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Effect of Pollination with Gamma Irradiated Pollen on In Vitro Regeneration of Ovule Culture in Cyclamen

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ABSTRACT

The obtention of homozygous lines through *in situ* parthenogenesis via gamma irradiation method is a comprehensive application in vegetables. However, there are a limited number of studies on *in situ* parthenogenesis in ornamentals. Therefore, *in situ* parthenogenetic capacity of more species needs to be examined. For this purpose, the effects of pollination with gamma-irradiated pollen on *in vitro* ovule cultured in *Cyclamen persicum* L. were evaluated in this study. Flower buds were collected before anthesis and irradiated at different doses 50, 100, 150, 200, 300, and 450 Gy of gamma-ray using a Co-60 source. The control group was pollinated with non-irradiated pollen. Fruits were harvested 30 days after pollination, and isolated ovule explants were cultured on four different mediums in *in vitro*. M0 was control group containing half-strength MS basal media; M1 media additionally has 10 g/L maltose, 1.0 g/L proline, 2.0 g/L peptone, 200.0 mg/L spermidine and 0.5 mg/L kinetin; in addition to basal medium, M2 media additionally contains 10 g/L maltose, 1.0 g/L proline, 2.0 g/L peptone, 200.0 mg/L spermidine, 0.4 mg/L gibberellic acid (GA_3) and 0.4 mg/L N6-benzyl adenine (BA). M3 media additionally contains 2.0 mg/L 2,4-D and 0.8 mg/L 6-(γ, γ-dimethylallylamino) purine (2iP). Plantlets started to form 10-12 weeks after the beginning of culture. The effects of nutrient media, irradiation dose, and their interactions on plant formation were statistically significant. The lowest plantlet regeneration (0.33%) was obtained from ovule explants exposed to 50 Gy gamma rays and cultured on M3 media while the highest plantlet regeneration (2.66%) was obtained from ovule explants cultured on M1 media 30 days after pollination with non-irradiated pollen grains. According to stomatal observations, there were no statistical differences between donor plant and *in vitro* regenerated plantlets.

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Introduction

Cyclamen is commercially cultivated worldwide for use as an ornamental pot plant. It is a perennial bulbous plant with attractive flowers and leaves (Curuk et al., 2015, Simsek et al., 2017). Tissue culture techniques were used for the propagation of cyclamen due to division of cyclamen tuber is not possible (Schwenkel and Winkelmann, 1998). Cyclamen is conventionally propagated expensive seeds due to hand pollination used in seed production. Moreover, cyclamen breeding is usually carried out with conventional crossing and phenotypic selection. Still, traditional breeding takes a long time and often fails due to cross-incompatibility and selfing depression at different levels of the breeding cycle (Kiviharju et al., 1992). Therefore, tissue culture-assisted breeding methods are valuable for cyclamen breeding to overcome these obstacles and obtention homozygous parental lines of F1 hybrids. The first haploid plant production was reported in *C. persicum* by Ishizaka and Uematsu (1993), and only a few haploid plants were obtained from anther culture. Sevindik (2018) reported that embryo and callus structures regenerated from anther culture in *C. persicum* were haploid, while plantlets obtained from these structures were spontaneous diploid.

In a classical plant breeding system, homozygous lines have an excellent value for developing novel cultivars. Homozygous lines, also called pure lines, are created after a recurrent selfing process in several generations and still may not be 100% homozygous (Germana, 2006). Homozygous pure lines can be obtained in a single generation via doubled haploidization, a two-step method known as haploidization and double haploidization. In the first step, haploid plants (n) with chromosome numbers the same as those in the species' gamete cells (n) are obtained. It can be induced using *in vitro* techniques (culturing male

and female reproductive cells on nutrient media in aseptic conditions) and *in situ* parthenogenesis (crossing between distant relatives, delaying pollination, pollination with abortive or irradiated pollen, chemical treatments, hormone applications, temperature shocks, etc.). In the second step, complete fertile homozygous lines or doubled haploids are obtained by doubling the chromosome numbers of the haploids (Ellialtıoğlu et al., 2021).

Haploids induced via gamma irradiation are common in vegetable breeding, particularly in some species of Cucurbits. Sauton and Dumas de Vaulx (1987), first time, successfully obtained haploid plants in melons. Later, the irradiated pollen technique was applied to cucumber (Sauton, 1989), watermelon (Sari et al., 1994), and squash (Kurtar et al., 2002). Obtention of haploid plants with irradiated pollen technique has also been studied in fruit species such as apples (Zhang and Lespinasse, 1991), pears (Bouvier et al., 1993), and cherries (Höfer and Grafe, 2003). However, it is a comprehensive application in vegetables and fruits, and there are a limited number of studies on *in situ* parthenogenesis in ornamental plant species such as petunias (Raquin, 1985; Raquin et al., 1989), rose (Meynet et al., 1994), carnation (Sato et al., 2000) iris (Grough et al., 2015).

The success of haploidy induction is affected by factors such as genetic predisposition, environment and plant growing conditions (Tütüncü et al., 2017). Induction of parthenogenesis through irradiated pollen technique differs from other haploidy techniques due to ray treatments, and determination of effects of pollination with irradiated pollen on *in vitro* culture is an important step. Therefore, this study aims to evaluate the pollen irradiation technique in cyclamen.

Material and Method

Plant Material and Irradiation of Pollen

Fifty commercial *C. persicum* plants (Maxora, Varinova, Holland) were cultivated in a plastic greenhouse. Irrigation was performed with tap water, and NPK 20-20-20+ME fertilizer (2 g/pot) was applied every two months. When needed, fungicide (Captan 50WP, Polsas¸ Chemical Co., Turkey) of a 250 g/100L concentration was applied. Flower buds were emasculated before the anthesis stage and irradiated with 50, 100, 150, 200, 300, and 450 Gy of gamma rays at 361 Gy/h using a Co^{60} source unit (Institute of Isotopes Co. Ltd., Hungary). At least twenty flower buds were irradiated for each dose, and a control group without irradiation was separated. Then, anthers were isolated and incubated in a desiccator overnight to obtain pollen grains. Emasculated flower buds were pollinated with irradiated pollen 24 h after emasculation.

In vitro Ovule Culture

Flower buds were collected at 30 DAP and washed with tap water for 20 min, then soaked in 0.1% $HgCl_2$ (mercuric chloride) for 25 min. After rinsing 3-4 times with sterile distilled water (SDW), samples were immersed in 70% ethanol for 1 min. Ethanol was removed by rinsing samples with SDW, and they were submerged in 30% Domestos® (NaOCl, 4.5% v/v) for 20 min. Finally, sodium hypochlorite was removed by rinsing the samples with SDW.

Ovules were isolated from ovaries and placed on four different tissue culture media (M). M0 was control group containing half-strength MS basal media including full-strength MS vitamin, 30 g/L sucrose, 2 g/L glucose; M1 media was M0 with the addition of 10 g/L maltose, 1.0 g/L proline, 2.0 g/L peptone, 200.0 mg/L spermidine and 0.5 mg/L kinetin; M2 media was M0 with the addition of 10 g/L maltose, 1.0 g/L proline, 2.0 g/L peptone, 200.0 mg/L spermidine, 0.4 mg/L gibberellic acid (GA_3) and 0.4 mg/L N^6-benzyl adenine (BA). M3 media was M0 with the addition of 2.0 mg/L 2,4-D and 0.8 mg/L 6-(γ, γ-dimethylallylamino) purine (2iP). All explants were cultured at 22–25°C in the dark for 8 weeks. Explants developed into shoots, embryos, or plantlets were cultured on M0 media supplemented with 0.5 g/L activated charcoal at 25 ± 2°C under a 16-h photoperiod under 75 µmol $m^{-2}s^{-1}$ cool white fluorescent lamps.

Experimental Design and Statistical Analysis

A completed randomized plot design was used as an experimental design. *In vitro* ovule culture experiment was designed with four media, seven irradiation doses, ten replicates, and four ovule explants in each replicate. Means were compared by analysis of variance, and significant differences were examined with the LSD test. Percentage values were arcsine transformed, and JMP® program (SAS Institute, Cary, NC) ver. 8.0 were used for statistical analyses.

Observation of Stoma Size and Density

A thin layer was taken from the abaxial surface of the leaf for stomatal observation. Three slides were prepared for each genotype, and a thirty stoma was observed in each slide under the light microscope. Stoma length and stoma width were measured with a software program (Digimizer 5.4.1, MedCalc Software, Belgium). Stoma density was determined by randomly selecting a 100 µm area for each slide and counting the stoma number per area. Means were separated by analysis of variance, and significant differences were examined with LSD test. Analyses were conducted with the JMP® program (SAS Institute, Cary, NC) ver. 8.0.

Results

Fruits were gathered 30 DAP (Figure 1A), and differences in ovule explants were observed (Figure 1B). Each explant showed a variable capacity to turn into callus formation *in vitro* ($P<0.01$). Friable callus was partly brownish and yellow (Figure 1C), and some callus structures turned black and hardened in the later stage of the culture. The highest (15%) and the lowest (1%) callus formation were obtained from ovules cultured on M3 and M1 media respectively in 50 Gy. Only the irradiation dose and media combinations of 50Gy+M1, 50Gy+M2, 50Gy+M3, 100Gy+M1, 100Gy+M3, and 100Gy+M3 resulted in callus induction. No growth response was observed in the control group (non-irradiated explants cultured on M0) (Table 1).

Shoot and micro bulb-like structures were formed either from callus tissues (Figure 1D) or directly from ovule explants (Figure 1E). After that, small leaves emerged from these fused structures (Figure 1F).

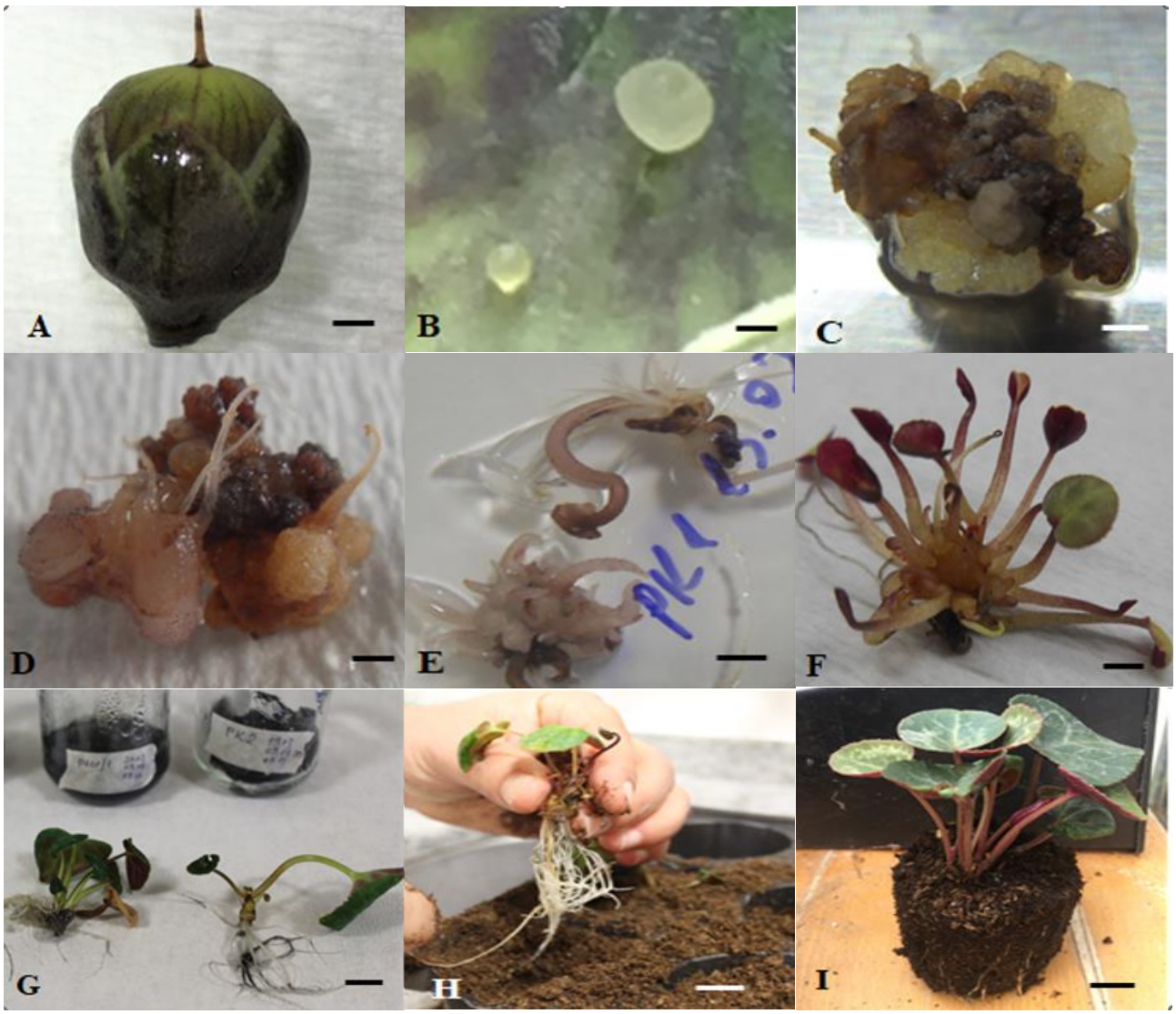

Figure 1. Developmental stages of *in vitro* regenerated plantlets. (A: Harvested fruit at 30 DAP, B: Isolated ovules, C: Friable callus tissue, D: Shoots and micro bulb-like structures regenerated from callus tissue, E: Shoots and micro bulb-like structures F: leaf structures, G: regenerated plantlets, Scale bars: 10 mm (B), 15mm (A, C, D, E, F), 1 cm (G, H, I)

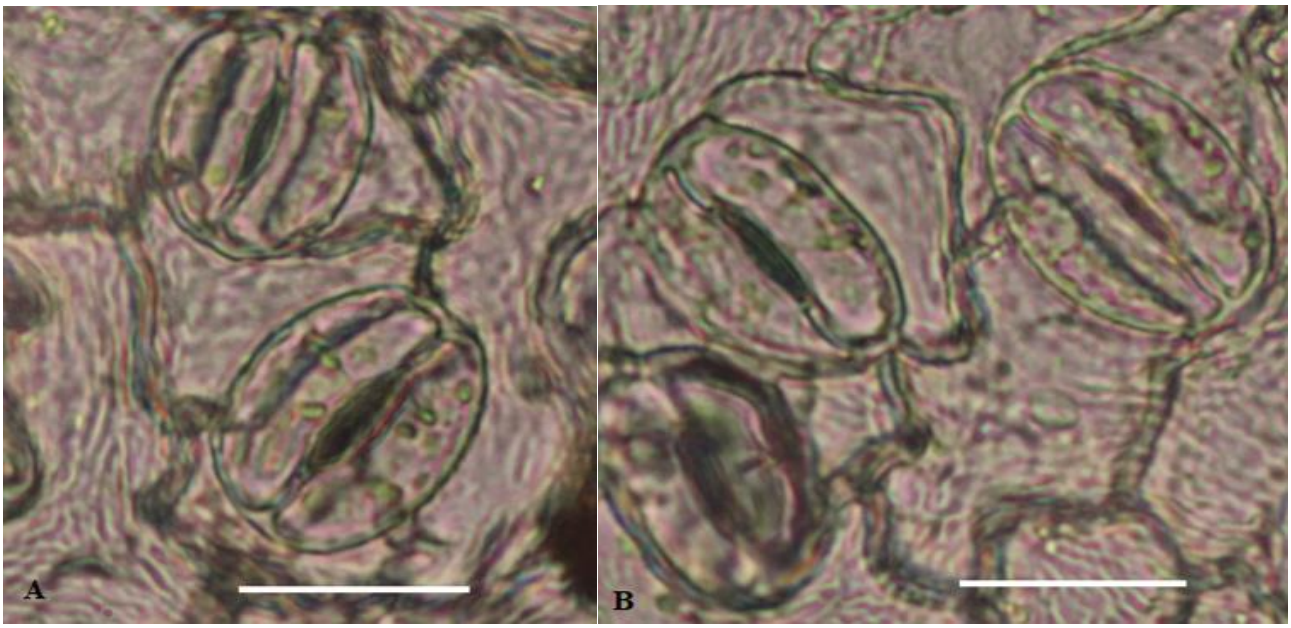

Figure 2. Stoma views of the regenerated plantlets A: control group, B: 100 Gy, scale bars: 10 µm

Table 1. Comparison effects of different media on callus formation.

Irradiation dose (Gy)	Callus induction rate (%)				Mean of dose
	M0	M1	M2	M3	
0	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}
50	0.00 (0.00)^{c}	1.00 (1.84)^{c}	11.00 (16.15)^{a}	15.0 (19.92)^{a}	6.75 (9.48)^{a}
100	0.00 (0.00)^{c}	3.00 (5.52)^{b}	0.00 (0.00)^{c}	3.00 (4.49)^{bc}	1.50 (2.50)^{b}
150	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	3.00 (4.49)^{b}	0.75 (1.12)^{bc}
200	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}
300	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}
450	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}	0.00 (0.00)^{c}
Mean of media	0.00 (0.00)^{c}	0.57 (1.05)^{bc}	1.57 (2.30)^{b}	3.00 (4.13)^{a}	

LSD_{dose}=1.583, LSD_{media}=1.197, $LSD_{dose\ x\ media}$ =3.167 (P<0.01). All values in parentheses were arcsine transformed. Statistically significan differences were shown with different letters.

Table 2. Effect of gamma irradiation and culture media (M) on plantlet regeneration from ovule explants.

Irradiation dose (Gy)	Plantlet regeneration rate (%)				Mean of dose
	M0	M1	M2	M3	
0	0.00 (0.00)d	2.66 (12.68)a	1.00 (5.52)bc	0.00 (0.00)d	0.91 (4.55)a
50	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d	0.33 (1.84)cd	0.08 (0.46)cd
100	0.00 (0.00)d	2.00 (8.99)ab	0.00 (0.00)d	0.00 (0.00)d	0.50 (2.24)bc
150	1.33 (7.37)b	0.00 (0.00)d	1.33 (6.34)b	0.00 (0.00)d	0.66 (3.42)ab
200	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d
300	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d
450	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d	0.00 (0.00)d
Mean of media	0.19 (1.05)d	0.66 (3.09)d	0.33 (1.69)d	0.04 (0.26)d	

LSD_{dose}=2.01, LSD_{media}=1.52, $LSD_{dose\ x\ media}$=4.02 (P<0.01). All values in parentheses were arcsine transformed. Statistically significan differences were shown with different letters.

Table 3. Stoma dimensions of the acclimatized plantlets obtained from *in vitro* ovule explants cultured 30 DAP.

Genotypes	Donor	50 Gy	100 Gy	150 Gy	Mean
Length (µm)	12.52	13.31	13.57	11.28	12.17
Width (µm)	9.76	8.47	10.71	9.29	9.55
Density	90	88	77	81	84

LSD_{stoma}: Not significant

When these structures were divided and cultured on M0 media supplemented with 0.5 g/L activated charcoal at 25±2°C under a 16h photoperiod, Some of them turned into plantlets (Figure 1G). The plantlets were directly regenerated from the non-irradiated explants cultured on M1 and M2 media, and the explants were exposed to 150 Gy gamma rays cultured on M0 and M2 media. On the other hand, plantlets were obtained from callus tissues regenerated from ovule explants irradiated with 100 Gy and 50 Gy gamma rays and cultured on M1 and M3 media, respectively. Plantlets started to form 10-12 weeks after the beginning of culture. Irradiation dose and their interactions were statistically significant (P<0.01). The lowest plantlet regeneration (0.33%) was obtained from ovule explants exposed to 50 Gy gamma rays on M3 media, while the highest (2.66%) was obtained from ovule explants on M1 media 30 DAP in control (Table 2).

Stomatal Observations

The stomatal observations were performed on regenerated plantlets from 0, 50, 100 and 150 Gy treatments. The visual observation under a light microscope showed similar stoma morphology (Figure 2).

The mean stoma density was found to be 84, and there were no statistical differences among treatments. Additionally, the mean stoma length was 12.17 µm, and the width was 9.55 µm. No statistical differences existed between donor plant and *in vitro* regenerated plantlets (Table 3).

Discussion

After pollination with irradiated pollen, the results of *in vitro* ovule culture showed diverse effects on callus induction and plant regeneration. *In vitro* ovule culture of *Cyclamen* was previously studied by different research groups aiming for embryo rescue after interspecific hybridization among incompatible species (Ewald, 1996; Takamura and Miyajima, 1996), somatic embryogenesis (Schwenkel and Winkelmann, 1998; Winkelmann, 2010; Koçak et al., 2014; Sevindik et al., 2017; Tütüncü et al., 2019) and micropropagation of endemic species (İzgü et al. 2016) and gene function analysis (Ratjens et al., 2018). In interspecific hybridization studies, ovules were mainly cultured on PGR- free MS media to induce embryo germination, while half-strength MS media supplemented with auxin (2,4-D) and cytokinin (2iP) were used to induce embryogenic callus regeneration from ovule explants (Winkelmann, 2010; Koçak et al., 2014; Sevindik et al., 2017 and Tütüncü et al., 2019). Our study is consistent with previous reports that the highest callus regeneration from ovule explants was observed on M3 media containing auxin (2,4-D) and cytokinin (2iP). It can be said that pollination with low doses of irradiated pollen increases callus stimulation, although the ratio of callus regeneration is lower than in previous studies.

On the other hand, the absence of callus stimulation in the control group suggested that it was due to the

regeneration ability of the plant from which the explants were taken. This situation may occur due to the genetic structure of the explant source. The physiological state in which the plant is biochemical affects the absorption of hormones from the nutrient media and cellular ability in callus regeneration from explants (Sharma and Nautiyal, 2009). Unlike previous studies, ovule culture was carried out 30 days after emasculated flower bud was pollinated. As it is known, the ripening of fruits, shedding of leaves and fruits, aging, and response to tissue injuries in plants are controlled by the ethylene hormone (Theologis, 1992; De Martinis and Mariani, 1999). The tissue injuries that occurred during the emasculation of flower buds for manual pollination may have caused the regeneration ability of explants to decrease. Indeed, ethylene production in flower buds affects ovule development and regeneration ability (Hedhyl, 2009).

There is a strong connection between stoma and ploidy level in many species such as melon (Abak et al., 1996), watermelon (Sari et al., 1999), winter squash (Kurtar and Balkaya, 2010), black wattle (Beck et al., 2003) and maize (Molenaar et al., 2019). Takamura and Miyajima (1996) reported that the stoma size of diploid and tetraploid plants was different. Therefore, the ploidy level of the donor and regenerated plants was evaluated, measuring stoma size and density. In the present study, there were no statistically significant differences in the stoma size of the regenerated and donor plants. It was thought that they had the same ploidy level. The results may explain that failure of *in situ* parthenogenesis induction is reasoned due to low regeneration frequency of ovule explants, inappropriate developmental stage, culture media, and other factors affecting the irradiated pollen technique.

Conclusion

According to our knowledge, this is the first study evaluating the irradiation pollen method in *Cyclamen*, although *in situ* parthenogenesis was not obtained. The lack of *in situ* parthenogenesis can be explained by the factors affecting the production of haploid plants with irradiated pollen. It is affected by the plant genotype, type of applied radiation, and amount of radiation dose. For future studies, 100-200 Gy gamma irradiations could be tested, and ovary culture could perform instead of single ovule culture to increase explant number and avoid damaging the integrity of ovule structure.

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