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ABNORMAL BEHAVIOUR OF TAPETAL MITOCHONDRIA LEADS TO POLLEN ABORTION IN BENZOTRIAZOLE TREATED *CYAMOPSIS TETRAGONOLOBA* L.

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Foliar sprays with the aqueous solutions of benzotriazole induced 93-100% pollen sterility in *Cyamopsis tetragonoloba*. A comparative light and transmission electron microscopic study on anther development in benzotriazole treated and untreated plants of *Cyamopsis tetragonoloba* L. showed that pollen sterility in treated plants was associated with abnormal behaviour of tapetal mitochondria. In sterile anthers, the tapetal cells not only remained intact throughout anther development but also enlarged radially. This intact and enlarged tapetal cell was highly vacuolated and all their organelles were in degenerated form. At pollen mother cell stage, the number of mitochondria in tapetal cells increased but they were small in size. At early vacuolate pollen grain stage, these mitochondria were in degenerated form as marked by abnormally thick outer as well as inner membranes. The cristae were also not discernable. Increase in the number of mitochondria reflects increased metabolic state of tapetum during early stages of development but their degeneration in later stage perhaps leads to failure of their normal breakdown and this finally leads to pollen sterility.

Keywords: Benzotriazole; *Cyamopsis tetragonoloba*; Mitochondria; Pollen sterility; Tapetum.

Introduction

Utilization of chemically induced male sterility has been considered desirable because it has unique potential to provide for the development of hybrids directly out of elite germplasm, without the time and effort required to regressively backcross male sterility genes and fertility restoration systems[1]. Benzotriazole ($C_6H_5N_3$), a copper chelator which acts as an inhibitor of microspore development has recently been used as a useful chemical hybridizing agent (CHA) in some crops e.g. *Helianthus annuus*[2], *Brassica juncea*[3], *Vicia faba*[4], *Gossypium hirsutum*[5,6], and *Datura alba*[7].

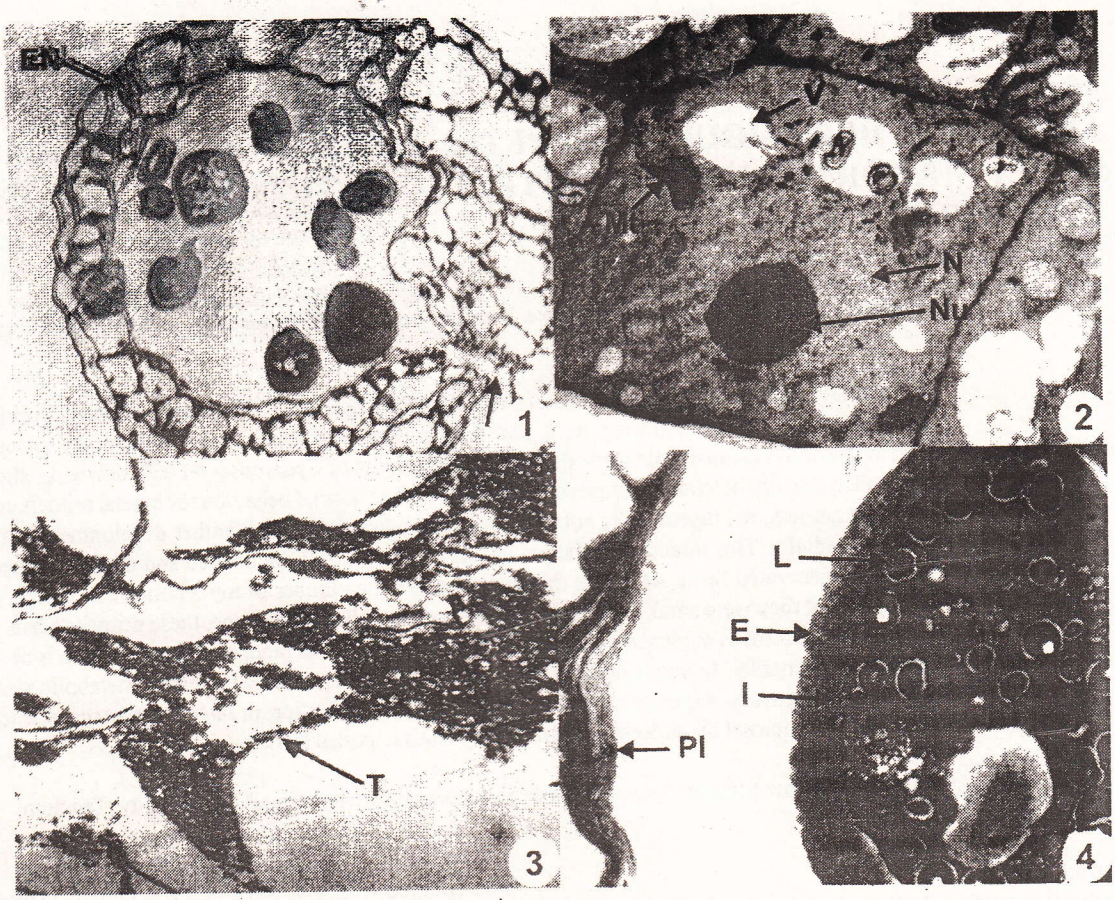
Numerous anatomical studies have been conducted primarily with light microscope to record the changes in the anthers of plants treated with various CHAs[1,8,9]. It is important to find out the involvement of a particular tapetal cell organelle in pollen abortion. This can be resolved by ultrastructural studies which have not received much attention. Keeping this in mind present investigation has been undertaken to study the ultrastructural changes in the anthers of *Cyamopsis tetragonoloba* L. plants treated with benzotriazole.

Materials and Methods

Cyamopsis tetragonoloba var. Deshi were sown at Botanical Garden, School of Life Sciences, Dr. B. R. Ambedkar University, Agra. A group of 60 plants each were sprayed with 0.4, 0.6 and 0.8% benzotriazole. A drop of liquid soap was added in each solution to serve as surfactant. The first spray was made a week before the initiation of floral buds at pre-meiotic stage(T_1). From 60 plants thus treated, 30 plants were sprayed again at the time of mature floral bud stage (T_2). A group of another 30 plants were sprayed with distilled water containing a drop of surfactant to serve as control (T_0). 25 ml of each solution was sprayed on one plant to run off. Pollen fertility was tested at regular internals with 1% TTC (Tetrazolium Chloride) in 0.15M Tris HCl buffer at pH 7.8.

For light microscopic studies, the floral buds of treated and untreated plants were fixed in formalin-acetic alcohol and these were dehydrated and embedded in paraffin by customary methods. The sections were cut at 5-12 µm and were stained with Delafield haematoxylin.

For TEM studies, anthers, at various developmental stages, were fixed in 3% glutaraldehyde in 0.1M PO_4 buffer at pH 6.8. Post fixation was done in 1% osmic acid. Samples were dehydrated in an ethyl propylene oxide series and embedded in spurr's low viscosity embedding media. Ultra thin sections were stained with lead citrate and uranyl acetate and were observed under electron microscope at All India Institute of Medical Sciences, New Delhi.



Figs. 1-4 : Photomicrographs of T.S. anthers of untreated plants.
Fig. 1. Light microscopic (LM) photograph of mature anther about to dehisce (arrow) showing mature pollen grains. Note the presence of fibrous thickenings in endothecium (En). 480 X; **Fig. 2.** Transmission electron microphotograph (TEM) of a tapetal cell with enlarged and organized nucleus (N) with a nucleolus (Nu) and dense cytoplasm with small vacuoles and organized mitochondria (Mt). 1650X; **Fig. 3.** TEM of anther at vacuolated pollen grain stage showing more or less degenerated tapetum (T). 1650 X; **Fig. 4.** TEM of part of mature anther showing engorged pollen grain with well developed exine (E) and thin intine (I). Note the presence of lipids (L) and tapetal plasmalema (Pl). 2050 X.

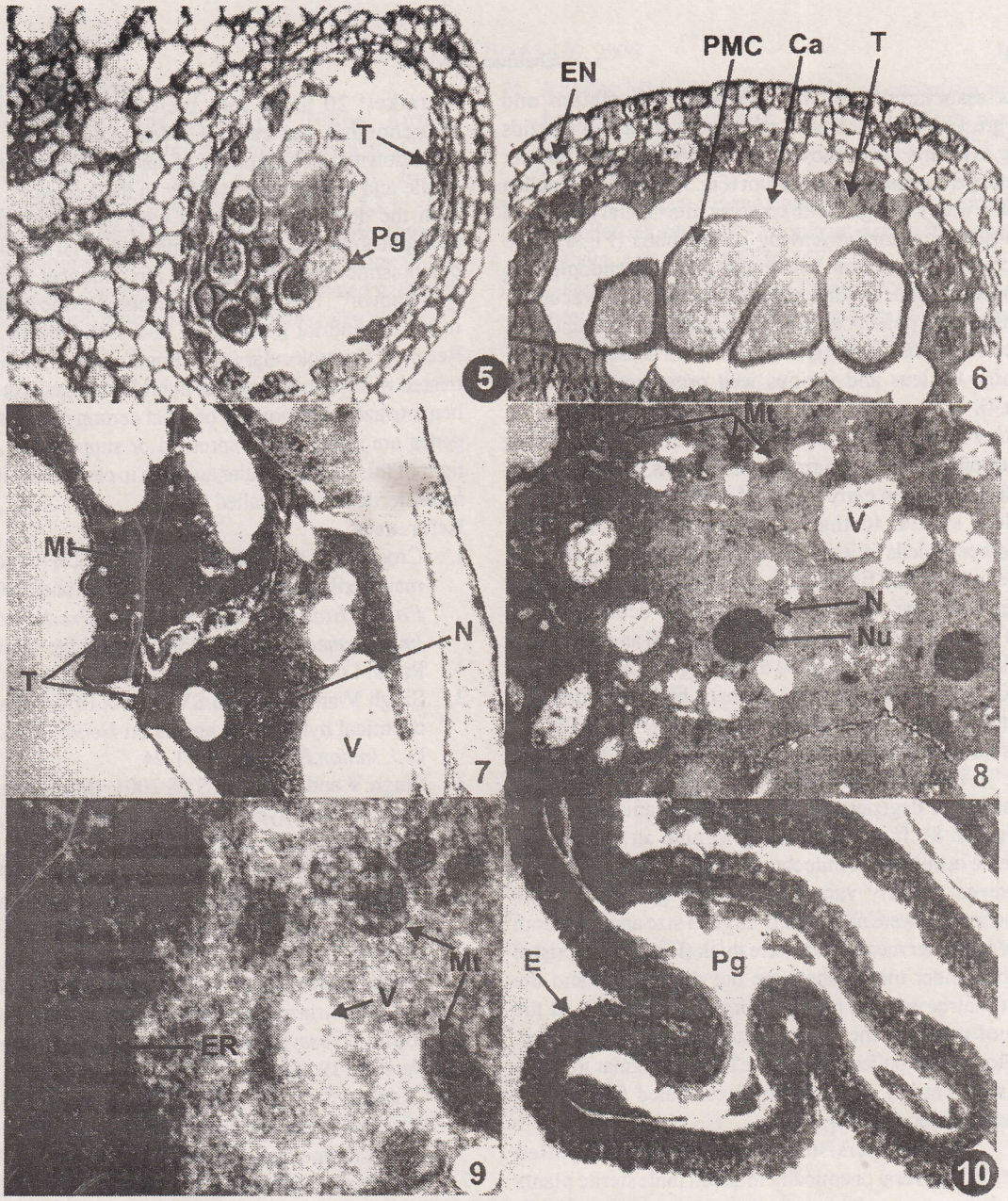
Results and Discussion

Foliar applications of benzotriazole effectively induced pollen sterility ranging between 93-100%. Single spray with 0.4 and 0.6 benzotriazole brought about 93-96.2% sterility. However, treatments with 0.6% and both the treatments with 0.8% benzotriazole caused 100% pollen sterility.

Anther development in control plants- The anther wall development in *Cyamopsis tetragonoloba* is of dicotyledonous type[10]. At sporogenous tissue stage, anther wall consisted of an epidermis, a middle layer, 2-3 layers of endothecium and a single layered glandular tapetum. The endothecial cells elongate tangentially during early stages of development and at early vacuolated pollen grain stage they elongate radially and at mature pollen grain stage, the characteristic fibrous thickenings appeared on their radial walls which help in anther dehiscence (Fig. 1). The cells of middle layers also elongated tangentially during early stages and at late vacuolated pollen grain stage the cells in this layer degenerated completely.

The tapetal cells were uninucleate at sporogenous tissue stage (Fig.2).The tapetal cell cytoplasm was dense and stained more intensely than those of sporogenous cells. Tapetal cell cytoplasm consisted of well organized organelles, viz. mitochondria, nucleus and plastids (Fig. 2).

Tapetal degeneration commenced at the microspore tetrad stage. Further degeneration of tapetum



Figs. 5-10 . LM and TEM microphotographs of T.S. anthers of treated plants.

Fig. 5. LM of mature anther showing degenerating pollen grains (Pg) and tapetal cells (T). 480X; **Fig. 6.** LM at pollen mother cell (PMC) stage showing thick callose (Ca) wall around them and radially enlarged, binucleate tapetal cells (T), poorly developed endothecium (En). 640X ; **Fig. 7.** TEM showing wall layers of mature anther showing highly vacuolated (V) intact tapetal (T) cells. Note the presence of large number of small mitochondria (Mt) and disorganized nucleus (N) in degenerated form. 2050X ; **Fig. 8.** TEM of enlarged tapetal cell showing several enlarged mitochondria, several small vacuoles (V) and degenerated nucleus(N) with a nucleolus (Nu). 2500 X; **Fig. 9.** TEM of tapetal protoplast showing enlarged and thick membraned mitochondria, disorganized endoplasmic reticulum (ER) and vacuoles (V). 2800 X; **Fig.10.** TEM of mature sterile pollen grain (Pg) of irregular shape showing thick walled exine (E) and degenerated protoplast. 2050X.

was associated with the increased vacuolation and progressive disappearance of mitochondria and plastids (Fig. 3). This was followed by degeneration of nuclear envelope, endoplasmic reticulum (Fig.3). By the time pollen grains were engorged with reserves, the tapetal cells were completely absorbed leaving plasmalema (Fig.4). The microspore cytoplasm contained plastids, endoplasmic reticulum, mitochondria and a number of small vacuoles. The exine of pollen consists of tectum, baculum and foot layer. The mature pollen grains were full of dense cytoplasm, a large nucleus and various well organized organelles (Fig.4).

Anther development in treated plants- The anther development in benzotriazole treated plants, showing 100% pollen sterility in early stages of development, was more or less similar to that of control plants. However, endothecial cells failed to elongate radially at any stage of development and fibrous bands on their radial walls also failed to appear, making the anthers non-dehiscent (Fig. 5). The PMC were abnormally enlarged and an abnormally thick callose wall surrounded them (Fig.6). Tapetal cells at PMC stages were intensely stained, highly vacuolated, radially enlarged and became binucleate (Fig.6). Tapetal cells failed to degenerate and remained intact till anthesis (Fig.7). The tapetal protoplast was highly vacuolated and consisted of degenerated nucleus, disorganized plastids (Figs.7 & 8). It was interesting to note an appreciable increase in number of mitochondria at PMCs stage (Fig.8). However, at early vacuolated pollen grain stage, the mitochondria were abnormally large in size and their both inner and outer membranes were thick (Fig. 9). The cristae on their inner membrane were not discernable and the inner matrix was not normally organized (Fig.9). At the time of anthesis, the tapetal mitochondria degenerated. The non-viable pollen grains were highly irregular in shape and size (Fig.10). They finally degenerated followed by tapetal degeneration (Fig.5).

Tapetal persistence associated with pollen abortion in several chemically induced male sterile plants have been observed recently by various workers[5,6,11-13]. These workers have also recorded an increase in the number of tapetal mitochondria in the anthers of treated plants. Increase in the number of tapetal mitochondria during early stages, seems to enhance metabolic activity in the tapetal cells and this in the opinion of present authors leads to radial enlargement of these cells. However, in subsequent stages, the thickenings of both outer and inner mitochondrial membranes indicate their degenerated form. This sudden degeneration seems to be responsible for the failure of tapetal degeneration which finally leads to pollen abortion. Metabolic activity of mitochondria plays an important role in male sterility[14]. According to Lee and Warmke[15] 20 to 40 fold increase in the number of mitochondria per cell precedes to failure of tapetal breakdown in sterile anthers of maize. They proposed that fertile and sterile anthers differ in their capacity to cope with the demands associated with rapid mitochondrial multiplication. Association of mitochondrial abortion with cytoplasmic male sterility in a large number of crops is well known[16]. This is largely attributed to the differences in mitochondrial genome of fertile and sterile plants. Recently, the molecular analysis in *Brassica juncea* plants treated with various chemical hybridizing agents including benzotriazole has pointed out that certain anther specific genes are either over expressed or suppressed[11]. In the light of this, the molecular analysis in chemically induced male sterile plants is called for.

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