

Full Length Research Paper

Induction of haploid embryo and plant regeneration via irradiated pollen technique in pumpkin (*Cucurbita moschata* Duchesne ex. Poir)

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The effects of gamma ray doses (50, 100, 200 and 300 Gray) at different times (July 9, 11, 15, 21 and 28) on the obtention of gynogenic haploid embryo and plant regeneration via irradiated pollen technique were investigated in pumpkin (*Cucurbita moschata* Duchesne ex Poir.). Production of haploid embryo and plant increased significantly at the lower irradiation doses of C⁶⁰ (50 and 100 Gy) and earlier irradiation periods (July 9 and 11). Different forms and stages of embryos were extracted from the seeds. Production of haploid embryo and haploid plant were also strongly influenced by forms and stages of embryos, genotypes and harvest times. Embryos and plants were obtained from only fruits pollinated with irradiated pollens at 50 Gy and 100 Gy and the highest parthenogenetic response was obtained from 50 Gy gamma ray doses. Frequency of haploid plant was 20% in torpedo and arrow-tip type embryos whereas cotyledon, hearth and amorphous type embryos produced only diploid plants. Necrotic embryos were the highest in delayed harvest times (5 and 6 week after pollination) and the best harvest time was determined about 3 weeks (between 18th and 24th days) after pollination. The frequency of haploid plants in per 100 seeds, 100 embryos and fruit were 0.24, 0.94 and 33.3%, respectively. The number of embryo per fruit was highest in "G9" (29.5) and "14YE02" (17.0) genotypes. Production of haploid embryo and plant was highest in the first (July 9) and second (July 11) irradiation times and 2 haploid plants were obtained.

Key words: Haploidization, irradiated pollen, pumpkin.

INTRODUCTION

Haploidization is the process of creating a haploid (n) cell, usually from a diploid (2n) cell. Haploid plants can be obtained spontaneously (androgenesis, gynogenesis or parthenogenesis, semigamy and polyembryony) or using with some haploidization techniques (*in vitro* androgenesis, *in vitro* gynogenesis and *in situ* parthenogenesis). These techniques facilitate the rapid production of pure lines from heterozygous plants in a single generation and represent advantages significant for breeders and geneticists.

Frequency of spontaneous haploids is very low in the Cucurbitaceae family (Pochard and Dumas de Vaulx, 1971; Savin et al., 1988). At the present, pollen irradiation (UV, gamma rays, X-rays) is the most widely used technique to induce *in situ* haploid plants. Gamma rays are commonly used in haploidy programmes because of their simple application, good penetration, reproducibility, high mutation frequency and less disposal problems (Chahal and Gosal, 2002). This technique consists of the induction of *in situ* gynogenic embryos with gamma-ray irradiated pollen and then followed by rescue of haploid embryos by *in vitro* culture. In this technique, irradiated pollen germinates on the stigma, but it cannot fertilize the egg-cell and the polar nuclei. Pollen stimulates the division of the egg cell and thus induces parthenogenesis and gynogenic haploid embryo. Induction of gynogenic

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Figure 1. Pumpkin genotypes.

haploid embryo and plant obtain has been achieved through irradiated pollen technique in melon (Sauton and Dumas de Vault, 1987; Sarı et al., 1992; Maestro-Tejada, 1992; Cuny, 1992; Abak et al., 1996; Lotfi et al., 2003), cucumber (Truong-Andre, 1988; Sauton, 1989; Niemirowicz-Szczytt and Dumas de Vault, 1989; Caglar and Abak, 1999; Faris et al., 1999; Lotfi et al., 1999; Dolcet et al., 2004), watermelon (Gursoz et al., 1991; Sarı, 1994; Moussa and Salem, 2009), snake cucumber (Taner et al., 2000) and summer squash (Kurtar et al., 2002). Pollen irradiation was proved to be effective for haploid induction, in melon but the embryo yield was found to be highly influenced by different factors such as genotype, environmental conditions and irradiation doses (Ficcadenti et al., 1995). To the authors' knowledge, induction of gynogenic haploid embryo and plant regeneration via irradiated pollen technique has not yet been investigated in pumpkin. This is the first report on haploid production in pumpkin.

The objective of the present study was to determine the effects of irradiation dose (Co^{60}), irradiation period and genotypes on induction of haploid embryo and plant in pumpkin.



Figure 2. Male flowers and anthers of pumpkin.

MATERIALS AND METHODS

Pumpkin

In this research, 5 pumpkin genotypes (55BA01, 14YE01, 14YE02, 14BO01 and G9) were used as plant materials (Figure 1). "55BA01", "14YE01", "14YE02" and "14BO01" were selected from the genetic materials of black sea region (project of TUBITAK-TOVAG - 104O144) and "G9" was provided from Turkish seed gene bank. The seeds were sown in pots filled with a mixture of peat moss and perlite (2:1, v/v) on April 20, 2008. Seedlings were grown in unheated glasshouse and planted in the open field condition with single row systems on May 11. 15 plants per genotype were grown in plots and spaces between and within rows were arranged 3 x 3 m. Plant nutrition components were applied by drip-irrigation system on the basis of soil analyses and plants were protected with fungicides and insecticides regularly throughout the growing season.

Pollen irradiation

One day before anthesis, female flowers were isolated with cloth bags to avoid open pollination and male flowers were collected in the mid-afternoon (Figure 2). Anthers with filaments were mixed equally and irradiated at 50, 100, 200 and 300 Gy doses of gamma rays at different times (July 9, 11, 15, 21 and 28) with a Co^{60} source of Theratron 780-C equipment (Radiation Oncology Department of Medical Science Faculty of Ondokuz Mayıs University). The following day of irradiation (0th day) in the morning (at 7⁰⁰ - 9⁰⁰ a.m.), irradiated pollens were placed in petri dishes and female flowers were pollinated at 0th and 1st days for each irradiation dose and irradiation time. Then, female flowers were isolated with cloth bags again to avoid pollen contamination. Cloth bags were removed 3 or 4 days after pollination. Pollens were kept in closed petri dishes and stored in a refrigerator at 4°C.

Table 1. Fruit number (FN), embryo number (EN) and mean embryo number in fruit (MN).

Irradiation date	Irradiation dose	Genotypes															General		
		55B01			14YE01			14YE02			14BO01			G9			FN	EN	MN
		FN	EN	MN	FN	EN	MN	FN	EN	MN	FN	EN	MN	FN	EN	MN			
July 9	50	1	38	38	1	22	22	0	0	0	0	0	0	1	27	27	3	87	29
	100	0	0	0	1	13	13	1	17	17	0	0	0	0	0	0	2	30	15
Means		1	38	38	2	35	17.5	1	17	17	0	0	0	1	27	27	5	117	23.4
July 11	50	1	8	8	1	16	16	0	0	0	0	0	0	1	32	32	3	56	18.7
	100	1	0	0	1	3	3	0	0	0	0	0	0	0	0	0	2	3	1.5
Means		2	8	4	2	19	9.5	0	0	0	0	0	0	1	32	32	5	59	11.8
July 15	50	0	0	0	1	9	9	0	0	0	1	25	25	0	0	0	2	34	17
	100	0	0	0	0	0	0	0	0	0	1	3	3	0	0	0	1	3	3
Means		0	0	0	1	9	9	0	0	0	2	28	14	0	0	0	3	37	12.3
General		3	46	15.3	5	63	12.6	1	17	17	2	28	14	2	59	29.5	13	213	16.4

In vitro embryo culture

Fruits were harvested after 3 to 6 weeks from pollination to determine the optimum harvest time. Harvested fruits were cleaned under tap water and were kept in 20% sodium hypochlorite solution for 30 min. Prior the commencing, surface of laminar air flow cabinet were disinfected with 70% ethanol and UV (Ultraviolet) light for 15 min. Then, the fruit surface was disinfected with 96% ethanol by on fire and seeds were extracted individually in a laminar air flow cabinet. Firstly, embryos were classified for embryo forms and stages and were initially cultured on E20A medium. 3-8 excised embryos were placed in magenta boxes or culture glasses containing approximately 25 ml of the medium. Magenta boxes and culture glasses were incubated in growth chamber [16 h photoperiod (3000 lux) at $28 \pm 1^\circ\text{C}$]. After 5 to 15 days, embryos having good roots and shoots were transferred to another magenta boxes or glasses.

Multiplication, transplantation and acclimatization

After 27 to 41 days, plantlets were adapted in the acclimatization. Acclimatization process was started while plantlets grown under *in vitro* conditions. Firstly, cover of magenta boxes and culture glasses were opened gradually for 8 days. Then, plantlets were removed from the magenta boxes and culture glasses. Plantlets were washed under tap water to remove the excess agar in the roots. Afterwards, the roots placed in a solution of "Maxim XL035FS" (0.25% v/v) for 10 min to prevent a possible contamination during the acclimatization. The seedlings were transplanted into white plastic cups (150 cm³) containing sterilised peat moss. Each cup was closed with a transparent cup and the plants were acclimatized in the growth cabinet [16 h photoperiod (3000 lux) at $28 \pm 1^\circ\text{C}$] under high relative humidity (approximately 95%). The transparent cups were gradually opened and removed completely for 6 days. Humidity of growth chamber was reduced at intervals 5% in each 2 days, periodically, till humidity reaches greenhouse or open field conditions.

Determination of ploidy level

The ploidy levels of haploid plants were determined by direct (chromosome counting in root tips) and indirect (stomata dimensions and

chloroplast number of the guard cells) methods. Chromosome counting in root tips was realised by Feulgen technique (Sari, 1994).

In order to measure stomata dimension and chloroplast number of the guard cells the 4th or 5th leaves were used. Lower leaf epidermal strips were placed onto slide after addition 1 drop of 1% AgNO₃ solution (Rouselle, 1992), then overlaid with a cover slip (Doré, 1986).

Experimental design

Responses to induction of haploid embryo and plant expressed in percentage (%) due to unequal test materials. Statistical analysis were applied for only stomata measurements as completely randomized experimental design and then the average values were compared by Tukey test's.

RESULTS

Effects of irradiation dose, irradiation period and genotype on embryo induction

Embryo induction was observed only irradiations at 50 Gy and 100 Gy and the highest embryo number was determined at 50 Gy in all irradiation times. Irradiated pollens at 200 Gy and 300 Gy were not effective on embryo induction (Table 1).

The periods of July 9 and July 11 gave the highest embryo number (117 and 59, respectively). The mean embryo number per fruit was determined 16.7 and 8.4 on July 9 and July 11, respectively. On July 21 and 28, embryo induction was not performed. In all periods, 13 fruits and 213 embryos obtained from genotypes and mean embryo number per fruit was determined 16.4. In all periods, embryo induction (number/per fruit) was changed between 12.6 (in 14YE01) and 29.5 (in G9). Among the investigated genotypes, "G9" demonstrated comparatively good gynogenic response.

Table 2. Effects of embryo development stages on transformation rate to plant (TR) (%), number of haploid plant (HPN); haploid plant rate (HPR) (%).

EF	ES	Genotypes										Σ		TR	HPN	HPR
		55B01		14YE01		14YE02		14BO01		G-9		EN	PN			
		EN	PN	EN	PN	EN	PN	EN	PN	EN	PN					
White	P	2	0	5	0	0	0	0	0	2	0	9	0	0.0	0	0.0
	G	3	0	4	0	3	0	0	0	3	0	13	0	0.0	0	0.0
	A	1	1	4	1	1	1	0	0	6	2	12	5	41.7	1	20.0
	T	3	1	2	1	1	1	2	1	3	1	11	5	45.5	1	20.0
	H	7	3	9	4	2	1	4	1	3	2	25	11	44.0	0	0.0
	C	5	3	3	3	2	1	3	1	5	4	18	12	66.7	0	0.0
	AM	1	0	2	0	0	0	1	0	0	0	4	0	0.0	0	0.0
Necrotic	P	2	0	8	0	0	0	2	0	7	0	19	0	0.0	0	0.0
	G	4	0	4	0	1	0	4	0	5	0	18	0	0.0	0	0.0
	A	4	0	7	0	2	0	4	0	4	0	21	0	0.0	0	0.0
	T	2	0	3	0	1	0	0	0	7	0	13	0	0.0	0	0.0
	H	6	0	7	0	3	0	5	0	9	0	30	0	0.0	0	0.0
	C	3	1	3	0	1	0	0	0	1	0	8	1	12.5	0	0.0
	AM	3	0	2	0	0	0	3	0	4	0	12	0	0.0	0	0.0
	ΣW	22	8	29	6	9	4	10	3	22	9	92	33	35.9	2	6.1
	ΣN	24	1	34	0	8	0	18	0	37	0	121	1	0.8	0	0.0
	Σ	46	10	63	9	17	6	28	6	59	9	213	34	16.0	2	5.9

EF: Embryo form; ES: Embryo stage; EN: Embryo number; PN: Plant number; P: Point; G: Globular; A: Arrow-tip; T: Torpedo; H: Hearth; C: Cotyledon; AM: Amorphous; W: White; N: Necrotic

Effects of embryo form and embryo stage on transformation to plant

Extracted seeds contained both white and necrotic embryos at different forms and stages. All embryos were classified into forms and developmental stages (Table 2 and Figure 3). In all season, 213 embryos were extracted from the 13 fruits and 34 embryos were transformed to plant (16.4%). Necrotic embryos (121) were determined higher than white embryos (92). Transformation rate to plant of white embryos was found 35.9% while transformation rate was 0.8% in necrotic embryos. The transformation rate to plants changed according to developmental stage of the embryo, also. In general, point and globular type embryos were not transformed to plants while cotyledon type embryos had the highest transformation rate (66.7%). Torpedo (45.5%), hearth (44.0%) and arrow-tip (41.7%) embryos gave better results also (Table 2).

Frequency of haploid production

Number of haploid plant in per 100 seeds, 100 embryos and fruit was investigated to determine the frequency of haploid production (FHP). These values fruit were 0.24, 0.94 and 33.3%, respectively. Frequency of haploid plant was the highest on July 9 and 11, but only 2 haploid plants were obtained at 50 Gy. Irradiated pollens were

not effective on embryo induction on July 21 and 28 (Table 3).

Ploidy determination

Chromosome counting in root tips

The results of chromosome counting showed that the ploidy levels of plants changed to embryo stage. Both arrow-tip and torpedo type embryos gave 20.0% haploid plants ($n = 20$). All the cotyledon and hearth type embryos were found to be diploid ($2n = 40$) (Figure 4).

Stomata size and chloroplast number of guard cells

The results indicated that stomata size, number of stoma per mm^2 and chloroplast number of guard cells could be alternative criteria to determine the ploidy level in pumpkin (Table 4, Figure 5). Chloroplast number was determined 6 or 8 for haploid plants and 10 or 12 for diploid plants. Average stomata length and stomata diameter were 30.13 and 20.08 μm in diploids and 21.47 and 17.12 μm in haploids, respectively. The non-linear relation was found between stomata dimension and stomata density (number/ mm^2). Although, diploid plants had bigger stomata than haploids, haploid plants had higher stomata density (407.3 number/ mm^2) than diploids (317.8 number/

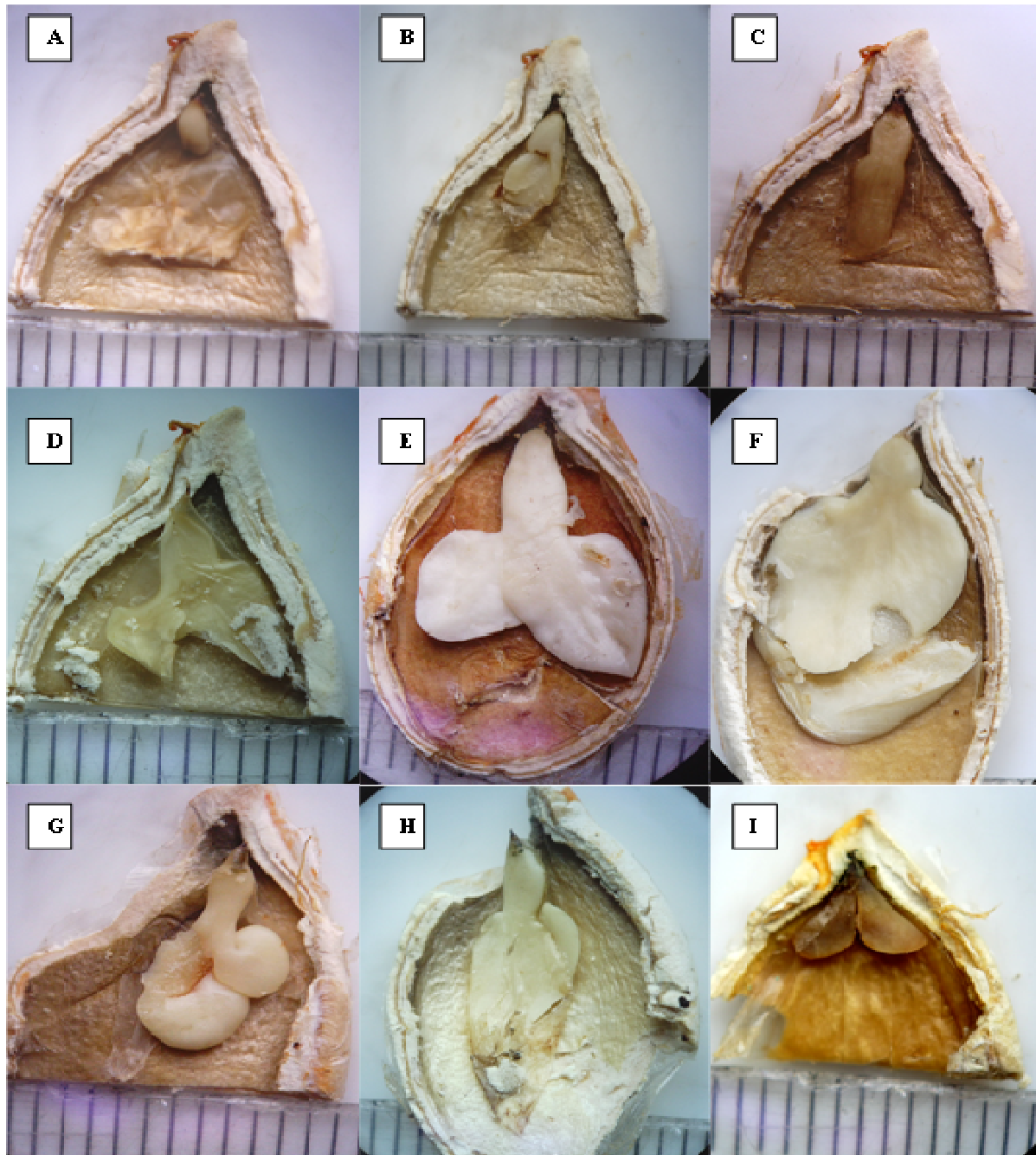


Figure 3. Some of the different forms and stages of embryos in pumpkin. **A)** Point. **B)** Globular. **C)** Arrow-tip. **D)** Torpedo. **E)** Heart. **F)** Cotyledon. **G, H)** Amorphous. **I)** Necrotic.

mm²). Chloroplast number in guard cells of stomata was counted 11.09 in diploids and 7.93 in haploids.

DISCUSSION

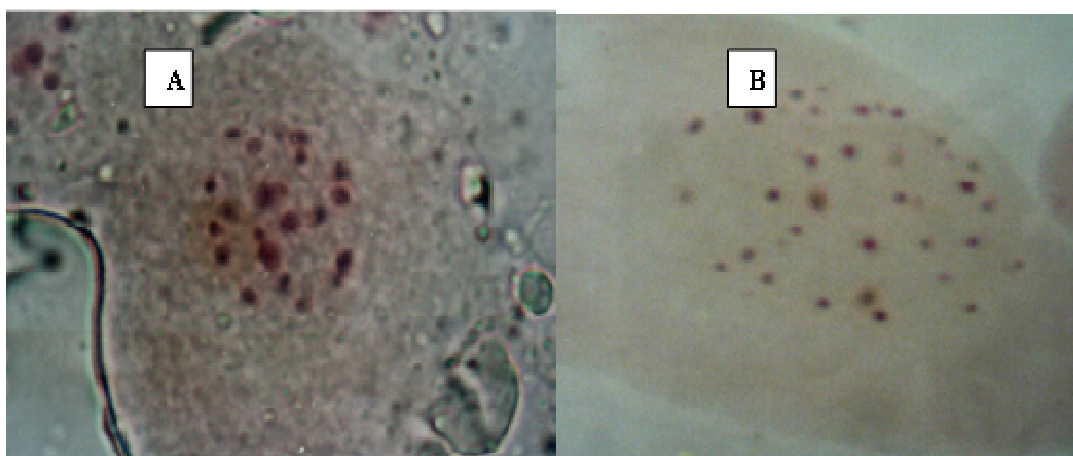
Our results indicated that embryo stimulation occurs only at 50 and 100 Gy in pumpkin as. The best result obtained from 50 Gy gamma ray dose. Embryos obtained at lower irradiation doses (25 and 50 Gy) in our previously works

in squash (Kurtar et al., 2002), also. On the other hand, haploid embryo induction was obtained in watermelon (Gürsöz et al., 1991; Sarı et al., 1994), melon (Sauton and Dumas de Vaulx, 1987; Sarı et al., 1992; Cuny, 1992; Abak et al., 1996) and cucumber (Niemirowicz-Szczytt and Dumas de Vaulx, 1989; Sauton, 1989; Çağlar and Abak, 1999) at relatively higher gamma ray doses (200-300 Gy). This is because of linear relationship between radio-resistance and pollen size

Table 3. Frequency of haploid production.

Irradiation date	FHP in 100 seeds			FHP in 100 embryos			FHP in a fruit		
	SN	HPN	Rate (%)	EN	HPN	Rate (%)	FN	HPN	Rate (%)
July 9	446	1	0.22	117	1	0.85	3	1	33.3
July 11	282	1	0.35	59	1	1.69	3	1	33.3
July 15	101	0	0.0	37	0	0.0	2	0	0.0
July 21	0	0	0.0	0	0	0.0	0	0	0.0
July 28	0	0	0.0	0	0	0.0	0	0	0.0
Σ	829	2	0.24	213	2	0.94	6	2	33.3

FHP: Frequency of haploid production; SN: Seed number; EN: Embryo number; FN: Fruit number; HPN: Number of haploid plant.

**Figure 4.** Chromosome number of haploid (A) ($n = 20$) and diploid (B) ($2n = 40$) plants.**Table 4.** Stomata characteristics of haploid (n) and diploid ($2n$) plants.

PL	SL	SDI	SD	CN
Haploid	21.47 b	17.12 b	407.3 a	7.93 b
Diploid	30.13 a	20.08 a	317.8 b	11.09 a
LSD %1	3.24	1.71	15.40	1.09

PL: Ploidy level; SL (μm): Stomata length; SDI (μm): Stomata diameter; SD (number/ mm^2): Stomata density; CN: Chloroplast number.

(Shridhar, 1992; Brewbaker and Emery, 1962; Alison and Casareff, 1968), and pumpkin pollens were one of the largest pollen (as in squash and winter squash) in vegetable species.

In all seasons, 213 embryos were extracted and necrotic embryos (121) were more than white embryos (92) due to delayed harvest times (5 and 6 weeks). The general parthenogenetic reaction of the genotypes was very low and no specific genotype reaction was detected in the investigated lines. Parthenogenetic embryos were obtained from all investigated genotypes, but with very low frequency. Although our results indicated low fre-

quency of haploids, this study is one of the first successful investigations on haploid embryo development in pumpkin via irradiated pollen technique.

The general evaluation, induction of parthenogenetic embryo (number/per fruit) was influenced from genotypes and changed from 12.6 to 29.5. Among the investigated genotypes, "G9" demonstrated comparatively good parthenogenetic response. This result indicated that embryo induction was influenced strongly on genotypes (Sauton, 1989; Bouvier et al., 1993; Faris et al., 1999). On the other hand, both the female genotype and the pollen source genotype were important on the parthenogenetic embryo induction (Musial and Przywara, 1998).

Size and densities of stoma and total number of chloroplasts in guard cells of haploid plants were compared with diploid plants determination by chromosomes counting. These differences were found to be significant at 1% level. Dorè (1986) in brussels sprouts, Rode and Dumas de Vaulx (1987) in carrot, Abak et al. (1996) in melon, Sarı et al. (1999a) in watermelon, Abak et al. (1998) in pepper and Kurtar et al. (2002) in squash found similar results. The results indicated that all methods can be used successfully, but chromosome counting is cumbersome while morphological observations take a long time

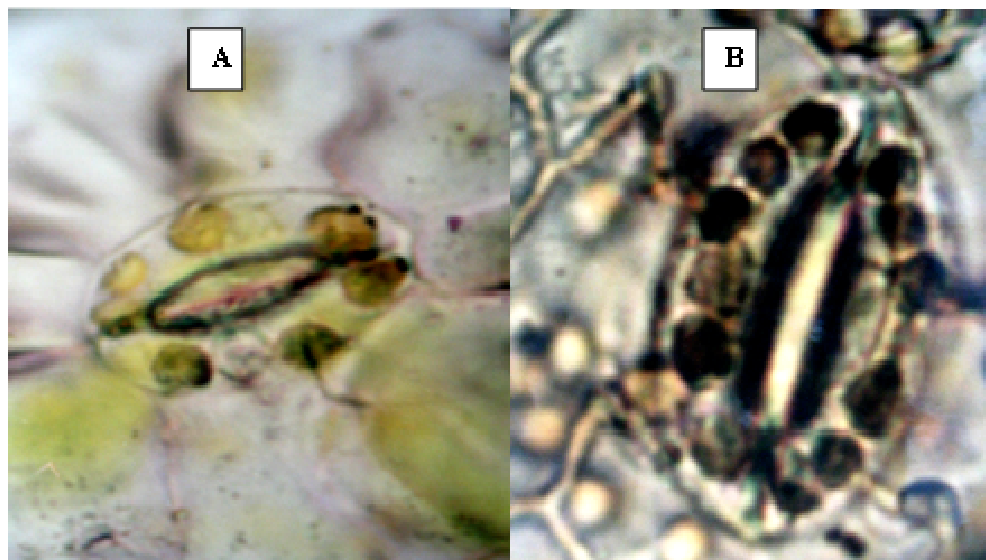


Figure 5. Stomata and chloroplast number in guard cells; **A)** haploid, **B)** diploid.

for plant development to reach on appropriate stage. Measurement of stoma and chloroplast counting are simple and more practical than the others methods.

In conclusion, irradiated pollen technique is proposed to induce of gynogenic embryo and obtained haploid plants in pumpkin as in melon (Sauton and Dumas de Vaulx, 1987; Sari et al., 1992), in cucumber (Sauton, 1989; Niemirowicz-Szczytt and Dumas de Vaulx, 1989; Çağlar and Abak, 1999), in watermelon (Gürsöz et al., 1991; Sari et al., 1994), in snake cucumber (Taner et al., 2000) and in squash (Kurtar et al., 2002). But, this technique must be improved for frequency of haploid plants. Therefore, the future works should be based on specific irradiation dose and irradiation time, culture conditions and productive genotypes.

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