DISCUSSION
5. DISCUSSION

In an effort to produce gynogenic haploids in *Cucumis melo* var. *acidulus* and *Cucumis melo* var. *momordica*, *in vitro* culture of ovule, ovary slice and embryo rescue after pollination of female flowers with gamma irradiated pollen techniques were attempted. The results of the above experiments have been discussed in the light of available literature.

5.1 Effect of cytokinins and auxins on ovule cultures of *Cucumis melo* cultivars

Ovules of *Cucumis melo* var. *acidulus* and *C. melo* var. *momordica* were inoculated onto MS medium containing 3% sucrose and medium supplemented with various cytokinins and auxins at concentrations varying from 0.25 to 10.0 µM individually for induction of gynogenesis.

The results indicated that in variety *acidulus*, lower concentration of TDZ caused the ovules to become green among the various cytokinins tested but they failed to induce either the callus or any organogenesis (Table 3, Fig. 3 A). Remaining cytokinins KN and BAP failed to induce any response in the ovules (Fig. 3 B) except similar bulging. Ovules cultured on medium supplemented with 2,4-D and NAA induced callus from the micropylar end (Fig. 3 C-F) whereas IAA and IBA failed to induce any change in the cultured ovules (Table 3). Ovules of variety *momordica* cultured on medium supplemented with all the cytokinins become green and swell in their size (Table 11, Fig. 11 A-B) without further response after 4 weeks of culture and non embryogenic callus formation was seen in auxins 2,4-D and NAA at different concentrations (Table 11).

In contrast to our results, in carrot, the *in vitro* cultured ovules bulged in size after 10 days of culture on medium supplemented with IAA, KN and 2,4-D. Few of them produced even embryos. Some ovules formed callus located most often near the micropylar region and IAA at 0.06 µM produced embryos when supplemented in MS
medium (Kielkowska and Adamus, 2010). In squash (*Cucurbita pepo*), 2,4-D at 1.0 and 5.0 mg/l induced gynogenesis (Metwally et al., 1998).

### 5.2 Effect of cytokinins in combination with auxins on ovule cultures of *Cucumis melo* cultivars

Ovules of *acidulus* and *momordica* were cultured on medium supplemented with TDZ in combination with 2,4-D or with NAA at different concentrations. Combination of TDZ with most of the 2,4-D concentrations resulted in callus formation in both varieties (Table 4 & 12, Fig. 4 & 11) whereas the TDZ and NAA combinations failed to induce the response in the cultured ovules (data not shown) in *acidulus*. The callus with profuse rooting was observed in the variety *momordica* (Table 13, Fig. 11 C-D). The callus obtained from variety *acidulus* in TDZ and 2,4-D combination was subcultured on 1.0, 2.0 and 5.0 μM concentrations of BAP and KN (Table 5). In BAP containing media, callus further proliferated without the differentiation into shoots (Fig. 4 B-C). But the media supplemented with 5.0 μM KN induced shoot formation followed by the rooting in the same media (Fig. 4 D-F). But for this response, repeated subculture was required. Cytology of the roots showed that the plantlets developed were diploids. No result except callus proliferation was observed in variety *momordica*.

Castillo and Cistue (1993) improved the frequency of haploid production from cultured ovaries of barley by the use of a combination of plant growth regulators. Muren (1989) used combination of 2,4-D + BAP for haploid induction in *Allium cepa* whereas Alan et al., (2003) used combination of 2,4-D and BAP or NAA and 2-ip for production of gynogenic plants from hybrids of *Allium cepa* and *A. roylei*. Tosca et al., (1999) used combination of IAA and IBA for gynogenesis in gerbera. Similarly in many genera combinations of growth regulators has been shown to influence haploid production (Keller and Korzun, 1996a).
5.3 Effect of cytokinins and auxins on ovary slice cultures of *Cucumis melo* cultivars

Ovary slices of *Cucumis melo* var. *acidulus* and *C. melo* var. *momordica* were prepared after surface sterilization and were cultured on MS medium containing 3% sucrose and medium supplemented with various cytokinins and auxins at different concentrations (0.25 - 10.0 µM) individually for induction of gynogenesis.

In both the varieties, slice cultures supplemented with TDZ at higher concentrations (1.0, 2.0, 5.0 and 10.0 µM) involved in bulging of the ovules which protruded out of the ovary surface within a week of culture (Fig 5 C) whereas KN and BAP failed to induce any change and the ovary starts to shrink within 10 days of culture and subsequently become brown (Table 6 & 15) after 4 weeks. Similarly, none of the auxins induce any change in the slices and gradually the ovules inside the ovary slice become dry indicating no response (Table 6) even after culturing for 6 weeks. In 2,4-D, the sporophytic tissue of the ovary formed profuse callus which was non embryogenic masking the ovules inside (Fig. 5 B). Mukhambetzhanov (1997) reported that during cultivation, ovaries and ovules often only increase their size by cell proliferation of somatic tissue around the female gametophyte without the embryo sac elements showing morphogenetic activity.

In the variety *acidulus*, the outer sporophytic tissue of the cultured ovary slice induced the organogenesis by forming multiple shoots after 4 weeks of culture in NAA and BAP combination. Shoots formed along with the ovary tissue were subcultured on the same media for another 4 weeks which subsequently grew into plantlets. However they were proved to be diploid based on cytology. Experiments of Lotfi et al. (2003) reported similar results in *Cucumis melo* spp. *melo* wherein the ovary slice cultures have developed only callus. These results suggest that gynogenesis is a less efficient way to obtain haploid plants than the culture of parthenogenetic embryos and that it may be more genotype specific (Lotfi et al., 2003).
In contrast, Ficcadenti et al. (1999) in melon, Gemes et al. (2002) and Diao et al. (2009) in cucumber reported successful ovary slice culture with direct production of doubled haploids. In onion, a total of 49 embryos were obtained from 2660 cultured flower buds and preconditioning stock ovaries significantly influenced gynogenic embryogenesis (Puddephat et al., 1999) and the highest embryogenesis ratio reported in ovary culture was 22.6% (Bohanec and Jakse, 1999). In tropical shallot, Cohat (1994) reported a 55.2% rate of gynogenic embryo induction. In sugar beet, the embryo frequencies ranged from 0 to 16.6%, depending on the genotype and the induction medium (Doctrinal et al., 1989).

In the variety *momordica*, as shown in the Fig. 12 B–D, many meristematic protuberances which looked like globular embryos and many leaf like protuberances were visible after 4 weeks of culture. They looked like emerging shoot buds but in spite of the growth hormone BAP, they failed to show any growth and even after subculture to fresh medium, shoots did not appear. Similar to the present results, the production of leaves and meristematic protuberances (“stipule-like leaves,” Leshem, 1989) without accompanying buds was reported in melon. Histological studies of Gaba et al. (1999) revealed that these organs have no shoot buds or shoot apical meristems.

5.4 Induction of parthenogenetic embryos after pollination by irradiated pollen in *Cucumis melo* cultivars

Pollen irradiation (γ-rays and X-rays) is the most widely used technique to induce *in situ* parthenogenetic haploid plants. Gamma rays are commonly used in haploid programs because of their simple application, good penetration, reproducibility, high mutation frequency, and low disposal (lethal) problems (Chahal and Gosal, 2002). This technique was used firstly with embryo culture on different species of *Nicotiana* (Pandey and Phung, 1982). Irradiated pollen can germinate on the stigma, grow within the style and reach the embryo sac, but cannot fertilize the egg-cell and the polar nuclei (Cuny et
Genetically inactive but germinable pollen can be used to stimulate the division of the egg cell, and thus induce parthenogenesis or development of parthenocarpic fruit, including gynogenic haploid production, minor cross-incompatibilities, and for physiological studies of incompatibility (Stairs and Mergen, 1964; Savaskan and Toker, 1991; Todorova et al., 2004), gene transformation (Pandey, 1978) and nucleus substitution (Raquin et al., 1989).

5.4.1 Effect of gamma irradiation dose of pollen and subsequent pollination on fruit setting and seed development in *Cucumis melo* cultivars

In our study, male flower buds collected one day before anthesis were irradiated with 150, 200, 250, 300, 350 Gy using cobalt-60 source and used for pollinating female flowers. At anthesis, the anthers from 3 irradiated male flowers were used for pollinating the female flowers which were protected from muslin cloth bags for preventing uncontrolled pollination.

Results revealed that 250 Gy of γ-irradiation was suitable for induction of parthenogenesis and fruit development (Table 7 & 16, Fig. 6 & 13). Lower (150 and 200 Gy) and higher doses (300 and 350 Gy) of γ-ray were not successful in inducing the fruit setting (Fig. 13 A-B). In cucurbits a similar procedure was adopted in melon haploid induction (Sauton and Dumas de Vaulx, 1987) with a dose of 30-100 kR (0.3-1.0 kGy). Embryos and haploid plants were also obtained from lower irradiation doses (25 and 50 Gy) in summer squash (Kurtar et al., 2002), pumpkin (50 and 100 Gy, Kurtar et al., 2009) and in winter squash (50 and 100 Gy, Kurtar and Balkaya, 2010). On the other hand, haploid embryo induction was obtained at relatively higher doses (200–300 Gy) in watermelon (Gursoz et al., 1991; Sari et al., 1994), melon (Sauton and Dumas de Vaulx, 1987; Cuny et al., 1992; Maestro-Tejada, 1992; Sari et al., 1992; Abak et al., 1996; Lotfi et al., 2003), cucumber (Niemirowicz-Szczytt and Dumas de Vaulx, 1989; Sauton, 1989; Caglar and Abak, 1999), and snake cucumber (Yanmaz et al., 1999; Taner et al., 2000).
Contrary to these reports, the best irradiation dose was found 100 Gy (Faris et al., 1999; Lotfi et al., 1999), 150 Gy (Xie et al., 2005), and 500 Gy (Claveria et al., 2005; Dolcet-Sanjuan et al., 2006) in cucumber, and 750 Gy in melon (Sun et al., 2006).

In variety *acidulus*, two fruits were formed at 250 Gy and a total of 376 seeds were harvested. Seventy six seeds were found to contain the embryos at different developmental stages. On the other hand, 200 Gy induced single fruit formation lacking any seeds. So even though 3 fruits have formed only 2 fruits with seeds were used for further studies. In *momordica*, a total of 3 fruits were harvested from 250 Gy group and 708 seeds were dissected out. Among them, 119 seeds found to contain embryos at various developmental stages and remaining seeds were empty (Table 16). Similar to present study, Ari et al. (2010) in melon reported formation of 204 fruits containing 280 haploid embryos of which 96% germinated.

### 5.4.2 Embryo culture

When 76 embryos from *acidulus* were dissected out from the seeds, they were at different developmental stages such as irregular, point, globular, heart shaped and mature cotyledonary embryos (Fig. 7 A-F). Point shaped embryos were at the highest number (50%) followed by globular (35.52%) and cotyledonary (11.84%) staged. Irregular and heart shaped embryos were too found to be present in seeds (1.32%; Table 8). Unlike *acidulus*, in *momordica*, only globular, heart shaped and cotyledonary embryos were found to be present (Table 17). Globular embryos again dominated by present in 99 seeds (83.19%) followed by cotyledonary (14 seeds, 11.76%) and heart shaped (6 seeds, 5.04%) embryos (Table 17). All the embryos were cultured on the embryo rescue medium and only cotyledonary embryos developed into plantlets (Table 17, Fig. 14 A-D) after 2 weeks of culture. Other embryos which were under-developed did not differentiate further and were thus irresponsive. Similar results were reported by Faris et al. (1999) and Faris and
Niemirowicz-Szczytt (1999) in cucumber wherein only matured embryo converted into plantlets.

In total, from both the varieties, three plants were produced which were multiplied by nodal cutting on modified E20A medium supplemented with 3% sucrose and 1 µM IAA for further analysis. The plantlets are easily propagated in vitro by nodal cuttings, but after colchicine treatment plant regeneration from nodes is much lower than from shoot tips (Lim and Earle, 2008). This might be due to the toxicity caused by the colchicine. Similar to the present results, induced parthenogenesis have been reported in watermelon (Gursoz et al., 1991), cucumber (Lotfi and Salehi, 2008), winter squash (Kurtar and Balkaya, 2010), summer squash (Kurtar et al., 2002), pumpkin (Kurtar et al., 2009), carrot (Rode and Dumas de Vaulx, 1987; Dore et al., 1995), citrus (Aleza et al., 2009; Yahata et al., 2010).

Lotfi et al. (2003) reported embryos at globular or white heart stage, and a low percentage of plants recovered from them (0.5% - 1.6%) which was in accordance with previous work (Sauton, 1988, 1989; Cuny et al., 1993; Sari et al., 1994; Ficcadenti et al., 1995), although precise comparisons are difficult because of differences in genotypes, seasonal effects. Other cucurbits exhibited even lesser percent of haploid embryos/plants e.g., 0.3% viable plants/seed in cucumber (Sauton, 1989) and 0.06–0.31% plants per seed in watermelon (Sari et al., 1994).

5.4.3 Transplantation and acclimatization

Acidulus plants were acclimatized by a simple method of hardening in the plastic cups containing a mixture of autoclaved vermiculite, sand and soil in the ratio 1:1:1 and covering the plantlets (Fig. 8) by transparent plastic bags which would be removed gradually. Initial three days the plastic cover was kept intact and on the 4th day, cover was tore at the 2 corners with the diameter of 1 cm each (Fig. 8 D). After one week, the size of the hole was increased to 3 cm and after 5 days, it was increased to 5 cm. Plants were
grown in this condition for a week and finally the cover was removed. Hardened plants were later transferred to pots (Fig. 8 E-F) kept in the green house.

In the variety *momordica*, the well developed plantlets (Fig. 15 A) were taken out of the culture vessel and transferred to plastic cup with potting mixture indicated above (Fig. 15 B). The plants were then grown in plant growth chamber for three weeks under controlled temperature (24 ± 2°C), relative humidity and light conditions (40 µmol m\(^{-2}\) s\(^{-1}\)). After hardening for three weeks the plantlets were transferred to pots (Fig. 15 C-D) kept in the green house. Both methods were found to be effective in hardening the plantlets. With *acidulus* being hardened with a simple method of plastic bags seemed to be economical and ease to perform without involving in much of the resources and instrumentation.

On comparison with the normal diploid plant, the haploid plant was very week in nature. Very thin stem, small sized leaves (Fig 15 C-D) and early appearance of the male flower buds (Fig. 16 A-C) (which never open) with sterile anther lobes (Fig. 16 D) were the common characters observed on the hardened plants. This is in accordance with the earlier reports wherein haploid plants were reported to be small and less vigorous than corresponding DH or inbred lines in maize (Chase, 1952; Chalyk, 1994; Lotfi et al., 2003). They were also much more sensitive to any kind of stress. Also the haploids were male sterile without the pollen grain (Chase, 1952; Chalyk, 1994; Lotfi et al., 2003). Kato et al. (1993) reported similar findings in various melon genotypes which had haploid pollen mother cells and absence of fertile pollen grains. Lotfi et al. (2003) described *in vitro* flowering of parthenogenetic melon plantlets without any pollen. *In vitro* flowering has been previously reported in several other cultured species, including cucumber (Kielkowska and Havey, 2012; Msikita et al., 1990; Mohammadi, 1996).

5.4.4 Ploidy analysis of the regenerated plants of *Cucumis melo* cultivars

Ploidy analysis of regenerated plants from both the varieties was done using Partec II flow cytometer. The analysis revealed that the plants were haploid in nature containing
1C DNA level when compared to diploids which had 2C DNA (Fig. 9 E & Fig. 17 E) and results are in accordance with Lim and Earle (2009).

The cytological studies of the root tips from regenerated plants from both the varieties showed haploid chromosome number (n = 12; Fig. 9 A & Fig. 17 A) whereas the cytology of root tips from control plant revealed diploid chromosome number (2n = 24; Fig. 9 B & Fig. 17 B) showing very small dot like chromosomes. Cytological studies of cucurbits have lagged behind other crop plants primarily because their chromosomes are relatively small and stain poorly (Wang et al., 2007). Cultivated melon is a diploid with 2n = 2x = 24 chromosomes. Melon chromosomes are small in size and cytogenetic studies are not easy to conduct. In spite of some attempts (Ramachandran and Seshadri, 1986; Dane, 1991; Ma et al., 1994), it is still proving difficult to obtain a good karyotype of melon. Thus in melon, ploidy analysis by means of chromosome counting is not only cumbersome but also tedious and time consuming. Similar obstacles are posed by plants with higher number of chromosomes as in the case of kiwifruit (Actinidia deliciosa) which contains 2n = 170 chromosomes (Przywara et al., 1988). Carrots also pose difficulty for cytological analysis as the chromosomes are tiny (Kielkowska and Adamus, 2010).

To separate plants with different ploidy levels the breeder requires methods which are simple, reliable, rapid and cost effective. Although chromosome counting is exact, it is time-consuming and requires trained personnel. Flow cytometry provides a direct measurement of the percentage of nuclei of different ploidy and can be applied to treated plants at any stage of growth. However, flow cytometry requires experience in complex sample preparation, is expensive and often not readily available. A further concern is that flow cytometry does not distinguish euploids from aneuploids so many plants with a diploid (2C) peak may actually have aneuploid chromosome numbers (Lim and Earle, 2008). Therefore, attempts have been made to find other methods of ploidy determination. Measurements widely used for the identification of ploidy levels are pollen grain diameter
and stomatal length. The number of plastids in guard cells is also often used as a selection
criterion in ploidy identification (Przywara et al., 1988).

Stomatal guard cell dimensions, density and the number of chloroplasts of the
stomatal guard cell can also be used for the determination of the ploidy level. Stomatal
guard cell of the haploid plants contained average of 4-5 chloroplasts while the control
plants showed average of 11 - 13 chloroplasts (Table 9 & 18; Fig. 9 C-D & Fig. 17 C-D)
in both the varieties. Stomatal length and diameter too varied between haploid and diploid
plants (Table 9 & 18).

Kato et al., (1993) compared the number of chloroplasts in leaf stomata of both
haploid and diploid melon plants. The average number of chloroplasts in haploid guard
cells was 2.40 – 3.00 while that in diploid cells was 4.95. Dore (1986) in brussels sprouts,
in watermelon, Abak et al. (1998) in pepper, Kurtar et al. (2002) in summer squash, Kurtar
et al. (2009) in pumpkin and Kurtar and Balkaya (2010) in winter squash found similar
results.

5.4.5 Effect of colchicine on induction of doubled haploids from haploid plants of
*Cucumis melo* cultivars

The frequency of spontaneous chromosome doubling of haploid plants is very low
so chromosome doubling is achieved by using colchicine or other antimitotic agents like
oryzalin, APM etc. to obtain fertile, homozygous plants (Lim and Earle, 2008, 2009;
Yetisir and Sari, 2003). Various methods can be used to apply colchicine in *in vitro* and in
*in vivo* growth conditions like adding colchicine to the growth media in *in vitro* culture,
immersing roots, plants and single node cuttings into colchicine solution, application of
colchicine to lateral buds by medicine dropper and immersing shoot tips of *in vivo* grown
plants (Yetisir and Sari, 2003). Besides the rate of *in vitro* chromosome duplication is low
in haploid melons, it was reported that shoot tip immersion into colchicine solution in
cantaloupe melon was the most efficient method (Koksal et al., 2002; Yetisir and Sari, 2003).

*In vitro* application of colchicine was tested for doubled haploid production in haploid plants of *Cucumis melo* var. *acidulus*. Due to high fungal contamination, plants did not survive (data not shown). So this method was not tried with the variety *momordica* and is proved to be ineffective in our system. Claveria et al. (2005) reported the survival rate of cucumber treated *in vitro* with 200 mg/l colchicine for 48 h was 20–60%, and the duplication rate was 30%.

*In vivo* application of colchicine at 250 mg/l concentration showed higher survival of the plantlets compared to colchicine at 500 mg/l in both the cultivars (Table 10 & 19). But the latter was much effective in doubled haploid production. Among 50 plantlets treated, 9 and 13 plants were found to be doubled haploids at 500 mg/l as compared to 6 and 2 plants at 250 mg/l concentrations respectively in *acidulus* and in *momordica*. This finding is in concordance with the previous studies carried out by different researchers. Solmaz et al. (2011) reported a similar result with *in vivo* colchicine application as more successful than doubling with *in vitro* colchicine application. It was reported that in melons dihaploidization rate was 89% in *in vivo* colchicine application and this rate was three times greater than *in vitro* colchicine application (Yetisir and Sari, 2003). Koksal et al. (2002) compared different *in vivo* methods for chromosome duplication in muskmelon. The results showed that immersing shoot tips into colchicine solution (0.5%) was determined the most successful technique. The dihaploidization efficiency was about 91.67%. On the contrary, Lim and Earle (2008) found *in vitro* exposure of shoot tip explants to 500 mg/l colchicine for 3 h as the most effective procedure. They obtained 83% survival rate of explants and 26% conversion to diploidy. In their study 132 plants were treated with colchicine *in vivo* dipping the tips and the first leaves of plants into colchicine solution (1000 mg/l for 12 h or 5000 mg/l for 2h or 4 h). They declared *in vivo*
technique resulted in less surviving rate (78%). This contrast may be caused by the higher dose of colchicine used or long exposure period (1 week) into colchicine solution. Same authors reported (Lim and Earle, 2009) that regeneration of nodal explants treated with 500 mg/l colchicine for 12 h increased dihaploidization from 40 to 88% by transferring the treated explants to a medium supplemented with growth regulators. In another study carried out by Lotfi et al., (2003) treating colchicine (250-500 mg/l) in vitro to the shoot tips of greenhouse grown plants for 3 to 6 h resulted in 10 diploid plants among 156 plantlets. Most were mixoploid and the remaining plantlets were still haploid. It was also reported that attempts of doubling by in vivo colchicine application to the greenhouse grown plants were unsuccessful.

Gayen et al. (1994) applied the colchicine at the coleoptiles stage of the maize seedlings, 2-3 days after germination. The authors cut off the tip of the coleoptiles and immersed the whole seedling into a 0.06% colchicine solution plus 0.5% DMSO (dimethyl sulfoxide) for 12 h at 18°C for successfully getting doubled haploids. Deimling et al., (1997) further increased the efficacy of the method by reducing the roots to 20-30 mm and placing the immersed seedlings in the dark. After the colchicine treatment, the seedlings were carefully washed in water and subsequently grown (during the first days under high humidity) in the greenhouse to the 5- to 6-leaf stage. A few weeks later the plants are transferred to the field. Eder and Chalyk (2002) applied the method to a broad range of donor genotypes and achieved an average doubling rate of 49%. For comparison, the authors tested a colchicine injection method applied in the 3-4 leaf stage and reached a doubling rate of only 16%. With both methods, 50 - 60% of the pollen-shedding plants could be selfed.

A gentler method of chromosome doubling was developed by Kato (2002). He treated haploid maize plants in the flower primordial stage with nitrous oxide gas (N02) for 2 days at 600 kPa. Averaged across donor genotypes, 44% of the treated plants
produced seed after self-fertilization. However, a very strong influence of the donor genotype on the doubling rate was observed. Furthermore, the method is very laborious and requires special equipment (safe gas chambers) and, therefore, is not easily adaptable to high-throughput applications.

In durum wheat, haploid plantlets obtained from ovary culture were extracted from the soil. After removal of old roots and leaves they were soaked for 4 hours in a 0.5% colchicine solution containing DMSO (10%) and KH$_2$PO$_4$ (100 mg/ml) with a pH of 5.7. During this treatment, plantlets were exposed to light. Authors reported doubling of the chromosomes with fertile spikes but this report lacks the data of percent success (Sibi et al., 2001).

Recent studies of chromosome doubling strategies for onion (Alan et al., 2007) showed that amipros methyl (APM) gave doubling rates comparable to those obtained with colchicine at substantially lower concentrations. Colchicine has higher mammalian toxicity than APM (Alan et al., 2007), so it would be worthwhile to test the effects of APM on melon haploids (Lim and Earle, 2008).

**5.5 Histological studies**

Melon ovules were anatropous and contained a monosporic, *Polygonum* type embryo sac as reported previously (Faris and Niemirowicz, 1999). Microscopic studies revealed several abnormal features along with few cases of normal initiation of embryo formation by the egg cell. The pollen tube was seen entering through the micropylar end and egg cell division was initiated only after 72 hours (Fig. 18 B-D). Abnormal development of embryos were observed in much of the sections most of them being arrested during embryogenesis or degenerating. Responding egg cell in many case seized its division and even at 10$^{th}$ day, embryos at different growth stages like 2 celled (Fig. 18 E, Fig. 19 A), 4 celled (Fig. 18 F, Fig. 19 F), globular (Fig. 19 B) and torpedo (Fig. 19 C) shaped were visible. Most of the sections revealed degenerating embryo sac (Fig. 19 D-E).
Most of the previous reports involving gynogenesis induced by irradiated pollen indicated occurrence of embryos at various developmental stages which is justified with the histological studies carried out in this study.

Irradiated pollen induces the embryo development by stimulating the egg cell which may not be triggering the central cell to divide to form the endosperm. Pro-embryo thus formed in its course of development gradually degenerate attributed to the absence of endosperm. In cucumber, the embryological tests confirmed that endosperm and embryo development was initiated within six days after pollination in 100% of embryo sacs at 0.1 kGy and in 70-80% at 0.3 kGy (Faris and Niemirowicz-Szczytt, 1999). After six days progressive degeneration of endosperm and embryo took place so that only 30-40% of embryos reached the globular stage 15 days after pollination. It can be supposed that the degeneration was further intensified as 3-5 weeks after pollination and 1.3-1.7 haploid embryos per 100 seeds were isolated (Przyborowski and Niemirowicz-Szczytt, 1994).

Embryo sacs possess haploid egg cells, but also other cells theoretically capable of forming a haploid embryo, such as synergids, antipodal cells or non-fused polar nuclei. The majority of records indicate that it is the egg cell that is the predominant source of haploid embryos: Beta vulgaris (Ferrant and Bouharmont, 1994), Allium cepa (Musial et al., 2001, 2005), Helianthus annuus (Gelebart and San, 1987), Hevea brasiliensis (Guo et al., 1982), Hordeum vulgare (Huang et al., 1982), Melandrium album (Mol, 1992) and Nicotiana tabacum (Wu and Chen, 1982). An antipodal or synergid origin of embryos has been proposed in Hordeum vulgare (San Noeum, 1979) and Oryza sativa (Zhou et al., 1986). A detachment of the egg cell at early stages of development has also been noted at least in onion and sugar beet (Ferrant and Bouharmont, 1994; Musial et al., 2001), and suspensor attached embryos have been found in sugar beet and mulberry (Pedersen and Keimer, 1996; Bhojwani et al., 2003). In addition to the major morphological differences, several other characteristics, such as early degeneration of antipodal cells or endo-
reduplication of the nuclei within synergids, clearly determine the egg cell as the predominant source of haploid embryos (Bohanec, 2009).

Although pollen irradiation has been used for many years, few studies have been made on double fertilization and early embryogenesis after pollination with irradiated pollen (Musial and Przywara, 1998). Embryological studies were carried out by Sniezko and Visser (1987) in pear, Zhang and Lespinasse (1991) in apple, Le Deunff and Sauton (1994) in cucumber, Falque (1992) in *Theobroma cacao*, Musial and Przywara (1998) in kiwifruit, Ogata et al. (2008) in *Citrus grandis* and Peixe et al. (2000) in plum. It was found that pollen irradiation disturbs double fertilization, and subsequently the development and interactions of embryo and endosperm, in a dose-related manner. Cytological events have been studied in detail by Nicoll et al. (1987) in apples and by Musial and Przywara (1998) in kiwifruit. Le Deunff & Sauton (1994) proposed in cucumber, irradiated pollen induces stenospermocarpy (fruit development after embryo abortion) rather than parthenocarpy. In their study, the nature of early embryogenesis whether gynogenetic or abnormally zygotic, has not been clearly determined. Only a few gynogenetic embryos develop and give rise to plants after *in vitro* culture of mature pseudo seeds. They observed 6-day-old ovules pollinated by irradiated pollen showing normal embryogenesis. However, the induced proembryos with or without endosperm were abnormal and abortive. Interestingly, Sugiyama et al. (2002) used X-ray for seedless watermelon production. They found sperm and egg cell alongside and central cell started dividing and even formation of endosperm. But after 7-10 days of pollination, the globular embryos formed degenerated. Authors concluded double fertilization might have taken place but the lethality of X-ray caused the paternal chromosome abnormalities and subsequent abortion of embryo.