditional medicines as a source of anti inflammatory compounds. The ethanolic extract of this plant contains coumarin that imparts its medicinal significance. This multipurpose potential forage legume needs sincere attention from plant biologists.

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