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Parthenogenetic Haploid Plant Production in Styrian Pumpkin by Gamma Irradiated Pollen

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ABSTRACT

Doubled haploid method via parthenogenesis induction still remained prominent in Cucurbitaceae breeding due to drastic reduction in time and cost of newly released lines. In this study, different doses of Gamma ray (25, 50, 75, 100, and 200 Gy) were used to irradiate pollen grains for induction of parthenogenetic haploid embryos in oilseed pumpkin (*Cucurbita pepo* var. 'Styriaca'). Parthenogenetic embryos at different developmental stages were rescued *in vitro* and 348 plants were obtained, of which 134 were recognized as haploid by ploidy analysis. The highest rate of haploid plants was obtained from globular (25.3%) and torpedo (23.8% plants) embryos followed by arrow-tip (13.4%), torpedo (10.5%), stick (10.5%), heart (9%), and cotyledonary (7.5%), respectively. All doses, except 200 Gy, were effective for induction of embryos and haploid plants; in a way that the highest number of haploids was obtained by 100 Gy. Our results indicated that parthenogenetic haploid embryos could be efficiently induced in *C. pepo* if proper Gamma ray dose and developmental stage of embryos are selected.

Introduction

Styrian, hull-less or oilseed pumpkin (*Cucurbita pepo* convar. pepo var. 'Styriaca') is an economically important horticultural plant, which is widely grown for its valuable seeds. The seeds contain abundant protein (particularly tryptophan) and lots of minerals such as zinc, manganese, magnesium, copper and iron that are often lacking in other foods (Yadav et al., 2010). Study-proven uses for pumpkin seed oil include lowering cholesterol, easing symptoms of benign prostatic hyperplasia in men, reducing hot flashes and hormone-related headaches in women, lowering blood pressure and providing other benefits to prevent heart diseases, treating overactive bladder, and reversing hair loss (https://www.verywellhealth.com/pumpkin-seed-oil-health-benefits).

The cultivated plants of *C. pepo* var. 'Styriaca' are not genetically uniform and appear to be highly heterogeneous, hence

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increased genetic homogeneity will certainly improve the cultivation of this plant and also extraction of components. Application of doubled haploid technique in a conventional breeding programme saves many generations that are normally needed to produce inbred lines (Germana, 2006). Doubled haploid production through the gametic embryogenesis allows the single-step development of complete homozygous lines from heterozygous parents (Germana, 2006; Ahmadi zet al., 2018). Homozygous lines are often perceived as a first step in genetic improvement of crops and can be promising for production of hybrids, synthetic cultivars and new cultivars in direct manner (Shariatpanahi and Ahmadi, 2016). Numerous methods such as wide hybridization, anther/microspore culture, ovule/ovary culture and *in situ* parthenogenesis by irradiated pollen followed by *in vitro* culture of immature embryos have been reported for production of haploids (Germanà 2011; Shariatpanahi and Ahmadi 2016). Being recalcitrant to androgenesis and gynogenesis, irradiating pollen grains with ultraviolet (UV), Gamma rays, or X-rays serves as the most common and efficient way of obtaining haploids in cucurbits (Gałązka and Niemirowicz-Szczytt 2013). Thanks to its simple application, good penetration, reproducibility, high mutation frequency, and low abortion problems, haploid programmes widely adopt Gamma rays for irradiation (Chahal and Gosal 2002). Irradiated pollen is able to germinate on the stigma, develop within the style and reach the embryo sac, but cannot fertilize the egg-cell and the polar nuclei (Cuny 1993). The genetically inactive but viable pollen can be exploited to trigger egg cell division, and hence induction of parthenogenesis or development of parthenocarpic fruit, including gynogenic haploid production; minor cross-incompatibilities as well as studying physiological incompatibility (Savaskan and Toker, 1991; Todorova et al., 2004), gene transformation (Pandey, 1978) and nucleus substitution (Raquin et al., 1989). Induction of haploid parthenogenetic embryos by *in situ* pollination with irradiated pollen has been successfully reported in Cucurbitaceae including *C. melo* (Sari et al. 1992; Taner et al., 2000; Lotfi et al., 2003) *C. sativus* (Niemirowicz-Szczytt and Dumas de Vaulx, 1989; Ebrahimzadeh et al. 2018), *Citrullus lanatus* (Gürsöz et al. 1991), *Cucurbita pepo* (Kurtar et al., 2002; Ebrahimzadeh et al., 2013), *C. moschata* (Kurtar et al., 2009), and *C. maxima* (Kurtar and Balkaya, 2010). The efficiency of this process is highly affected by genotype, growth condition, irradiation source and dose, and developmental stage of embryos at the time of excision, exhibiting different regeneration capacity (reviewed by Dong et al., 2016). Košmrlj et al. (2013) induced parthenogenetic haploid embryos in various accessions of *C. pepo* via pollination with X-ray (at 50-350 Gy) irradiated pollen grains for the first time and no further effort has been made to improve the efficiency of the procedure. In this study, the effects of various doses of Gamma ray (25, 50, 75, 100, and 200 Gy) and also different developmental stages of derived embryos were assessed on efficiency of haploid parthenogenetic embryo induction in *C. pepo* var. 'Styriaca' for the first time.

Materials and Methods

Plant materials

Local open-pollinated pumpkin cv. 'Styriaca' was provided by Zardband pharmaceuticals company. The seeds were sown in plastic trays (45 cells) containing mixture of peat-moss: perlite (1:1 v/v). Thirty seedlings at 3-4 true leaf stage were planted in rows spaced in 40 × 150 cm under natural light condition and day/night temperature of 25/20 °C. Plants were irrigated on every second day; commercial fertilizers were applied as per irrigation schedule and insecticides (for aphids and red spiders) and fungicides (for mildew) applied if needed throughout the growing season.

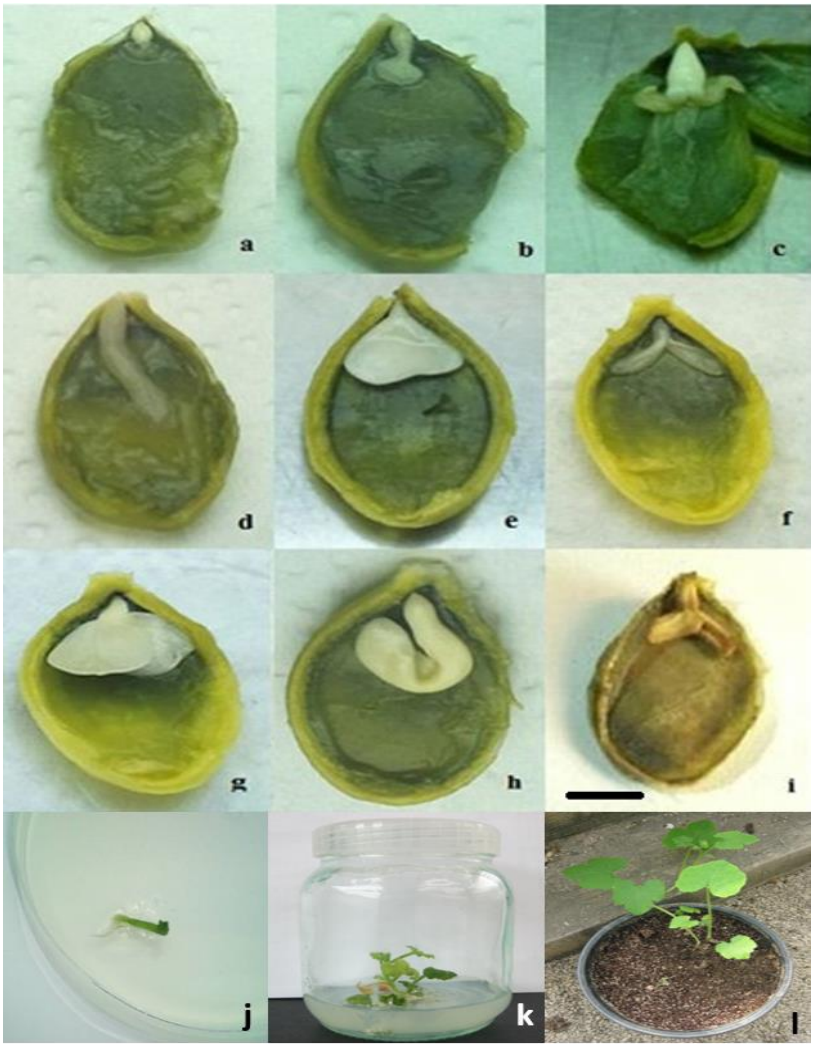

Fig. 1. Morphologically different haploid parthenogenetic embryos regenerated following pollination with irradiated pollen in *Cucurbita pepo* var. 'Styriaca'; a globular, b arrow-tip, c torpedo-tip, d stick, e heart, f torpedo, g cotyledon, h amorphous, i necrotic, j rescued embryo, k regenerated plantlet, and l acclimatized plant.

Pollen irradiation and pollination

Female flowers were isolated with paper bags (150 × 100 mm), and male flower buds were collected one day before anthesis. The petals were dissected and the anthers were placed in Petri dishes and irradiated at 25, 50, 75, 100, and 200 Gy using a Cobalt-60 (Co^{60}) as Gamma ray source with the output of 12 Gy/min at the Nuclear Agriculture Research School (NARS), Karaj, Iran. Irradiated anthers were then incubated at room temperature (25 ˚C) overnight. Next day, isolated female flowers were pollinated by irradiated pollen and were isolated again to avoid undesired pollen contamination. Three male flowers were used to ensure the pollination of each female flower. Paper bags were then removed 3-4 days after pollination. Control plants were hand pollinated with non-irradiated pollen grains.

Embryo detection, excision, and culture

Four weeks after pollination, the fruits were harvested and washed with running tap-water and then immersed in 40% commercial bleach (2% sodium hypochlorite solution) for 30 min. Seeds were extracted under aseptic conditions and sterilized with sodium hypochlorite (1%) for 12 min followed by three times washing with sterile distilled water each 3-5 min. In order to detect embryos more easily, seeds (approximately 20-40) were displaced in Petri dishes containing 25 mL of liquid E20 medium (Sauton and Vaulx, 1987)

supplemented with 3% sucrose, 0.01 mg L^{-1} indole-3-acetic acid (IAA), and pH 6.0. The Petri plates were then sealed with parafilm and placed in a growth chamber with a fixed temperature of 24 ºC, 16-h photoperiod, and light intensity of 30 µmol m^2 s^{-1}. About 10-20 days later, the seeds were checked over a light box equipped with a light-emitting diode (Lotfi et al., 2003). Excised embryos were classified according to the stages of development (Fig. 1a-i), then cultured on E20A medium supplemented with 2% sucrose and 0.01 mg L^{-1} IAA. The rescued embryos (Fig. 1j) were transferred to a growth chamber at 28 ± 2 ºC and 16-h photoperiod for embryo germination and plant regeneration (Fig. 1k). For optimizing the growth, regenerated plantlets were colonized by nodal cuttings on E20A medium.

Acclimatization

After 30-50 days, regenerated plantlets were transferred to vermiculite and gradually were adapted to greenhouse temperature between 28 ± 5 °C and 16 h photoperiod (Fig. 1, l). Transparent glasses were also placed on each plants to keep moisture. The glasses were gradually perforated and completely removed after 10 days. Once acclimatized, plantlets were transferred in pots containing field soil, peat and perlite (1:1:1 v/v/v).

Determination of ploidy level

Morphological observations

After vegetative growth of acclimated plants, their floret length and width (cm) were measured and also the existence of pollen in male flowers was assayed based on the procedure previously described by Kurtar et al. (2002).

Chloroplast counting in the guard cells of stomata

Chloroplast number in each stomatal guard cells were determined in the 5th or 6th leaves of putative haploid and control diploid plants (Sari et al., 1992). Lower epidermal strips of leaves were placed onto a microscope slide, adding 10% lugol's iodine solution (ICN, Costa Mesa, CA) for 4 min, and observed by light microscope at ×1000 magnification (Guimarães and Stotz, 2004; Rêgo et al., 2011).

Flow cytometry analysis

Specimens from leaves of *in vitro* cultivated haploid and diploid (control) plants were prepared. Nuclei were extracted from 0.5 cm^2 of leaf samples by chopping with a razor blade in 600 mL of modified Galbrith's nuclei isolation buffer (Galbrith et al. 1983) containing 300 mM Tris, 4 mM $MgCl_2.6H_2O$, pH 7.5, 0.5% Triton X-100 (Partec, Munster, Germany). 4,6-Diamino-2-phenylindole (DAPI, 1400) was added for DNA staining for 2 min. Then, the derived suspension was passed through a 30-mm nylon filter to exclude the cell debris and the ploidy level of samples was analyzed by flow cytometer (Partec, Munster, Germany) using a 488 nm Argon laser to excite the PI fluorochrome, and FL-2 detector with a 585/42 band pass filter.

Statistical analysis

All experiments were carried out based on a completely randomized design with six replications. The entire experiments were repeated two times. Statistical analyses including analysis of variance (ANOVA) and means comparison were performed using Duncan's multiple range test (DMRT) at 5% probability level using SAS® software (SAS Institute Inc., Cary, NC).

Results

Embryo induction and growing of haploid plants

Approximately 6% of the total extracted seeds from pollination with irradiated pollen contained embryo and the rest were almost empty. Therefore, 850 embryos were achieved out of 14258 inspected seeds. Different doses (25, 50, 75, 100, and 200 Gy) of pollen Gamma ray irradiation significantly affected the rate of embryo induction (Table 1).

Table 1. Number and rate of embryos in *Cucurbita pepo* var. 'Styriaca' obtained after pollination by irradiated pollen with different doses.

Dose (Gy)	Fruit number	Seed number	Embryo number	Produced embryo/fruit	Produced embryo /total embryo (%)
25	6	2890	84	14.00^{bc*}	9.88
50	6	2598	212	35.33^{b}	24.94
75	6	2156	188	31.33^{b}	22.12
100	6	3092	364	60.67^{a}	42.82
200	6	3522	2	0.33^{c}	0.24
Total	30	14258	850	28.33	100%

*Means followed by the same letters are not significantly different according to DMRT ($P = 0.05$).

Irradiating with 100 Gy resulted in the highest number of parthenogenetic embryo per fruit (60.67 and 42.82% of total embryos) and significantly fewer embryos were achieved following irradiation with lower doses (25-75 Gy). In the light of foregoing results, 200 Gy was found to be detrimental for embryo induction and only 2 embryos were obtained from total seeds extracted.

A total of 348 seedlings were grown from the 850 rescued embryos (40.9%) subsequently. Dose of pollen irradiation not only affected the rate of parthenogenetic embryo induction but also the rate of obtained plantlet (Table 2). The highest number of plants (168 out of 364 embryos) were obtained using 100 Gy, whereas no plantlet was regenerated in 200 Gy. Such morphologically different embryos i.e. globular (Fig. 1-a), arrow-tip (Fig. 1-b), torpedo-tip (Fig. 1-c), stick (Fig. 1-d), heart (Fig. 1-e), torpedo (Fig. 1-f), cotyledonary (Fig. 1-g), amorphous (Fig. 1-h), and necrotic (Fig. 1-i) revealed different regeneration capacity so that the highest rate of plant regeneration along all tested doses was attributed to arrow-tip (54.55%), cotyledonary (46.21%), amorphous (45%), and heart shape (41.93%), respectively. Few necrotic embryos were also produced in the doses of 25 and 75 Gy, none were able to germinate.

A total of 134 haploid plants from regenerated plantlets (348) were obtained from 25, 50, 75, and 100 Gy dosages of Gamma rays. Almost all diploid plants were regenerated from morphologically abnormal embryos, while normal embryos such as cotyledonary, heart-shaped, torpedo-shaped, arrow-tip, torpedo-tip, globular, and sticky embryos produced 8.2, 23.1, 48.4, 75, 87.5, 94.4, and 100% haploid plants, respectively (Table 2).

Table 2. Effects of embryo type and different doses of gamma irradiation (Gy) on embryo production and regeneration rate in *Cucurbita pepo* var. 'Styriaca'

Embryo stage	Gamma dose (Gy)										Total				
	25		50		75		100		200						
	PE	RP	PE	RP	EP	RP	EP	RP	PE	RP	PE	RP	R (%)	RH	RH (%)
Globular	12	2	22	10	16	8	60	16	2	0	112	36	32.14	34	94.4
Arrow-tip	2	0	14	8	16	10	12	6	0	0	44	24	54.55	18	75
Torpedo-tip	2	0	10	6	2	2	30	8	0	0	44	16	36.36	14	87.5
Stick	0	0	8	4	2	0	34	10	0	0	44	14	31.82	14	100
Heart	10	0	20	6	22	10	72	36	0	0	124	52	41.93	12	23.1
Torpedo	16	8	42	18	42	6	72	34	0	0	172	66	38.37	32	48.4
Cotyledonary	40	16	86	28	76	30	62	48	0	0	264	122	46.21	10	8.2
Amorphous	0	0	10	6	8	2	22	10	0	0	40	18	45.00	0	0
Necrotic	2	0	0	0	4	0	0	0	0	0	6	0	0.00	0	0
Total	84	26	212	86	188	68	364	168	2	0	850	348	40.9	134	38.5

Produced Embryo (PE); Regenerated Plant (RP); Regenerated Haploid (RH); Regeneration (%R = PE/RP × 100); Regenerated Haploid (%RH = RH/RP × 100).

Table 3. Effect of pollen gamma irradiation dose on fruit (F), seed (S), embryo (E), regenerated plant (RP) and haploid plant (HP) in *Cucurbita pepo* var. 'Styriaca'

Dose	F (No.)	S (No.)	E (No.)	RP (No.)	HP (No.)	HP/Total (%)	HP /Fruit	HP/100 Seeds	HP/100 Embryos	HP/100 Plants
25	6	2890	84^{bc*}	26^{bc}	6^{b}	4.48%	1	0.21	7.14	23.08
50	6	2598	212^{b}	86^{b}	32^{b}	23.88%	5.33	1.23	15.09	37.21
75	6	2156	188^{b}	68^{bc}	24^{b}	17.91%	4	1.11	12.77	35.29
100	6	3092	364^{a}	168^{a}	72^{a}	53.73%	12	2.33	19.64	42.86
200	6	3522	2^{c}	0^{c}	0^{b}	0%	0	0	0	-
Total	15	14258	850	348	134	-	4.47	0.94	16.18	38.51

*Means followed by the same letters are not significantly different according to DMRT ($P = 0.05$).

In total, 36 haploid plants were obtained by 100 Gy of Gamma rays that was the highest percentage of haploid production (53.73%) and significantly more than other doses (Table 3).

According to the results, irradiation with dose of 100 Gy also led to relatively higher rate of haploid plant per fruit (12), per 100 seeds (2.33), per 100 embryos (19.64) and per 100 plants (42.86) compared to the other doses. However, higher intensity of irradiation was detrimental for embryo induction; in a way that no embryo was obtained from flowers pollinated with irradiated pollen grains with 200 Gy.

Ploidy determination

Generally, the growth of haploid plants was relatively slow and they had smaller leaf and flower size compared to those in diploids (Fig. 2). In this study, male flowers of haploid plants failed to release pollen and female flowers did not set fruit at all when pollinated.

Chloroplast number in the guard cells of stomata was also differed significantly between haploid and diploid plants (Fig. 3). The average chloroplast number in guard cells of haploid and diploid plants were 6.87 and 11.21, respectively, which was almost doubled in diploid plants in comparison with their numbers in haploid plants.

Flow cytometer analysis also validated polidy determination of haploid plants by chloroplast counting and morphological assays (Fig. 4).

Fig. 2. Male flowers of diploid (2n, left) and haploid (n, right) plants at the day before anthesis in *Cucurbita pepo* var. 'Styriaca'

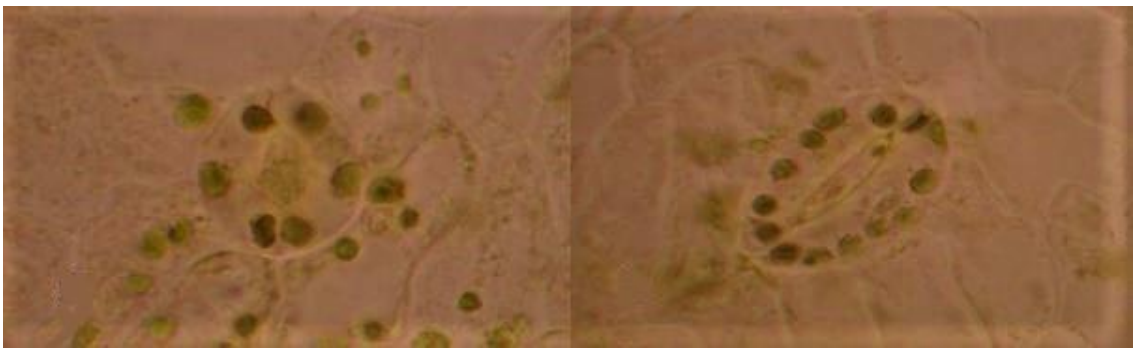

Fig. 3. Stomatal guard cell on the leaf of haploid plant (left) containing 6 chloroplasts and on diploid plant (right) containing 12 chloroplasts in *Cucurbita pepo* var. 'Styriaca'

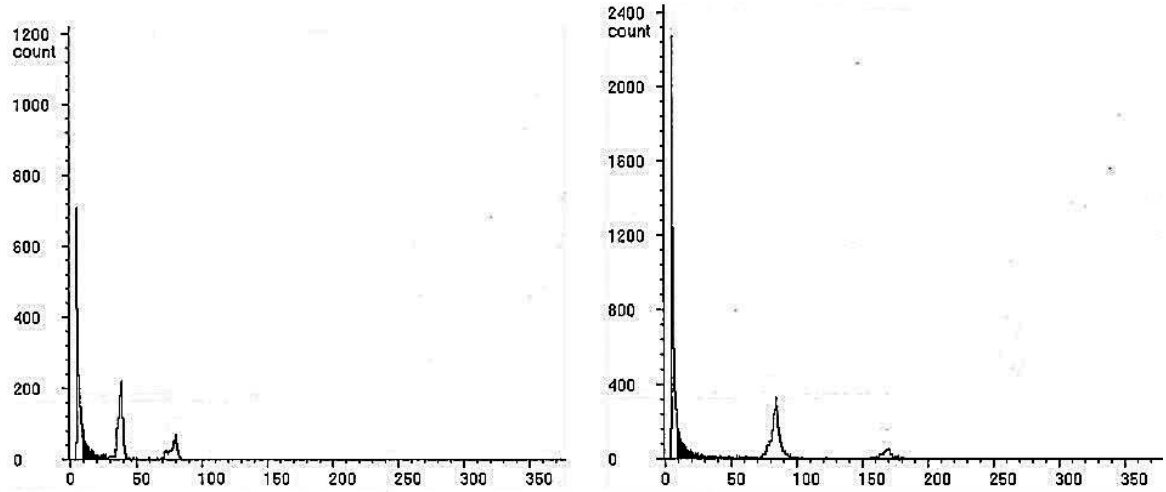

Fig. 4. Flow cytometry histograms of diploid and haploid nuclei status in styrian pumpkin; intensity of 4,6-diamidino-2-phenylindole (DAPI) fluorescence (x-axis) and the number of nuclei (y-axis) in haploid (left) and diploid (right) plants.

Discussion

The satisfactory rate of embryo induction in this trial (6% of the total extracted seeds) is attributed to appropriate maternal plant condition and time of pollination in June. Positive effect of the spring season on the yield of haploid embryos in *C. sativus* (Caglar and Abak, 1999), *C. lanatus* (Gürsöz et al., 1991), and *C. pepo* (Kurtar et al., 2002; Ebrahimzadeh et al., 2013a) have already been reported. Number of necrotic embryos was also very low (less than 1%) in this trial, which is respectable compared with similar works, showing the right time of fruit harvesting. Based on Ebrahimzadeh et al. (2013a), delaying in harvest of fruits (5-6 weeks after pollination) increases the number of necrotic embryos in *C. pepo*.

The rate of derived plants (40.9%) was closed to the previously report in *C. moschata* (47.22%; Berber et al. 2010). The frequency of derived haploid plants was affected by the intensity of Gamma irradiation and embryo developmental stage. It has been previously reported that the developmental stage of embryos at the time of excision is one the major factors affecting the efficiency of plant regeneration (Jaskani et al. 2005). Cotyledonary embryos have been reported as the most favourable stage in different species such as *C. sativus* (Caglar and Abak 1999) with 81.8% regeneration and *C. melo* with 14.29% (Godbole and Murthy, 2012). It has been proposed that unlike premature and developing embryos, cotyledonary embryos, display the potential capacity to synthesize their own hormones for successive function during further development and regeneration (Thiruvengadam et al. 2013).

Taken together, the best results for production of haploid plants in this trial was obtained by 100 Gy of Gamma ray. In different species of *Cucurbita*, irradiating with the doses of 25 and 50 Gy (Kurtar et al. 2002) and 50 Gy for *C. pepo* (Ebrahimzadeh et al., 2013a), 50 Gy for *C. moschata* (Kurtar et al., 2009), 50 and 100 Gy for *C. maxima* (Kurtar and Balkaya, 2010) have been reported as the best treatment for induction of haploid parthenogenetic embryo. Košmrlj et al. (2013) working on different doses of X-ray, recognoized the intensity of 200 Gy as the best dose for induction of parthenogenetic embryos in hull-less pumpkin, that is double dosage of the best rate (100 Gy) in this trial. Applying gamma (Godbole and Murthy, 2012) and X-ray (Košmrlj et al., 2013) for pollen irradiation in melon also showed similar result. Lower dose of Gamma ray compared to the X-ray is attributed to its more energy and permeability due to its shorter wavelength.

It was revealed that chloroplast counting in the guard cells of stomata could be considered as a convenient and reliable criterion for verification of haploid and diploid plants. Similar results have been found in *Brassica oleracea* and carrot (Dore 1986), *Cucumis melo* (Abak et al., 1996; Godbole and Murthy, 2012), *Capsicum annum* (Abak et al., 1998), *Cucurbita moschata* (Kurtar et al., 2009) and *C. maxima* (Kurtar and Balkaya, 2010).

Conclusion

In conclusion inducing parthenogenetic embryo by irradiated pollen followed by *in vitro* embryo rescue is an effective technique for haploid production in Styrian pumpkin. In the present study, the highest percentages of haploid plants (23.8-25.3%) were achieved when pollen grains were irradiated by 100 Gy Gamma ray and embryos were rescued at globular and torpedo developmental stages. Liquid culture of seeds also has advantages for embryo rescuing.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Author contribution statement

All authors whose names appear on the submission have contributed sufficiently to the scientific work and, therefore, share collective responsibility and accountability for the results. Informed consent was obtained from all individual participants included in the study.

References

1. Abak K, Sari N, Paksoy M, Yılmaz H, Aktas H, Tunalı C. 1996. Genotype response to haploid embryo induction with pollination by irradiated pollen in melon, obtaining of dihaploid lines, determination of haploid plants by different techniques. Turkish Journal of Agriculture and Forestry 20: 425-430.
2. Abak K, Çömlekçioglu N, Büyükalaca S, Sarı N. 1998. Use of stomatal characteristics to estimate ploidy level of haploid and dihaploid pepper plants. Xth EUCARPIA Meeting Capsicum and Eggplant, Avignon – France, pp 179-182.
3. Ahmadi B, Ahmadi M, Teixeira da Silva J.A. 2018. Microspore embryogenesis in *Brassica*: calcium signaling, epigenetic modification, and programmed cell death. Planta 248(6): 1339–1350.
4. Berber M, Yildiz M, Abak K. 2010. Effects of irradiation doses on haploid embryo and plant production in naked and shelled seed pumpkins, XXVIII International Horticultural Congress on Science and Horticulture for People (IHC2010): International Symposium on 929, pp 381-384.
5. Caglar G, Abak K. 1999. Obtention of in vitro haploid plants from in situ induced haploid embryos in cucumber (*Cucumis sativus* L.). Turkish Journal of Agriculture and Forestry 23: 283-290.
6. Chahal G, Gosal S. 2002. Principles and procedures of plant breeding: biotechnological and conventional approaches. Alpha Science Int'l Ltd, United Kingdom.
7. Cuny F. 1993. Processus d'induction d'embryons haploïdes par du pollen irradié chez le melon (*Cucumis melo* L.) Réponses du pollen à l'irradiation gamma. These de Docteur, Universite´ d'Avignon et des Pays de Vaucluse, Avignon.
8. Dore C. 1986. Evaluation du niveau de ploidie des plantes d'une population de choux de Bruxelles (*Brassica oleracea* L. ssp. gemmifera) d'origine pollinique. Agronomie 6: 797-801.
9. Ebrahimzadeh H, Lotfi M, Azizinia S, Ghanavati F. 2013. Production of haploids in *Cucurbita pepo* L. through parthenogenesis induced by gamma-irradiated pollen. Crop Biotechnology 3: 99-108.
10. Ebrahimzadeh H, Shariatpanahi M.E, Ahmadi B, Soltanloo H, Lotfi M, Zarifi E. 2018. Efficient parthenogenesis induction and in vitro haploid plant regeneration in cucumber (*Cucumis sativus* L.) using putrescine, spermidine, and cycocel. Journal of Plant Growth Regulation 37(4): 1127-1134.
11. Gałązka J, Niemirowicz-Szczytt K. 2013. Review of research on haploid production in cucumber

and other cucurbits. Folia Horticulturae 25: 67-78.

12. Galbrith D, Harkins K, Maddox J, Ayres N, Sharma D, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220: 1049-1051.

13. Germanà M.A. 2006. Doubled haploid production in fruit crops. Plant Cell, Tissue and Organ Culture 86: 131-146.

14. Germanà M.A. 2011. Gametic embryogenesis and haploid technology as valuable support to plant breeding. Plant Cell Reports 30: 839-857.

15. Godbole M, Murthy H.N. 2012. Parthenogenetic haploid plants using gamma irradiated pollen in snapmelon (*Cucumis melo* var. momordica). Plant Cell, Tissue and Organ Culture 109: 167-170.

16. Guimarães R.L, Stotz H.U. 2004. Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. Plant Physiology 136: 3703-3711.

17. Gürsöz N, Abak K, Pitrat M, Rode J, Dumas de Vaulx R. 1991. Obtention of haploid plants induced by irradiated pollen in watermelon (*Citrullus lanatus*). Cucurbit Genetics Cooperative 11: 109-110.

18. Jaskani M.J, Khan I.A, Khan M. 2005. Fruit set, seed development and embryo germination in interploid crosses of citrus. Scientia Horticulturae 107: 51-57.

19. Košmrlj K, Murovec J, Bohanec B. 2013. Haploid induction in hull-less seed pumpkin through parthenogenesis induced by X-ray-irradiated pollen. Journal of the American Society for Horticultural Science 138: 310-316.

20. Kurtar E, Sarı N, Abak K. 2002. Obtention of haploid embryos and plants through irradiated pollen technique in squash (*Cucurbita pepo* L.). Euphytica 127: 335-344.

21. Kurtar E.S, Balkaya A, Ozbakir M, Ofluoglu T. 2009. Induction of haploid embryo and plant regeneration via irradiated pollen technique in pumpkin (*Cucurbita moschata* Duchesne ex. Poir). African Journal of Biotechnology 8: 5944-5951.

22. Kurtar E.S, Balkaya A. 2010. Production of *in vitro* haploid plants from in situ induced haploid embryos in winter squash (C*ucurbita maxima* Duchesne ex Lam.) via irradiated pollen. Plant Cell, Tissue and Organ Culture 102: 267-277.

23. Lotfi M, Alan A, Henning M, Jahn M, Earle E. 2003. Production of haploid and doubled haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. Plant Cell Reports 21: 1121-1128.

24. Niemirowicz-Szczytt K, Dumas de Vaulx R. 1989. Preliminary data on haploid cucumber (*Cucumis sativus* L.) induction. Cucurbit Genetics Cooperative. 12: 24-25.

25. Ondrej V, Navratilova B, Lebeda A. 2001. *In vitro* cultivation of *Cucumis sativus* ovules after fertilization, II International Symposium on Cucurbits 588, pp 339-343.

26. Pandey K.K. 1978. Gametic gene transfer in *Nicotiana* by means of irradiated pollen. Genetica 49: 53-69.

27. Raquin C, Cornu A, Farcy E, Maizonnier D, Pelletier G, Vedel F. 1982. Nucleus substitution between *Petunia* species using gamma ray-induced androgenesis. Theoretical and Applied Genetics 78: 337-341.

28. Rêgo M, Rêgo E, Bruckner C, Finger F, Otoni W. 2011. *In vitro* induction of autotetraploids from diploid yellow passion fruit mediated by colchicine and oryzalin. Plant Cell, Tissue and Organ Culture. 107: 451-459.

29. Sari N, Abak K, Pitrat M, Dumas de Vaulx R. 1992. Induction of parthenogenetic haploid embryos and plant obtention in melon (*Cucumis melo* L. var. inodorus Naud ve *C. melo* L. var. reticulatus Naud). Turkish Journal of Agriculture and Forestry. 16: 302-314.

30. Sauton A, Vaulx R.D. 1987. Obtention de plantes haploïdes chez le melon (*Cucumis melo* L.) par gynogenèse induite par du pollen irradié. Agronomie 7: 141-148.

31. Savaskan C, Toker M. 1991. The effects of various doses gamma irradiation on the seed germination and root tips chromosomes of rye (*Secales cereals* L.). Turkish Journal of Botany 15: 349-359.

32. Shariatpanahi M.E, Ahmadi B. 2016. Isolated microspore culture and its applications in plant breeding and genetics. In: Anis M, Ahmad N (eds), Plant Tissue Culture: Propagation, Conservation and Crop Improvement. Springer Science+Business Media, Singapore, pp 487-507.

33. Song Y, Yang Y, Zhang Y, Duan L, Zhou C, Ni Y, Liao X, Li Q, Hu X. 2013. Effect of acetylation on antioxidant and cytoprotective activity of

polysaccharides isolated from pumpkin (*Cucurbita pepo,* lady godiva). Carbohydr Polym 98: 686-691.

34. Taner K, Yanmaz R, Kunter B. 2000. The effects of irradiation dose and harvest period on haploid plant formation via irradiated pollen in snake cucumber (*Cucumis melo* var. flexuosus Naud.). IIIrd National Vegetable Culture Symposium, 11-13 September 2000, Isparta-Turkey. pp 177-181.

35. Thiruvengadam M, Jeyakumar J.J, Kamaraj M, Lee Y.J, Chung I.M. 2013. Plant regeneration through somatic embryogenesis from suspension cultures of gherkin (*Cucumis anguria* L.). Australian Journal of Crop Science 7(7): 969-977.

36. Todorova M, Ivanov P, Nenova N, Encheva J. 2004. effect of female genotype on the efficiency ofγ-induced parthenogenesis on sunflower (*Helianthus annuus* L.). Helia 27: 67-74.

37. Yadav M, Jain S, Tomar R, Prasad G.B.K.S, Yadav H. 2010. Medicinal and biological potential of pumpkin: an updated review. Nutrition Research Reviews 23: 184-190.