Research Article / Araştırma Makalesi

Uluslararası Tarım ve Yaban Hayatı Bilimleri Dergisi

International Journal of Agriculture and Wildlife Science

2022, 8(3): 414 – 422, doi: 10.24180/ijaws.1145144

Assessment of The Effect of Gamma Ray Treatments on Pollen Behavior in Cyclamen*

Siklamende Gama Işını Uygulamalarının Polen Davranışı Üzerine Etkisinin Değerlendirilmesi*

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Geliş Tarihi (*Received*): 18.07.2022 **Kabul Tarihi (*Accepted*):** 21.09.2022 **Yayın Tarihi (*Published*):** 15.12.2022

Abstract The aim of the study is to evaluate the effects of gamma irradiation on pollen grains of *Cyclamen persicum* L. which is a valuable ornamental pot plant. Pollens were irradiated at different doses (0, 50, 100, 150, 200, 300, 450 Gy) of gamma-ray using Co-60 source and pollen viability and *in vitro* pollen germination test were carried out and tube length of *in vitro* germinated pollens was measured. Additionally, *in vivo* pollen development was visualized by performing an aniline blue fluorescence method. Average pollen viability was ranged from 82.02% to 87.03%. The highest pollen germination rate (66.13%) was observed in the control group at 24 h after irradition, while the lowest rate (28.51%) was determined in 450 Gy treatments at 72 h after irradition. The shortest pollen tube length (48.02 µm) was observed at 72 h old non-irradiated pollen grains, while the most extended pollen tube length (79.37 µm) was determined in pollen grains irradiated with 300 Gy at 24 HAI. When the irradiation dose increases from 150 Gy to 450 Gy, inhibition on pollen germination and pollen tube elongation within the style were observed.

Keywords: Irradiation, Pollen tube elongation, germination, viability, lethal dose

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Öz: Bu çalışmanın amacı önemli bir saksılı süs bitkisi olan *Cyclamen persicum* L.'nin polenleri üzerine gama ışınlamasının etkileri değerlendirilmiştir. Polenler Co-60 kaynağı kullanılarak farklı dozlarda (0, 50, 100, 150, 200, 300, 450 Gy) gama ışını ile ışınlanmış ve polen canlılığı, polen çimlenme oranı ve *in vitro*'da çimlenen polenlerin tüp uzunluğu ölçülmüştür. Ek olarak, anilin mavisi floresan yöntemi uygulanarak *in vivo* polen gelişimi gözlenmiştir. Ortalama polen canlılığı %82.02 ile %87.03 arasında değişmiştir. En yüksek polen çimlenme oranı ışınlamadan 24 saat sonra kontrol grubunda (%66.13), en düşük oran ise ışınlamadan 72 saat sonra 450 Gy uygulamada (%28.51) belirlenmiştir. En kısa polen tüpü uzunluğu (48.02 µm) 72 saatlik ışınlanmamış polenlerde gözlenirken, en uzun polen tüpü (79.37 µm) ışınlamadan 24 saat sonra 300 Gy ile ışınlanmış polenlerde belirlenmiştir. Işınlama dozu 150 Gy'den 450 Gy'ye yükseldiğinde, polen çimlenmesi üzerinde ve stil içerisinde polen tüp uzamasında durağanlık gözlemlenmiştir.

Anahtar Kelimeler: Işınlama**,** polen tüpü uzaması, çimlenme, canlılık, letal doz

Atıf/Cite as: Tütüncü M. & Mendi N Y Y. (2022). Assessment of The Effect of Gamma Ray Treatments on Pollen Behavior in Cyclamen. Uluslararası Tarım ve Yaban Hayatı Bilimleri Dergisi, 8 (3), 414-422. Doi: 10.24180/ijaws.1145144.

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*This study respresents first author's PhD thesis titled "Siklamende (*Cyclamen persicum* L.) ışınlanmış polen yöntemiyle parthenogenesis'in *in vitro* ve histolojik teknikler kullanılarak araştırılması".

INTRODUCTION

Cyclamen is commercially cultivated worldwide for the use as ornamental pot plant. It is a perennial bulbous plant with attractive flowers and leaves (Curuk et al., 2015, Simsek et al., 2017). 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 (Winkelmann et al., 2011). Therefore, it is important to obtain superior parental lines by haploid methods. Pollination with irradiated pollen is one of the standard methods to induce in situ parthenogenesis to get haploid plants. The principle of the technique is based on hand pollination with pollens exposed to ionizing (X-ray, Gamma-ray, etc.) or non-ionizing (UV) rays to get parthenogenetic haploids. In this method, pollen germination occurs on stigma, and the pollen tube elongates to reach the embryo sac within the style. Nevertheless, fertilization cannot happen, but egg cell division and embryo development are stimulated (Grough et al., 2015).

The applications of irradiated pollen began with Rontgen's discovery of X-rays. The first studies investigated the effects of irradiation on pollen germination and pollen tube elongation. The production of haploid plants with irradiated pollen was first performed in the *Triticum monococcum* species. This technique has been successfully applied to other economically important species. In the latest studies, gamma rays are widely used to irradiate pollens due to their simple application, efficient penetration, repeatability, high mutation frequency, and low lethality of the samples (Kurtar and Balkaya, 2010). There are different opinions about the effect of radiation on parthenogenic development. However, these views agree that pollination with irradiated pollen mainly consists of cytological abnormalities during mitotic divisions during pollen tube formation and deviations in the normal sexual fertilization process. A haploid embryo or haploid endosperm nucleus is formed after pollination with irradiated pollen is explained in two different ways. The first of these; is the fertilization of the egg cell by the damaged male reproductive cell by eliminating the chromatin from the cytoplasm. The second opinion is the stimulation of the egg cell by the male reproductive cell with a pycnotic nucleus (Sestili and Ficcadenti, 1996).

Although the factors affecting haploid plant production with irradiated pollen are similar to other haploidization techniques, they show differences due to irradiation. Therefore determination effects of radiation doses on pollen behaviour is one of the most important factor in in situ parthenogenesis. In this study, we aimed to evaluate the effects of different gamma irradiation doses (Co60) on pollen viability, pollen germination, pollen tube development within the style.

MATERIAL AND METHOD

Irradiation of Pollen and Pollination with Irradiated Pollens

C. persicum plants (Maxora, Varinova, Holland) were cultivated in a plastic greenhouse in Ondokuz Mayis University, Faculty of Agriculture in Türkiye for the duration of the experiments. Unopened flower buds were emasculated two days before the anthesis stage early morning. For the emasculation process, the petals of the flowers were held tightly with the thumb and forefinger so that they would not be crushed, and the receptacle was fixed by holding it with the thumb and forefinger finger of the other hand without crushing it. Afterwards, the petals were gently moved back and forth and sideways to separate the petals from the point where the inner anthers were attached to the flower base. In the last stage, the petals are pulled upwards by turning clockwise and in this way they are separated from the receptacle without damaging the ovary and stigma. Then petals with inner anther were transported to the Turkish Energy, Nuclear and Mineral Research Agency (Ankara, Türkiye) in the cool box (+4 °C). They were irradiated with 50, 100, 150, 200, 300, 450 Gy of gamma rays at 361 Gy/h using a Co60 source unit (Institute of Isotopes Co. Ltd., Hungary). Petals with inner anthers from at least twenty flower buds for each irradiation dose were irradiated, and a control group without irradiation was separated. Anthers were isolated from petals after irradiation and incubated in a desiccator overnight at 30 °C and 75% RH to obtain pollen grains. Finally emasculated flower buds were pollinated with irradiated pollen grains 24 h after emasculation.

Pollen Viability, In Vitro Pollen Germination, and Pollen Tube Growth

Pollen viability and pollen germination tests were carried out with irradiated pollen grains according to the protocol reported by Tütüncü and Mendi (2020). 2,3,5-Triphenyltetrazolium chloride (TTC, 0.1%) were used for pollen viability tests. TTC solution was dropped on a microslide. Then, a small amount of pollen were distributed homogeneously in the drop, and a coverglass was applied. Incubation of the samples were performed at 30±2 °C in the dark. After 60 min incubation period, pollen rates were estimated counting viable pollens under a light microscope. Four microslides were used for each replication and viability test was repeated twice. In each experiment, pollen grains in randomly selected at least four areas (1x1 mm) in each microslide were counted. A basic media supplemented with sucrose (10%) and solidified with 1.0% agar were used for in vitro pollen germination. Pollens were distributed with paintbrush on solid media and incubated for 60 min at 30±2 °C. Then, the germinated pollen grains were counted under a light microscope (DM1000, Leica, Wetzlar, Germany) and the germination test repeated twice in three Petri dishes for each replication. Three square (1×1 cm) slices were cut out randomly from the media for each Petri dish (90 × 15 mm), and four areas (1×1 mm) for each slide were randomly selected and analyzed. The photographs of the elongated pollen tubes which are at least three times longer than their diameter, from the each area were taken, and tube length was measured with a software program (Digimizer 5.4.1, MedCalc Software, Belgium). The remaining pollen grains were stored within the lightproof boxes at +4 °C and pollen viability and in vitro pollen germination tests were repeated at 24, 48 and 72 hours after irradiation (HAI) to evaluate irradiation effect.

In Vivo Pollen Tube Growth

The pollen tube growth (PTG) within the stylus were analyzed using the aniline blue fluorescence method according to Karabiyik et al. (2018). Emasculated flower buds were pollinated with irradiated pollens (50, 100, 150, 200, 300, 450 Gy γ-ray). The pollinated flower buds were harvested 24 h intervals after pollination until the 9th day. A three flower buds were collected for each time-dose combination and stored in FPA-70 solution (formaldehyde-propionic acid-alcohol) at +4 °C until analysis. Firstly, flower buds were rinsed under tap water overnight to remove FPA-70 and then transferred into softening solution (NaOH, 8N) for 8 hours. Flower buds rewashed overnight under tapwater before staining step. An aniline blue stock solution (0.4%) was prepared by dissolving 1 g aniline blue diammonium salt (Sigma-Aldrich, USA) and 11.28 g tri-potassium phosphate (Sigma-Aldrich, USA) in 250 distilled water. The staining solution was prepared by diluting stock solution with distilled water (1:3 v/v) before staining. Thereafter, samples were immersed to staining solution and incubated at 4°C for three days. At the last step, pistils were cut longitudinally from the mid-axis, and transferred to a slide to scan PTG under a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

RESULTS

Pollen Viability, In Vitro Pollen Germination, and Pollen Tube Growth

The effects of gamma rays irradiation were evaluated to reveal pollen viability at 24, 48 and 72 h after irradiation (HAI). 24 h after irradiation (HAI), stainability of the pollen grains with TTC was ranged from 78.00 to 88.67 % at 450 Gy and 50 Gy irradiation doses, respectively. At 48 HAI, the irradiated pollen grains' viability rate increased in 300 and 450 Gy treatments and the highest rate (89.25%) was observed in 300 Gy. However, pollen viability slightly decreased at 24 HAI and 72 HAI as the irradiation dose increased. A similar decrease compared to 48 and 24 HAI was determined at 72 HAI, except for 450 Gy treatment. The viability rate of the pollen irradiated with 450 Gy increased at 48 HAI, then viability rate placed between 24 and 48 HAI levels (Table 1).

The irradiated pollen grains' *in vitro* germination ability was determined at 24, 48 and 72 h after irradiation and *in vitro* germination rates were lower than the stainability of the pollen grain with TTC to overall results. The germination rates of the non-irradiated pollen grains were higher than that of irradiated pollen. The highest pollen germination rate (66.13%) was observed in the control group at 24 HAI, while the lowest rate (28.51%) was determined in 450 Gy treatments at 72 HAI. *In vitro* germination rates decreased continuously depending on the increase of irradiation doses and the elapsed time after irradiation treatments. The percentage of the germinated pollen was approximately 50% for 150 Gy doses at 24, 36 and

72 HAI and germination rates were found to be below 50% for 200, 300 and 450 Gy doses at 24, 36 and 72 HAI (Table 2).

Table 1. Pollen viability rates at 24, 48 and 72 hours after irradiation (HAI).
Çizelge 1. Işınlamadan 24, 48 ve 72 saat sonraki polen canlılık oranları.

Irradiation doses (Gy)	Pollen viability rate (%) ± Stdv.			
	24 HAI	48 HAI	72 HAI	Mean
0	83.46 ± 2.75	82.67 ± 6.28	79.98 ± 2.98	82.03 ± 4.00
50	88.67 ± 1.72	85.59 ± 3.16	79.04 ± 7.27	84.43 ± 4.05
100	87.05 ± 2.39	86.46 ± 1.70	87.58 ± 1.54	87.03 ± 1.87
150	83.25 ± 1.50	82.41 ± 1.70	81.01 ± 3.56	82.22 ± 2.25
200	87.51 ± 2.34	84.48 ± 5.18	86.60 ± 0.88	86.19 ± 2.80
300	84.93 ± 3.93	89.25 ± 2.34	81.54 ± 1.64	85.24 ± 2.63
450	78.00 ± 5.64	87.45 ± 3.79	83.19 ± 2.24	82.88 ± 3.89

Table 2. *In vitro* germination percentage pollen grains in 24, 48 and 72 h after irradiation (HAI).
Çizelge 2. *Işınlamadan 24, 48 ve 72 saat sonraki in vitro polen çimlenme oranları.*

Irradiation doses (Gy)	Pollen germination (%) ± Stdv.			
	24 HAI	48 HAI	72 HAI	Mean
0	66.13±3.54	65.19±3.14	65.40±2.30	65.57±2.99
50	63.19±4.22	61.18±4.86	60.09±1.95	61.48±3.67
100	60.78±3.85	60.24±1.18	59.47±2.11	60.16±2.38
150	50.58±1.66	51.25±1.86	49.72±3.05	50.52±2.19
200	50.63±2.01	48.91±1.22	46.78±1.32	48.77±1.52
300	46.94±2.40	43.63±1.18	40.56±3.64	43.71±2.40
450	35.03±1.71	30.92±1.78	28.51±1.74	31.48±1.74

In vitro pollen tube growth determined measuring tube length *in vitro* germinated pollens 24, 48 and 72 h after irradiation. The effects of irradiation doses and HAI on pollen tube length were statistically significant ($P<0.01$). The shortest pollen tube length (48.02 µm) was observed at 72 hours old non-irradiated pollen grains, while the most extended pollen tube length (79.37 µm) was determined in pollen grains irradiated with 300 Gy at 24 HAI (Table 3). The pollen tube lengths increased in parallel with the increase in irradiation doses except for 450 Gy. Moreover, pollen tube length was restricted by the duration after irradiation treatments.

Table 3. Comparison *in vitro* pollen tube elongation in 24, 48 and 72 HAI.
Çizelge 3. *Işınlamadan 24, 48 ve 72 saat sonra in vitro polen tüpü uzamasının karşılaştırılması.*

Irradiation doses (Gy)	Pollen tube length (µm)			
	24 HAI	48 HAI	72 HAI	Means of doses
0	50.55 hij	50.24 ij	48.02 j	49.60 d
50	54.68 g-j	53.25 g-j	55.81ghi	54.58 c
100	64.74 de	66.2 cd	55.62 ghi	62.19 b
150	56.19 f-i	53.58 g-j	63.53def	57.77 c
200	66.64 cd	67.87 cd	58.02 e-h	64.18 b
300	79.37 a	78.89 a	58.9 efg	72.12 a
450	73.34 abc	75.57 ab	69.73 bcd	72.88 a
Means of HAI	63.65 a	63.66 a	58.40 b	

LSD_{dose}=4.325, LSD_{HAI}=2.831, $LSD_{dose \times HAI}$ =7.491 ($P<0.01$), Different letters within a column (for each HAI: hours after irradiation) or within a row (for the mean of irradiation doses) indicate significant differences.

In Vivo Pollen Tube Growth

The aniline blue fluorescence method was used to evaluate pollen tube growth within the style after pollination with irradiated pollen grains. Pollen grains in the control group (non-irradiated) started to germinate on stigma from the first day after the pollination, and the pollen tube elongated within the style (Figure 1A-1B: germination of non-irradiated pollen grains on stigma at 1 DAP and 4 DAP respectively, 1C: elongation pollen tube within the style at 4 DAP). Elongation of the pollen tubes continued during the first week after pollination and reached ovule at 8 DAP (Figure 1D). Similar growth behaviors were determined for pollen germination, pollen tube elongation, and duration for pollen tubes to reach ovules, along with the increasing dose from 0 to 150 Gy of gamma radiation.

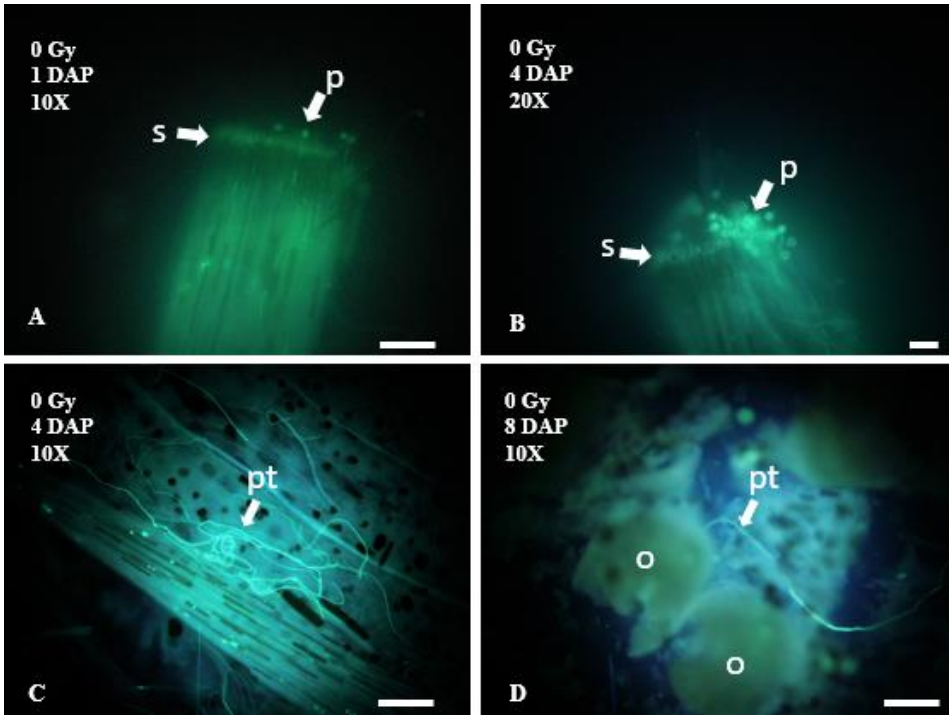

Figure 1. Pollen tube development of non-irradiated pollen grains (o: ovule, p: pollen, pt: pollen tube, s: stigma, scale bars: 100 µm).

Şekil 1. Işınlama yapılmayan pollerin polen tüpü gelişimi (o: ovül, p: polen, pt: polen tüpü, s: dişicik tepesi, ölçü çizgisi: 100 µm).

At the dose of 150 Gy, pollen grains started to germinate on stigma at 1 DAP, and the pollen tube started to elongate within the style (Figure 1A). Although pollen tubes elongated within the style from the first day after pollination, it was observed that the pollen tube did not reach the ovary at 4 DAP (Figure 2B). At 8 DAP, it has been seen that the pollen tube elongated through the ovary and the pollen tube reached the ovule (Figure 2C, 2D).

A small amount of pollen germination on stigma was observed at the higher doses (200, 300 and 450 Gy). When the irradiation dose increases from 150 Gy to 450 Gy, inhibition on pollen germination and pollen tube elongation within the style were observed. At the dose of 450 Gy, a few germinated pollen grains were determined on stigma (Figure 3A), but any elongated pollen tube was not observed within the style or ovary (Figure 3B-3C: longitudinal section of style at 4 DAP, Figure 3D: ovary and ovules at 8 DAP).

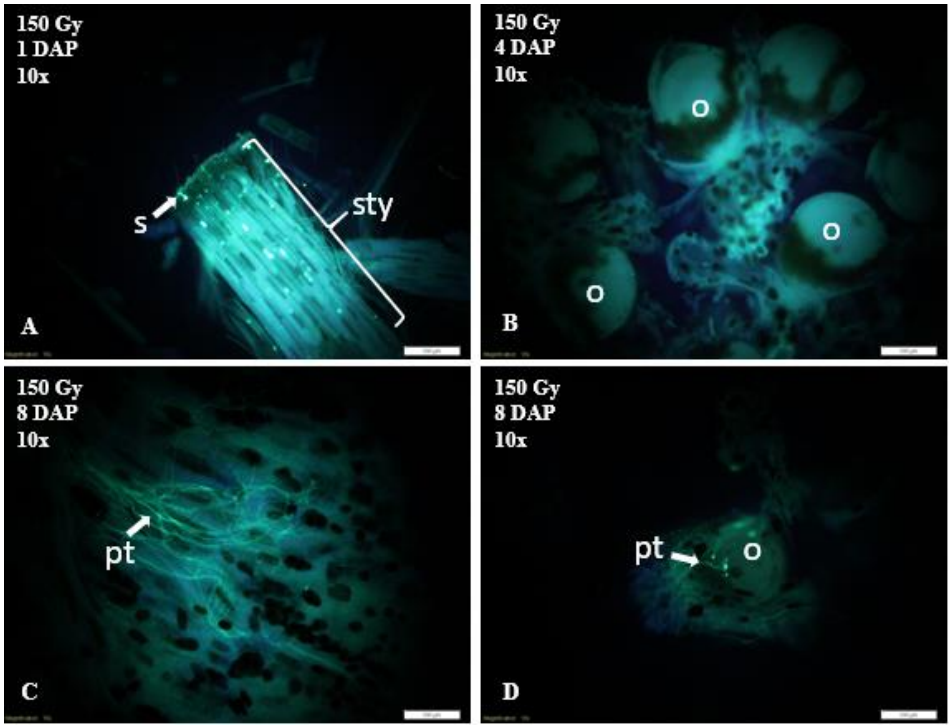

Figure 2. Pollen tube development of pollen grains irradiated with 150 Gy gamma rays (o: ovule, p: pollen, pt: pollen tube, s: stigma, sty: style, scale bars: 100 µm).

Şekil 2. 150 Gy gama ışını uygulanan polenlerde polen tüpü gelişimi gelişimi (o: ovül, p: polen, pt: polen tüpü, s: dişicik tepesi, sty: dişicik borusu, ölçü çizgisi: 100 µm).

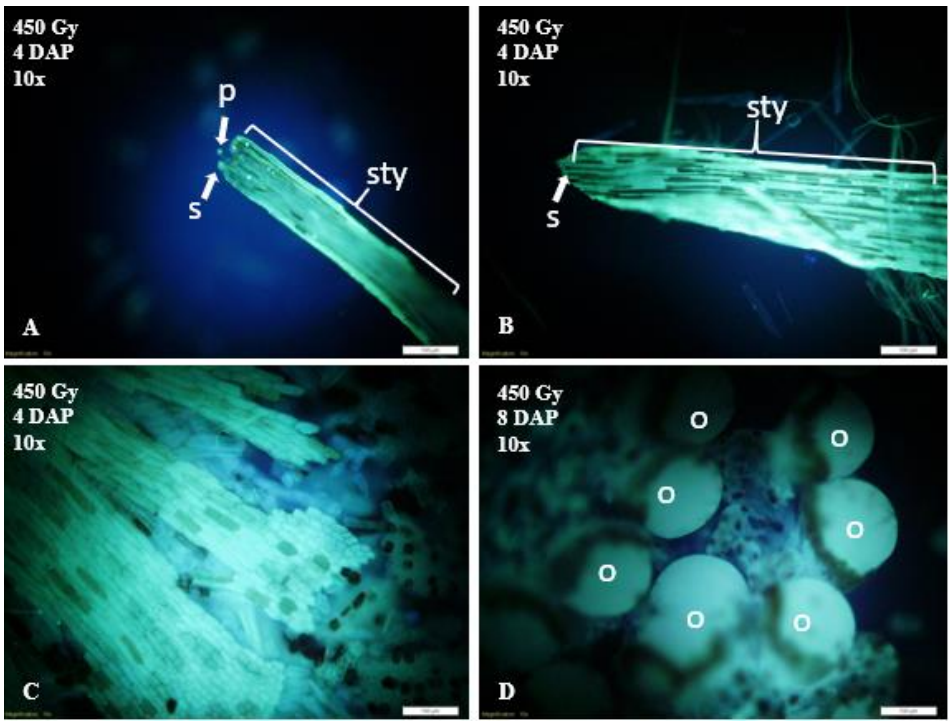

Figure 3. Pollen tube development of pollen grains irradiated with 450 Gy gamma rays (o: ovule, p: pollen, pt: pollen tube, s: stigma, sty: style, scale bars: 100 µm).

Şekil 3. 450 Gy gama ışını uygulanan polenlerde polen tüpü gelişimi gelişimi (o: ovül, p: polen, pt: polen tüpü, s: dişicik tepesi, sty: dişicik borusu, ölçü çizgisi: 100 µm).

DISCUSSION

The pollen viability rate decreased from the irradiation doses increased from 50 Gy to 200 Gy, while an increase was observed in pollen viability in 300 and 400 Gy doses at 24 HAI compared to the non-irradiation group. The pollen viability were found to be lower than the initial values in all treatments except control and 50 Gy. A steady decline were observed in control and 50 Gy. Schwartz-Tzachor et al. (2007) reported that pollen viability gradually decreased in *C. persicum* in the following days of their research. The highest survival rate was reported to be approximately 80% in 1-day-old pollens. On the 4th day, it was seen that the pollen vitality decreased to 70-75%. Additionally, Cordea and Triplica (2019) examined the pollen viability rates in 7 different cyclamen varieties and found that the pollen viability rates were between 85-95%. According to the previous studies, it is seen that the pollen viability rate in cyclamen is higher than 70%, and the results are consistent with the results of the control group of our study.

Akhtar (2014) reported that 5 kr gamma irradiation dose in tomato cv. 'Rio Grande' increased the pollen viability rate compared to the control. Kurtar (2009) reported viability rate of non-irradiated pollens in squash was 90%, this rate decreased as the irradiation dose increased and the pollen viability rate decreased to 17% in the 300 Gy dose applications. On the other hand, no significant differences were observed in different irradiation doses on pollen viability in 'Majia' pomelo by Yang et al. (2020) and trifoliate orange by Wang et al. (2016). These results suggest that increase or decrease in pollen viability is primarily due to the different reactions of plant species, cultivars or genotypes according to the amount and duration of the applied irradiation dose.

The pollen germination rate is relatively lower than the pollen viability rate in non-irradiated pollens. Cordea and Triplica (2019) reported that the lower *in vitro* germination rate than pollen viability was 4-20% in seven cultivars of *C. persicum*. These results were consistent with our results. However, the mean of the pollen germination rates decreased as the radiation dose increased from 0 to 150 Gy, and the germination capability of the pollen fell below 50% in irradiation applications above 150 Gy. The reduction in pollen germination also occurred at 48 and 72 HAI suggesting irradiation dose and time after pollen exposed irradiation were detrimental for pollen grains. Pollen viability and germination are affected by many factors. Besides the development period of the anthers, the relative humidity, the amount of oxygen and carbon dioxide and the ambient temperature are other factors that determine the viability and germination capability of the pollen (Sidhu, 2019). Additionally, Ali et al. (2015) stated that different doses of gamma radiation in various crops causes an increase in cellular enzymatic activity. It may suggest that differences in pollen vitality and germination rate may also result from cellular stress caused by radiation applications.

In vitro pollen tube length was strictly affected by gamma radiation. Pollen tube length increased as the radiation dose increase from 0 to 300 Gy. It was observed that the pollen tube length tended to decrease at 450 Gy gamma radiation. In a previous study conducted by Yiğit (2008) in apple a positive correlation between pollen tube length and radiation level was reported. Pandey and Kumar (2013) reported that germination rates in pollen increased by irradiation dose up to 300 Gy in *Linum*. At the 400 and 500 Gy doses, a decrease in pollen viability, germination rates, and pollen tube length was determined. Therefore, the effects of irradiation doses on *in vitro* pollen tube length may differ among plant species in a dose-dependent manner. Additionally, Stephan (2021) indicated that ionization radiation affects pollen tube growth and nuclear function. Ionization radiation causes dramatic impairment of the genome entity, but its effects on polar growth are minor.

CONCLUSION

The results showed that irradiation doses and time after irradiation affect pollen viability, germination and pollen tube length. Although increasing radiation doses up to 300 Gy had a positive effect on *in vitro* pollen tube length but limited the development of the pollen tube within the style. Pollen tube formed in the first two days within the style after pollination with irradiated and non-irradiated pollen and reached the ovary in 5-7 days. However, in increasing doses from 150 Gy to 450 Gy, it was observed that the number of pollen tubes reaching the ovary decreased or could not reach the ovary due to the retention of the pollen tube within the style after germination. This limitation in pollen tube development within the style is related to

irradiation doses and detrimental effects of the radiation. It can be inferred that 150 Gy gamma irradiation is LD_{50} dose for pollen grains in our study considering germination and *in vivo* pollen development. Therefore, *in vivo* pollen tube development could be inhibited in gamma irradiation above 150 Gy. On the other hand, the inhibition of *in vivo* pollen tube development above 150 Gy while *in vitro* pollen tube length increases are thought to be due to the pollen-pistil interactions are genetically regulated. Another possible explanation could be the inhibition of novel RNA synthesis, required for continued pollen tube growth, due to disruption of the genome by radiation.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DECLARATION OF AUTHOR CONTRIBUTION

Mehmet Tütüncü and Yeşim Yalçın Mendi designed the study, participated in experiment, and drafted the manuscript.

ACKNOWLEDGMENT

This study supported by The Scientific and Technological Research Council of Turkey (TUBITAK; project number 118O728). We thank for the technical support during gamma irradiation to Dr. Yaprak Taner Kantoğlu (Turkish Energy, Nuclear and Mineral Research Agency) and Prof. Dr. Sinan Eti and Dr. Şenay Karabıyık for technical support during histological analysis.

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