# POST-FERTILIZATION OVULE ABORTION IN *VIGNA RADIATA* (L) WILCZEK

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**Abstract:** *Vigna radiata* (L.) Wilczek is a promising and widely used pulse crop in India. Unfortunately, the yield of this crop has been low. One of the reasons for this low yield has been the occurrence of as much as 50 per cent ovule abortion. The position of the abortive ovules varies from the first to the fourteenth position in the pod. To gain an insight into the cause(s) that lead to ovule abortion, developmental changes in ovules have been studied at light and ultramicroscopic level. Besides, behaviour of chromosomes in two sex mother cells i.e. embryo-sac mother cell and pollen mother cell has also been studied. Although the chromosomal studies in female sex mother cells did not reveal any abnormality in the behaviour of chromosomes thus ruling out its involvement in inducing abortion, the detailed ultrastructural studies revealed ovule abortion in *V. radiata* to be taking place at proembryo stage. Degeneration of cellular components particularly in integumentary cells was a common feature observed in these ovules. This study reveals that besides the endosperm failure, which is generally regarded as the main cause of ovule abortion, changes in integumentary cells may also lead to abortion of ovules.

**Keywords:** *Vigna radiata,* Ovule abortion, TEM, Autophagy, Integument degeneration, Female meiosis, Myelin bodies

## INTRODUCTION

Represented by about 150 species all over the world, the members of genus *Vigna* occur mostly in old world particularly the African subcontinent (Kochhar, 2009). Some of its species are economically important and one among those is *Vigna radiata* (L) Wilczek. *V. radiata* is popularly known as green gram or 'Mung' in India and is an important pulse crop. Together with other legumes, it occupies an important place in the overall scheme of solving the problem of protein malnutrition. *V. radiata* has been an excellent source of protein, mineral and vitamins. Its seeds are used as pulse and 4-day old seedlings are eaten as vegetable. In view of its tremendous utility, several attempts have been made by plant breeders and plant physiologists to improve the yield of this pulse crop (Baldev and Jain, 1986). One of the major reason(s) for sharp decline in yield has been the failure of some ovules to mature into a well developed seed after fertilization in most of the pods. Detailed account of embryological changes that lead to normal seed development is available for a number of legumes (Marinos, 1970; Salgare, 1973; Deshpande and Bhasin, 1974; Johri, 1984; Tilton *et al.*, 1984). It has been a known fact that integumentary cells transport nutrients to developing embryo (Kapil and Tiwari, 1978; Offler *et al.*, 2003). The events occurring after fertilization is the proliferation of endosperm, which promotes the transfer of nutrients from integuments to the embryo. During embryo development, nucellar cells degenerate. The nutrients from these cells as well as endosperm cells are consumed by developing embryo (Mansfield and Briaty, 1992).

Crop yield affected by abiotic stresses has been reported as much as 20 fold below their optimum (Boyer, 1982; Bray *et al.*, 2000). The ovule abortion results occasionally from developmental program that limits the number of seeds/fruits (Pimienta and Polito, 1982, 1983; Raju *et al.*, 1996). It may also results from resource limitations attributed to environmental conditions ( Miller and Chourey, 1992; Stephenson, 1992; Suzuki *et al.*, 2001). In Glycine max, pre-anthesis water deficits caused defects in ovule development (Kokubun *et al.*, 2001). The hypothesis which explains that plants maximize maternal fitness by aborting a portion of developing flowers, ovules or seeds (Lloyd, 1980) is derived from the observation of stress-induced senescence or PCD (Liu *et al.*, 2010) of reproductive organs in many plant species. In view of the lack of information on developmental changes that lead to ovule abortion, in *Vigna radiata*, the present study was initiated. Moreover, during the present course of investigations, meiotic studies, particularly the female meiosis, were conducted to find out the probable role of chromosomal behavioral changes, if any, in inducing seed abortion.

## MATERIAL AND METHODS

Seeds of *Vigna radiata* (L.) Wilczek var. PS 16 were obtained from Indian Agricultural Research Institute (IARI), New Delhi and sown in the Botanical Garden of Delhi University. To find out the time of anther dehiscence and anthesis, a large number of fully grown buds were tag marked during the day and fixed in the evening on the same day at one hour intervals till the midnight hour. Anther dehiscence was first encountered in the buds fixed between 10.30-11.00 p.m. Pollination followed immediately after anther dehiscence. Anthesis took place early next morning. The open flowers picked up during that day were taken to be one day old after pollination (l DAP).

For ultrastructural studies, the ovules were dissected out from ovaries collected 1-3 days after pollination. They were transferred to a freshly prepared solution of 5% glutaraldehyde (Taab laboratories, Reading, U.K./Fluka, AG Buchs SG, Switzerland). The fixative was made by diluting the commercially available 25% glutaraldehyde with 0.05 M Sörensen's phosphate buffer (pH 6.8). The material was placed under vacuum in order to remove air from the ovules to facilitate the penetration of the fixative. The material was washed in cold buffer (4 changes at 3 hours intervals) and post-fixed in 2% w/v aqueous osmium tetroxide (Ladd Research Industries, Vermont, U.S.A.) solution for 2 hours at 4°C. Specimens were then washed in phosphate buffer (3 changes of 30 min. each) at 4°C prior to dehydration in order to remove traces of osmium.

The material was dehydrated in a graded acetone series, infiltrated and embedded in resin/Epon 812 (Ladd Research Industries, Vermont, U.S.A.) using 'Beem ' polyethylene capsules. Thin sections (500 – 800 Å) were cut on a Sorvall Porter Blum MT -2 ultramicrotome. The sections were stained with alkaline lead citrate (Reynolds, 1963). The stained grids were observed in Philips EM 300 transmission electron microscope operated at 80 KV. Photographs were taken on 35 mm Kodak roll film (Type 5302) on Kodak Sheet Film (Type 4489).

To know whether the chromosomes behaved normally during the microsporogenesis and megasporogenesis or showed abnormal behaviour that could lead to defective development of seed, both male and female meiosis was studied in the anthers and ovules of young unopened buds, respectively. The buds of appropriate size, taken from 5-6 plants, were fixed separately in 1:3 acetic alcohol for 24 hrs. These were then preserved in 70 percent alcohol till further use.

For meiotic studies anthers and ovaries, isolated from the same plant used for TEM studies, were hydrolyzed together in 5N HCI at room temperature for 20 min and then stained in Leucobasic Fuchsin. While male meiotic studies were carried out by squashing feulgen stained anthers in a drop of 1 percent acetocarmine, for female meiotic studies ovules were first dissected out of ovaries using dissection microscope and then stained in a drop each of acetocarmine and 45 percent acetic acid. All the ovules taken out together from a single ovary, were gently tapped under the coverslip ensuring that the large sized megaspore mother cell did not break and/or slipped under nucellar tissue. All the observations were made from the temporary slides and photomicrographs of selected cells were taken using Olympus CH30 microscope with photomicrographic attachment.

## RESULT

*Vigna radiata* var. PS 16 is an erect, annual, profusely branched plant which produces yellow flowers in clusters on axillary and terminal racemes. The flowers are self-pollinated. Pollination occurs immediately after anther dehiscence and fertilization takes place a few hours after pollination with zygote dividing immediately after its formation. The ovary initially measures 0.9-1.2 cm on one day after pollination (DAP) and its size increases to 7-10 cm by 12 DAP. As the growth of pods proceed, some of the ovules attached to marginal placenta, abort on the way and fail to reach maturity. The difference in the size of normally growing ovule and the one destined to abort is easily discernible even at early stages during pod development. Interestingly, the aborted ovules are not restricted to the proximal or distal ends of the fruit. The ovary generally produces 10-14 ovules and abortive one could be located anywhere between the first and the fourteenth position (Fig. 1). To gain an insight into the events leading to abortion of ovules, anatomical studies were carried out at light and ultrastructural level. Male and female meiotic studies were also carried out to find out if any anomalous chromosomal behaviour had led to ovule abortion.

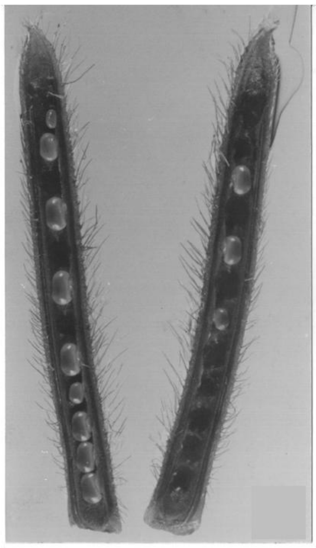

**Figure 1:** Split open pods 2DAP showing normal and abortive ovules . x 6.

### Ultrastructural Studies

Detailed account of embryological changes leading to the development of normal seed is very well documented in a number of legumes and more or less follow same course of events in all legumes. In *V. radiata* cellular changes occurring during the development of normal ovule very much synchronize with the observations made earlier on *Phaseolus* species (Lorenzi *et al.*, 1978; Mishra and Sahu, 1970; Salgare, 1973; Savithri and Ganapathy, 1978; Yeung and Clutter, 1979). However, wherever necessary a brief mention of anatomical changes occurring in normal ovules has been made to facilitate comparison of cellular events leading to abortion of ovules.

Study of anatomical changes at ultrastructural level revealed that ovule abortion was occurring at proembryo stage. The ovule abortion was characterized by the presence of following features:

The ovules collected from pods 1 DAP showed zygote and primary endosperm nucleus (Fig. 2).

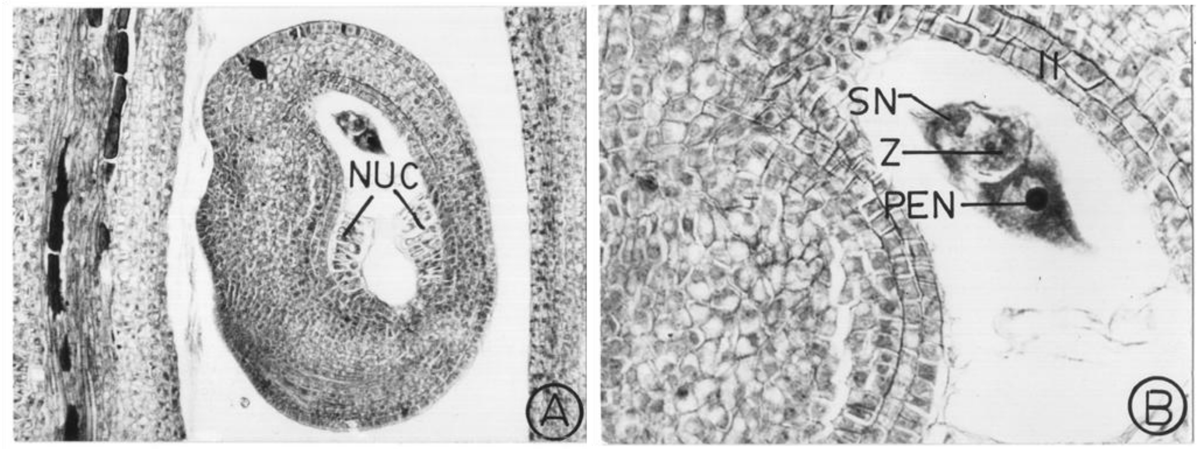


**Figure 2: A.** L.S. of abortive ovule 1 DAP showing zygote, primary endosperm nucleus and synergid cell . x 140. **B.** A portion enlarged. x 280.

However, in this type of aborting ovule on 2 DAP, though the zygote divides to form a few-celled proembryo, the primary endosperm nucleus remains undivided (Fig. 3). The cells in the outer integument (inner layers) showed vacuolated cells with thin walls and plasmodesmatal connections. Besides, cells also showed the presence of multivesicular structures; the cell organelles other than nucleus were not discernible.

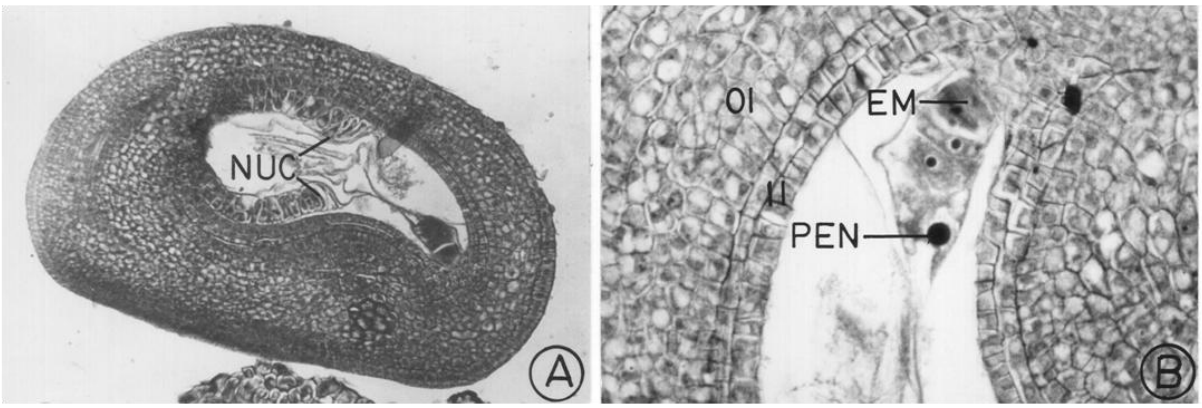


**Figure 3: A.** L.S. of abortive ovule 2 DAP showing a few-celled proembryo and undivided primary endosperm nucleus. x 160. **B.** A portion enlarged. x 450.

A very common and curious feature of the cells of the outer and inner layers of outer integument was the formation of concentric, membranous bodies (myelin-like bodies) in the vacuoles (Fig. 4). The various stages in the formation of complete myelin-like bodies could be traced in these cells. In some cells, cytoplasmic contents were seen included in the vacuoles. On 2 DAP, the cells had fully formed myelin-like bodies with many concentric rings of membranes (Fig. 5) and later these became highly vacuolated (Fig. 6). These type of myelin-bodies were also observed in degenerating nucellar cells of normal ovule (Fig. 7).

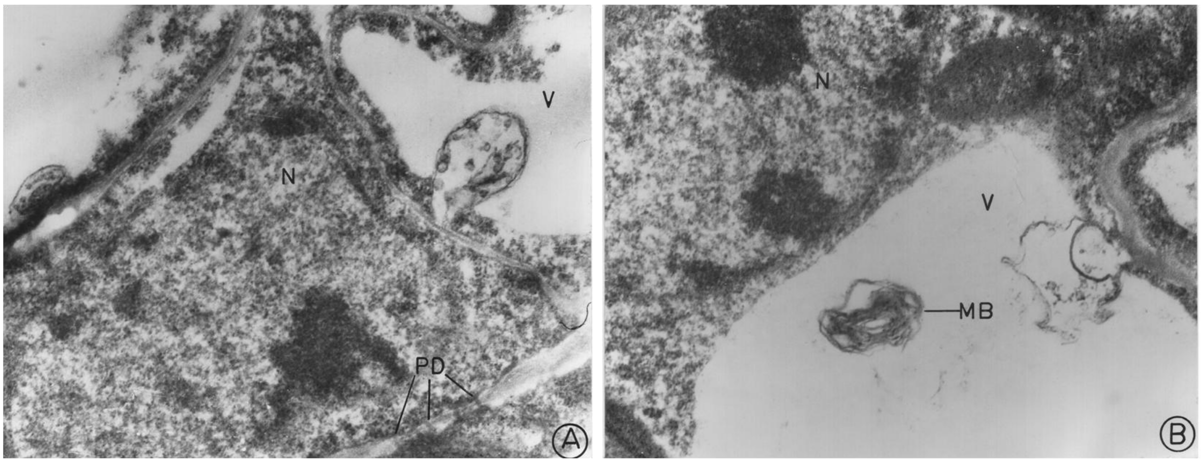


**Figure 4:** Longisections of portions of post-fertilization abortive ovules (1 DAP) to show cellular details of outer integument. **A.** A cell from the outer layer shows the release of cytoplasmic contents into the vacuole (autophagy) in the form of a multivesicular body. x 36,000. **B.** A cell from the inner layers showing the presence of a myelin-like body in the vacuole. x 36.000. (MB – Myelin-like body, N – Nucleus, PD – Plasmodesmata, V – Vacuole).

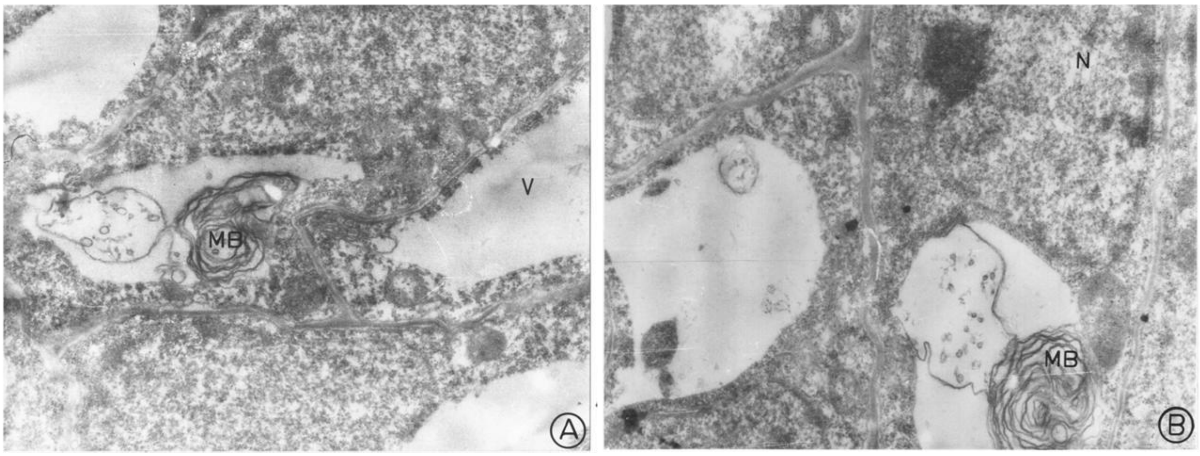


**Figure 5:** Longisections of portions of post-fertilization abortive ovules (2 DAP) to show changes in the outer integument. **A.** Cells from the outer layers of the outer integument (2 DAP) show well-formed myelin-like bodies and pronounced vacuolation. x 28,200. **B.** Inner layers of outer integument also have vacuolated cells with myelin-like body formation. x 28,200. (MB – Myelin-like body, N – Nucleus, V – Vacuole).

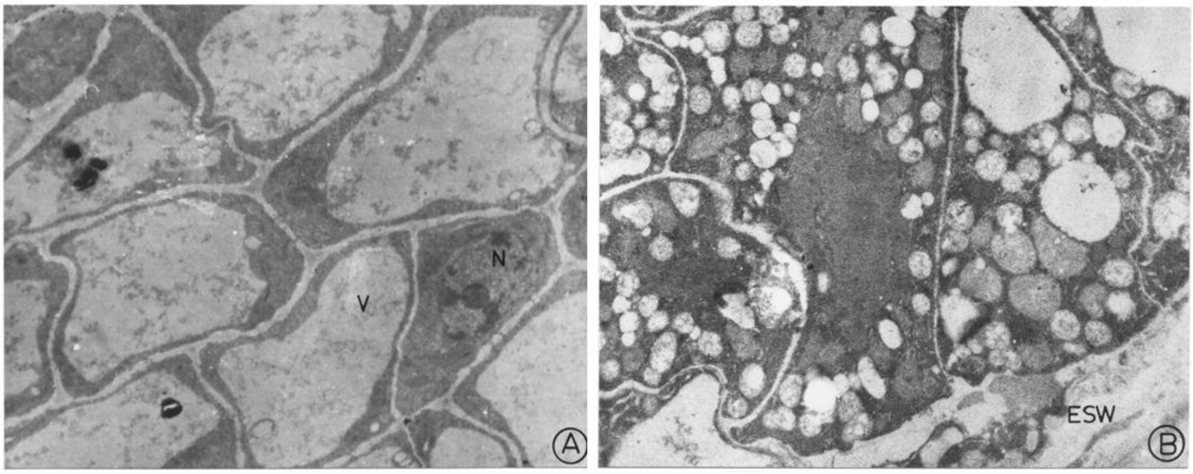


**Figure 6:** **A.** Longisection of portion of post-fertilization abortive ovule (3 DAP) to show cellular details of outer integument. Low-power profile of a group of cells from the outer integument showing highly vacuolated cells. x 6,600 (N – Nucleus, V – Vacuole). **B.** Longisection of portion of post-fertilization abortive ovule (2 DAP) to show embryonal cells. Some of the vacuoles are filled with cytoplasmic contents. Embryo sac wall

shows only a few small wall ingrowth. Remnants of endosperm remain as a thin parietal layer in the embryo sac cavity. x 9,600. (ESW – Embryo sac wall).

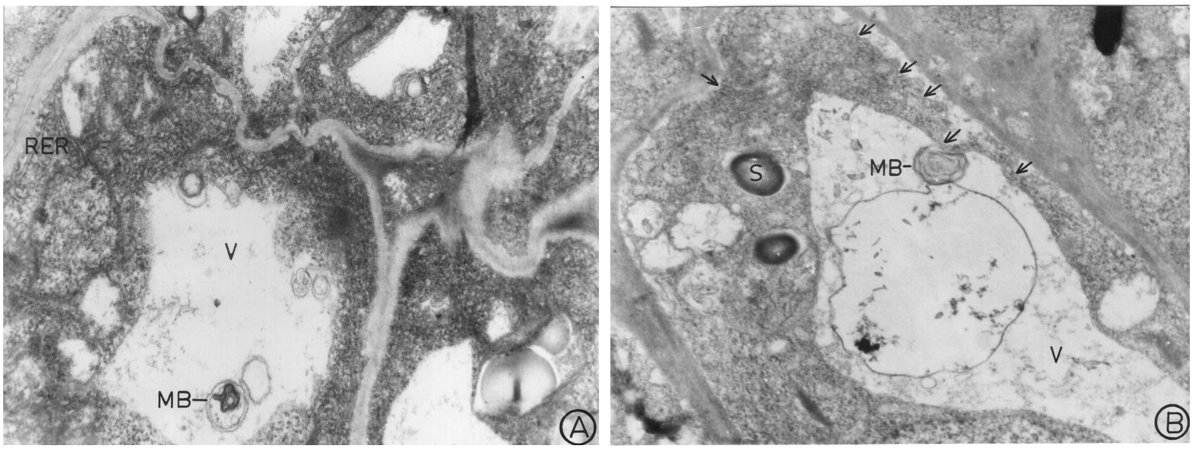


**Figure 7:** Longisections of chalazal portion of post-fertilization normal ovule (1-2 DAP) showing nucellar cells. **A.** Nucellar cells (1 DAP) at chalazal end.showing most of the plasmodesmatal connections obliterateed. Myelin-like bodies can be seen in the vacuoles. x 16,000. **B.** A nucellar cell (2 DAP) shows myelin-like body in the large vacoule. The cytoplasm has started shrinking (arrows). A few dictyosomes can be seen. Plastids are loaded with starch. x 14,400. (S – Starch, RER – Rough endoplasmic reticulum, MB – Myelin-like body, V – Vacuole)

Some abnormalities were also seen in the nucleus of integumentary cells. Many dark, round, membrane-bound bodies were seen protruding from the nucleus which was and pinched off as vesicles having nuclear material into cytoplasm (Fig. 8). The cytoplasm was very scant and only a thin layer could be seen surrounding the embryo.

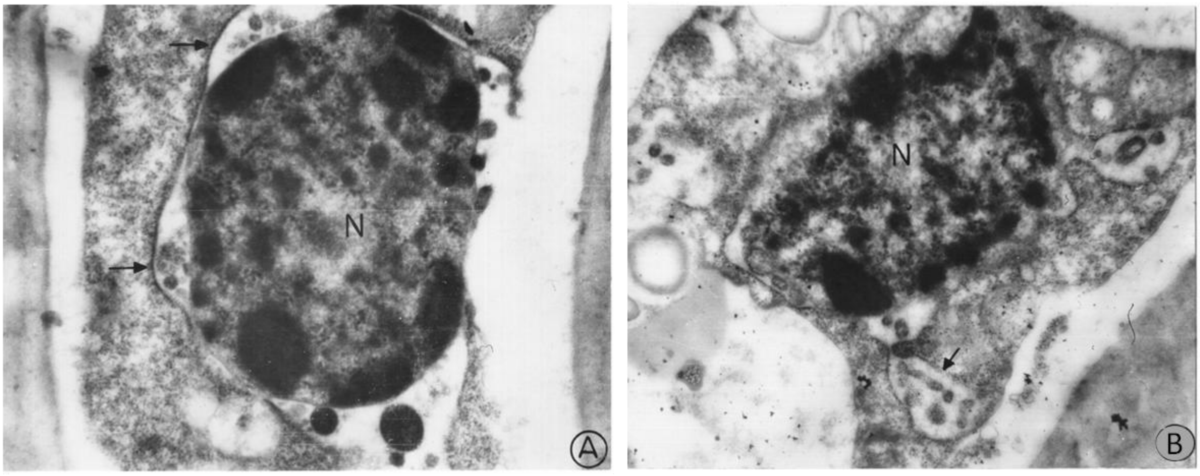


**Figure 8:** Longisections of portions of post-fertilization abortive ovules (2 DAP) to show cellular details of inner integument. **A.** The nucleus of cells of the inner layer of inner integument enlarged to show the nuclear blebs attached to the nucleus and further out in the cytoplasm (arrows). x 36,000. **B.** Part of the nucleus in the cell of the inner integument shows the extensive separation of membranes. Protuberances of these membranes are producing vesicles which on detachment lie in the cytoplasm (arrows). x 27,600. (N – Nucleus)

**Cytology**

Meiotic studies conducted on male and female sex mother cells revealed the presence of 22 chromosomes which resolved into 11 perfect bivalents at diplotene/diakinesis and metaphase-I stage of meiosis in the two sex mother cells. The chromosome behaviour was normal and similar in the two sex cells with respect to the position, distribution and frequency of chiasmata. The average chiasmata frequency in the pollen mother cells (PMC) and embryosac mother cell (EMC) at diplotene and metaphase-I, respectively, work out to be $22 \pm 2.13$ and $15 \pm 1.26$ (for EMC) and $21 \pm 2.06$ and $15 \pm 1.09$ (for PMC). The anaphase-I segregation of chromosomes was normal i.e. 11 chromosomes moved to each pole. None of the sex mother cells studied showed any abnormality (Fig. 9).

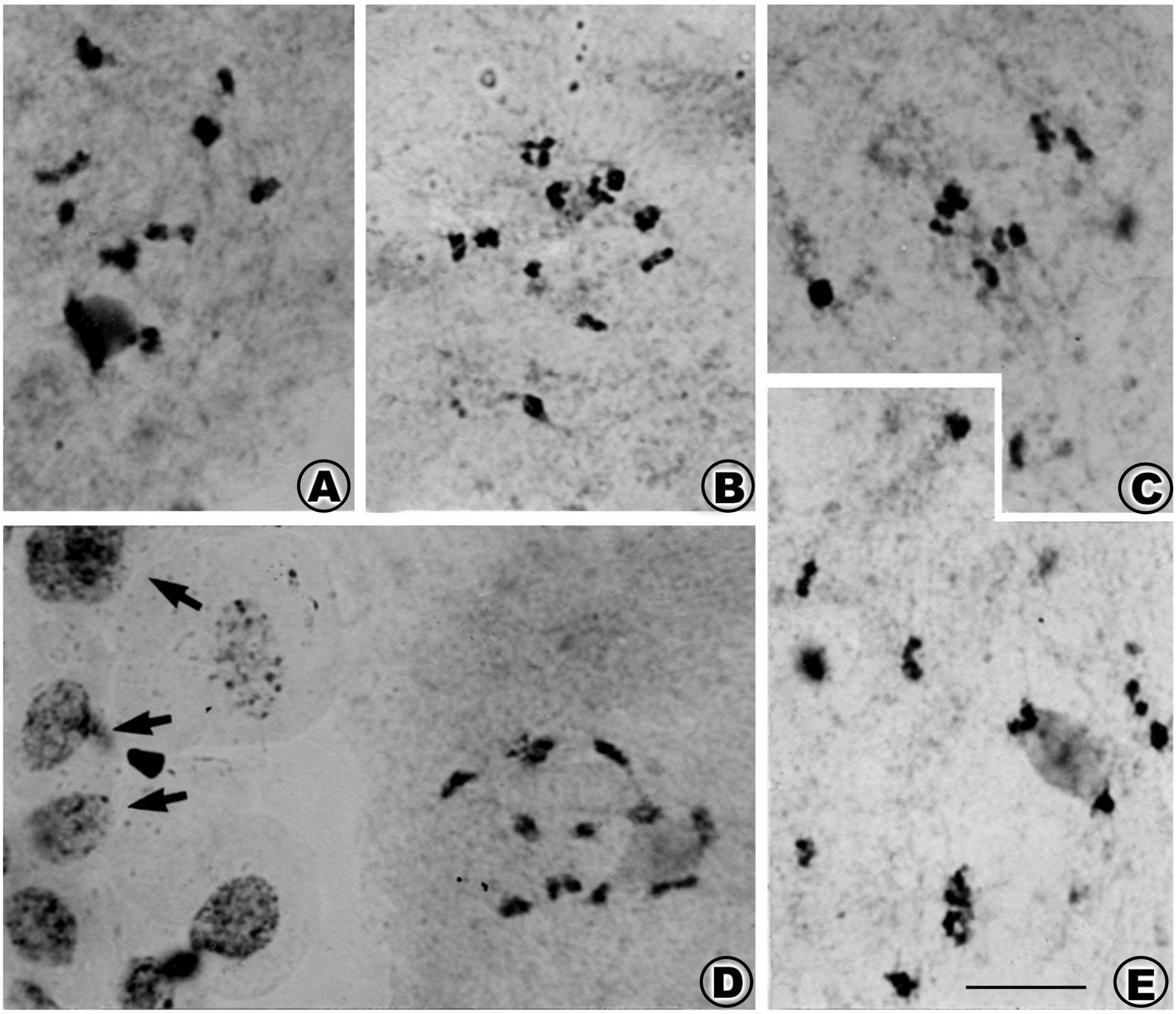


**Figure 9:** Male and female meiosis in *Vigna radiata* (2n=22). **Figs. A-C**. pollen mother cells at diplotene (Fig. A), diakinesis (Fig. B) and metaphase-I (Fig. C) showing 11 bivalents. **Figs. D-E**. embryo-sac mother cells at diplotene stage. Note the nucellar tissue at arrows. Scale = 10 µm.

## DISCUSSION

Most plants do not experience optimal growth conditions, and as a result ovules abort. In such cases resource limitation is the most common cause of ovule abortion. In a number of species/hybrids ovule abortion is mainly attributed to the retarded growth of endosperm (Atwood and Grun, 1951; Beasley, 1940; Brink and Cooper, 1941; Cooper *et al.*, 1937; Sansome *et al.*, 1942; Williams and White, 1976), proliferation of nucellus cells (Renner, 1914) and overgrowth of endothelium (Kostoff, 1930; Sansome *et al.*, 1942). Sometimes stress conditions, which lead to the accumulation of reactive oxygen species (ROS) in the cells of the gametophyte, result in programmed cell death (PCD) of the tissue (Hauser *et al.*, 2006; Liu *et al.*, 2010). Sun *et al.* (2004), reported the DNA fragmentation as a possible cause of PCD and ovule abortion in *Arabidopsis*.

As far as the present material is concerned this report of ovule abortion is the first one and highlights the involvement of degenerating integument in the ovule abortion in *Vigna radiata* (L.) Wilczek. Our observation is supported by the ultrastructural studies which revealed abortion to be taking place at pro-embryo stage. The ovular cells showed features characterizing degeneration processes. The degeneration process begins with the lysis of integumentary cells as evidenced by an increase in the number of myelin-like bodies, the tonoplast invaginations and sequestration of cytoplasmic portions along with cellular organelles as seen in degenerating nucellar cells of normal ovule.

The nuclear degeneration in integuments was recognized by separation or dilatations of the nuclear membranes, at first along small sectors of the periphery but later increasing and widening to produce broad cisternae which appeared as extensive vacuoles within the cytoplasm. These cisternae are angular, and always contained inclusions of various sizes, some of which appeared to originate as evaginations from the inner nuclear membrane and invaginations of outer nuclear membrane. These single membrane-bound blebs got detached as vesicles and appeared within the widening vacuolar system of the cell. The continued budding-off of fragments from these vesicles progressively reduced their size and definition. Later, in the advanced stages of degeneration, the cells were filled with a network of small vacuoles suspended in a fibrillar-like matrix which in turn was subjected to lysis.

The involvement of integuments in inducing abortion of embryo/seed has been reported in *Medicago sativa* (Cooper and Brink, 1940a), *Nicotiana* hybrid seed (Cooper and Brink, 1940b) and in 2n x 4n and 4n x 2n hybrids of *Lycopersicon pimpinelifolium* (Cooper and Brink, 1945), in all such cases proliferation of integument has been the root cause of abortion and in none of these cases integument degeneration was observed. Presence of myelin-like bodies, though not seen in integument cells, has earlier been reported in autolysis of embryo-suspensor of *Phaseolus* and *Trapaeolum* (Nagl, 1976, 1977). Further, vacuole disorganization has been reported in senescing corolla of *Ipomoea* (Matile and Winkenbach, 1971). During the degeneration process the hydrolases diffuse in the whole enclave and perform lysis of its contents. The sequestration vacuole becomes an autophagic vacuole and digestive reactions sometimes give rise to rearrangements of transitory products such as myelin-like bodies (Buvat, 1968; Coulomb, 1968; Coulomb and Buvat, 1968). Finally the internal membrane disappears, thus the autophagic vacuole is bound only by the external membrane. As hydrolysis gives rise to smaller and more numerous molecules, a water deficit is created which determines hypertrophic growth, and fusion of new vacuoles occurs (Buvat and Robert, 1979).

According to Mesquita (1972), in *Allium cepa* and *Lupinus albus* besides the normal vacuoles which seem to be empty, or else contain a very light precipitate, there are other autophagic vacuoles which display dense and heterogeneous contents that are either amorphous or formed by ribosome-like granules or multi lamellar bodies. Sometimes these contain cellular debris. While studying these residues one may be able to recognize several organelles in several stages of degradation/disorganization.

After the degeneration of all the sequestered organelles by the lytic enzymes of the membrane components of vacuoles, the appearance of myelin figures in the autophagic vacuoles is in agreement with the detection of lytic activity (Coulomb, 1973). After the degeneration of the mitochondrial population of a cell has occurred, all the other organelles quickly disorganize and the cell becomes reduced to an empty cavity (Oliviera, 1976). Matile (1975) suggested that the loss of compartmentalization was considered initially as an indicator of the moment of cell death. According to Gahan (1981) a series of events such as the loss of control lead to loss of compartmentalization. Events leading to loss of compartmentalization include ultrastructural changes such as those related to nucleus, nucleolus, ribosomes, endoplasmic reticulum and golgi bodies. Inactive golgi bodies are abundant in cells of the aborting ovules of *V. radiata.* So relative inactivity of the dictyosomes and ER in these cells suggests failure of nitrogen metabolism and transport in all nutritive tissues including nucellus, integumentary tapetum and endosperm as reported by Sangduen *et al*. (1983a, b).

The abnormal behavior of chromosome in the female sex mother cell may also lead to ovule abortion (Koul *et al.*, 2000). Thus, chromosomal studies were also conducted in the two sex mother cells to assess their possible role, if any, in inducing ovule abortion; the studies did not reveal anything that could help in pinpointing a cause for ovule abortion.

In view of the foregone account, it can be concluded that the embryo/seed abortion does not always point towards the involvement of endosperm; instead developmental abnormalities in other mother tissue, like integument, can also be instrumental in inducing seed abortion.

## ACKNOWLEDGEMENT

We gratefully acknowledge Indian Agricultural Research Institute (IARI), New Delhi for providing the seeds of *Vigna radiata* (L.) Wilczek var. PS 16. We also thank Dr. Hemant Kumar Singh for his help in formatting the manuscript.

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