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ULTRASTRUCTURAL STUDIES ON ANTHER DEVELOPMENT IN ETHREL INDUCED MALE STERILE *VICIA FABA* L.

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A comparative light and transmission electron microscopic study on anther development in ethrel induced male sterile and male fertile plants of *Vicia faba* L. was undertaken. Foliar sprays of ethrel induced complete pollen sterility associated with abnormal tapetal behaviour. In fertile anthers, at pre-meiotic stages, the tapetal cells consisted of large number of organelles associated with smooth ER and a number of pro-orbicular bodies. During meiosis, the organelle population increased indicating an increase in the metabolic activity. The disorganization of tapetum at microspore tetrad stage was accompanied by increased vacuolation and progressive disappearance of mitochondria and plastids. Towards the end, nuclear envelope, ER and the plasmalemma were disrupted and finally the tapetal cells were completely absorbed when pollen grains were about to shed. The exine in pollen grains differentiated into foot layer, tectum and bacule. On the other hand, in the anthers of ethrel treated plants, the first abnormality in the tapetum was detected at microspore tetrad stage and the differences in organelle structure first became discernible at mid- or late-microspore stage. Mitochondria in the tapetum displayed structural disorganization of the inner membrane. The other organelles including the plastids, nuclei and ribosomes were also seen in disorganized form. At pollen grain stage, the tapetal protoplast was fully disorganized and dark stained and their organelles were not discernible. These cells finally degenerated at the time of anthesis followed by degeneration of non-viable pollen grains. The exine was poorly developed without any differentiation of foot layer, tectum and bacule and intine was absent.

Key words: Ethrel, Male sterility, Ultrastructure, *Vicia faba L.*

Male sterility has potential uses in production of mass-scale hybrids or in promoting cross-pollination in naturally self-pollinated crops. Considerable success has been achieved through chemicals known as male gametocides or chemical hybridizing agents [CHAs] that selectively retard or inhibit the development of anthers (Cross and Schulz, 1997). Ethrel is an ethylene-releasing plant hormone that also acts as microspore inhibitor. The complete pollen sterility has been induced by treatments with ethrel in *Hordeum vulgare* (Colhoun and Steer, 1983), *Brassica juncea* (Banga *et al.*, 1986), *Triticum aestivum* (Keys and Sorrels, 1990) and *Vicia faba* (Chauhan and Chauhan, 2003).

A large number of light microscopic studies have been undertaken to understand the mechanism of pollen abortion in chemically treated plants (Chauhan and Kinoshita, 1982; Chauhan, 1986). However, ultrastructural changes in the anthers of chemically treated plants have received less attention. According to Laser and Lersten (1972) it is important to find out the exact stage at which organelles show the first sign of disintegration and whether there is a definite sequence or simply a simultaneous collapse.

Keeping this in view, the present investigation has been undertaken to find out the mechanism of male sterility through ultrastructural studies in chemically induced male sterile plants of *Vicia faba* L.

The seeds of *Vicia faba* var. ILB 1817 obtained from National Bureau of Plant Genetic Resources, New Delhi were grown at Botanical Garden, School of Life Sciences, Dr. B. R. Ambedkar University, Agra maintaining a distance of 35 cm between rows and 15 cm between plants.

A group of 60 plants were sprayed with 0.1, 0.2 and 0.3 % (v/v) ethrel containing 2-3 drops of liquid soap as a wetting agent only once (T_1). Thirty plants were sprayed again (T_2) at time of floral bud initiation. A group of 30 plants were sprayed with distilled water containing a few drops of the wetting agent to serve as control.

Pollen viability of treated and control plants was

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tested at regular intervals throughout flowering period with 1% Tetrazolium chloride in 0.15M Tris-HCl buffer at pH 7.5 and flurochromatic reaction (FCR) test as described by Shivanna and Rangaswamy (1992).

For both light microscopic (LM) and transmission electron microscopic (TEM) studies, anthers of treated plants exhibiting 100% pollen sterility and control plants (84 % pollen fertility) at various developmental stages were fixed in 3% glutaraldehyde in 0.1MPO_4 buffer at pH 6.8. The samples were rinsed twice in the same phosphate buffer for 5 minutes each. Post fixation was done with 1% osmium tetraoxide in the same buffer for 2 hours. Samples were dehydrated in an ethyl-propylene oxide series, embedded in Spurr's low viscosity embedding media and were polymerized at 60^{0}C overnight.

For LM studies semi-ultrathin sections were cut at 1mm and stained with a solution of 0.5% w/v toluidine blue in 1% w/v sodium borate.

Sections for TEM studies were cut at 60-80nm and picked up on gold-coated copper grids. These were stained with uranyl acetate and alkaline lead citrate. Observations on chosen anther locules were made on Philips Cryo CM10 electron microscope at All India Institute of Medical Sciences, New Delhi.

Foliar sprays with different concentrations of ethrel were found to be significantly effective in inducing complete pollen sterility. All the treatments with 0.1, 0.2, & 0.3 % ethrel induced 100% pollen sterility. The sterility induced was of permanent nature that lasted throughout the flowering period. The results on the extent of pollen sterility induced and other yield parameters have been published earlier (Chauhan and Chauhan, 2003).

Anther development

A. Male fertile plants

During the early stages of development, the tapetal cells were made up of thin walls In several plants, tapetal walls are known to have plasmodesmatal connections between them (Mascarenhas, 1975), but such connections could not be recorded in the present material. At late pre-meiotic stages, ribosomes with associated profiles of smooth and elaborate ER-system, large number of mitochondria, plastids, several small vacuoles and a number of pro-orbicular bodies were identified in the cytoplasm. The cytoplasm was dense and nuclei were prominent. Beginning with synizesis, these cells enlarged radially and became bi-nucleated. Pro-orbicular bodies acquired a coat of electron dense material and deposition of sporopollenin in the orbicular wall. At microspore tetrad stage, the tapetal cells attained their maximum development, but by the time microspores were liberated, the tapetal cells started degenerating. This was accompanied by increased vacuolation and progressive disappearance of mitochondria and plastids. Tapetal cells completely disappeared by the time pollen grain became engorged with reserve food (Fig. 1E).

The primary sporogenous cells divided to generate a mass of secondary sporogenous cells, to function as pollen mother cells [PMCs]. The PMCs after meiosis, resulted in the production of microspores which were released by the digestion of callose (Fig.1C). Fertile microspores possessed well organized nucleus and well developed cell organelles including mitochondria, plastids and golgi complex. A well developed exine with foot layer, bacule and tectum and intine differentiated in each microspore to convert them into oval shaped vacuolated pollen grains. They were well equipped with developed mitochondria, plastids and nucleus. Finally the vacuole from pollen disappeared, as they became bi-celled and engorged with reserve food (Fig. 1F).

B. Male sterile plants

Development of anther and microsporogenesis in ethrel treated male sterile plants in pre-meiotic stage was more or less normal and similar to control plants (Fig. 1A). However, in post-meiotic stages, the behaviour of endothecium, tapetum and microspores was abnormal. The cells in the endothecial layer failed to elongate radially and characteristic fibrous thickenings failed to appear on their radial walls.

The tapetum in sterile anthers remained intact through the major course of anther development. At

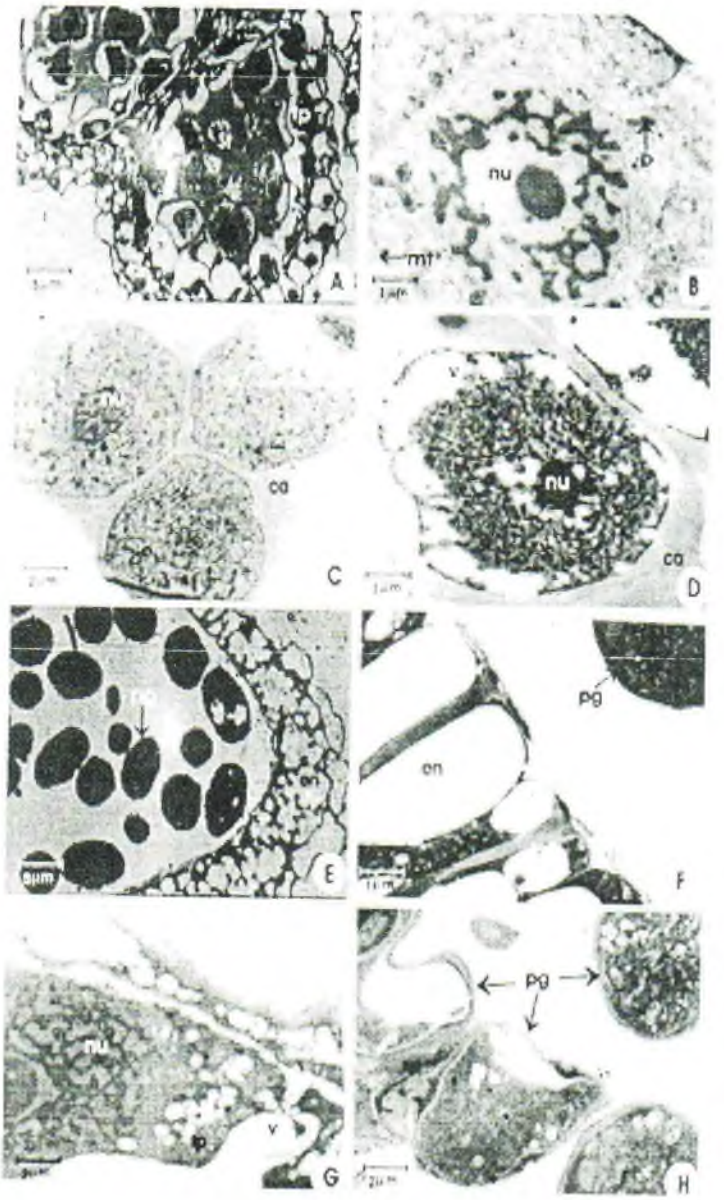

Figure 1. LM and TEM photographs showing anther development in male fertile (MF) and ethrel treated male sterile (MS) plants of Vicia faba L. : A. LM photograph of MS anther at sporogenous tissue (st) stage showing degenerated tapetum (tp), B. TEM photograph of sporogenous tissue of MS anther. Note the presence of well-developed mitochondria (mt), plastids (p) and nucleus (nu), C. TEM photograph of MF anther showing microspore tetrad with well developed nucleus (nu) and other cell organelles, D. TEM photograph of MS anther showing a single microspore of a tetrad enclosed within a thick callose wall (ca) exhibiting high vacuolation (v) and degenerated nucleus (nu), E. LM photograph of MF anther at engorged pollen grains (pg) stage. Note the disintegrated tapetum and developed endothecium (en), F. TEM photograph of MF anther at mature pollen grain (pg) stage. Note the presence of fibrous thickenings in endothecial cells (en), G. TEM photograph of MS anther showing a single tapetal cell (tp) with high vacuolation (v) and degenerated cell organelles and nucleus (nu), H. TEM photograph of MS anther of highly vacuolated non-viable pollen grains (pg) showing degenerated nucleus and other organelles.

sporogenous tissue stage, well-developed mitochondria and nucleus were seen in the tapetal cells (Fig. 1B). However, these organelles showed signs of degeneration with the commencement of meiosis in PMCs and were completely degenerated by the end of meiotic division and tetrad formation. The degeneration of mitochondria commenced with the dissolution of mitochondrial cristae. Concurrent with mitochondrial deterioration in the tapeum, about mid- or late vacuolate pollen stage, whorls of endoplasmic reticulum were seen. A number of pro-oribicular bodies were seen in tapetal cytoplasm and synthesis of sporopollenin was more or less normal. Fair amount of ribosomal population made the cytoplasm dense. At mature pollen grain stage, tapetal cells became deformed, irregular, highly vacuolated and showed the presence of degenerated cell organelles (Fig. 1G) and at the time of anthesis, the tapetal cells degenerated followed by the degeneration of pollen grains.

Microspores in tetrads were surrounded by a well-developed thick callose wall (Fig. 1D). The microspores released from tetrads on disintegration of callose wall showed variation in their size and shape. Cytoplasm of microspores was highly vacuolated and showed degenerated cell organelles particularly mitochondria and plastids. Nucleus was also in the degenerated form (Fig. 1D). The primexine also failed to develop around the microspores even after their release. The pollen grains exhibited poorly developed exine and intine (Fig. 1H). The exine failed to differentiate into tectum, bacule and foot layer. These pollen grains were highly vacuolated and the nucleus was in degenerated form (Fig. 1H). Cell organelles present in the cytoplasm degenerated.

Pollen abortion in ethrel treated plants was found to be associated with delayed degeneration of tapetum. Similar abnormal behaviour of tapetum has also been reported in several chemically induced male sterile plants (Chauhan and Kinoshita, 1982; Shivanna and Johri, 1985; Worall *et al.*, 1992; Cross and Schulz, 1997; Chauhan *et al.*, 2003).

Present ultrastructural studies indicated that the cell organelles particularly mitochondria and

plastids in tapetum were in degenerated form. Lalonde *et al.*, (1997) observed abnormal vacuolization and degeneration of cell organelles in water deficit wheat. The resulting sterile pollen grains lacked both starch and intine. Alterations in mitochondrial ultrastructure may be associated with changes in the energy requirement of the cell (Smith *et al.*, 2002). Degeneration in mitochondria seem to indicate low metabolic activity in tapetal cells. The pollen grains of treated plants exhibited degenerated nucleus and cell organelles, particularly mitochondria. In CMS plants, pollen abortion is the result of structural mutations in the mitochondrial genomes of fertile plants (Mikami *et al.*, 1985; Chauhan and Kinoshita, 1995). Thus, the mechanism of pollen abortion in treated plants seems to be similar to that of CMS plants.

Another interesting finding of present study recorded is that development of endothecium was inhibited in sterile anthers. The failure of endothecium development is attributed to the inhibition caused by the persistent tapetum (Chauhan, 2001). Present observations also lend support to the hypothesis of DeFossard (1969) that the development of endothecium is related with the disintegration of the tapetum and fibrous bands develop within the endothecium only after the tapetal breakdown. Thus, the persistent tapetum in sterile anthers of *Vicia faba* not only failed to provide nutrition to the developing microspores, but also seems to inhibit endothecial development to makes the anther indehiscent.

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