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CEMALETTİN Y. ÇİFTÇİ

ASLI DİVANLI TÜRKAN

KHALID MAHMOOD KHAWAR

MEHMET ATAK

SEBAHATTİN ÖZCAN

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Cemalettin Yaşar ÇİFTÇİ[1], Aslı DİVANLI TÜRKAN[2], Khalid Mahmood KHAWAR[3*],
Mehmet ATAK[4], Sebahattin ÖZCAN[1]

[1]Department of Field Crops, Faculty of Agriculture, University of Ankara, Dışkapı, Ankara - TURKEY

[2]Central Field Crops Research Institute, Ministry of Agriculture and Rural Affairs, Eskişehir yolu, Ankara - TURKEY

[3]Central Laboratory, Institute of Biotechnology, Behind Rektorluk, University of Ankara, 06000, Tandoğan, Ankara - TURKEY

[4]Department of Field Crops, Faculty of Agriculture, Mustafa Kemal University, Hatay - TURKEY

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Abstract: The study reports a 3-step optimization to find out the effects of cytokinins BAP and TDZ on seed germination and γ rays to induce mutations in 4 pea cultivars: Winner, Sprinter, Bolero and Karina. It was observed that germination was independent of the dose of γ rays and was mainly affected by the germination capability of the genotypes and doses of BAP and TDZ in the germination medium. Seed germination was better on germination medium containing 50 µM BAP than 10 µM TDZ. Variable rooting was observed on shoots obtained from non-irradiated seeds germinated on MS medium containing 50 µM BAP. However, shoots of 2 cv. Winner irradiated with 60 Gy and Karina irradiated with 140 Gy γ rays and germinated on 50 µM BAP showed reduced rooting.

Key Words: Cytokinins, Gamma radiations, mutation, pea, *Pisum sativum* L, seed germination.

Abbreviations: γ radiations: gamma radiations, BAP: 6 Benzylaminopurine, TDZ: Thidiazuron, NAA: α Naphthalene acetic acid, IBA: Indole 3 butyric acid.

Gamma Işınlarının Bezelye Çeşitlerinde Mutagenik Etkileri

Özet: Bu çalışmada Winner, Sprinter, Bolero ve Karina bezelye çeşitlerinde üç aşamalı optimizasyon ile sitokininler (BAP ve TDZ)'in tohum çimlenmesinde ve gamma ışınlarının gelişen sürgünlerde meydana getirdiği mutasyon belirlenmiştir. Çimlenmenin gamma ışınlarıyla bağlantılı olmadığı bezelye çeşitlerinin çimlenme kabiliyetleri ile çimlenme ortamında BAP ve TDZ dozları ile bağlantılı olduğu görülmüştür. Tohum çimlenmesinde BAP içeren MS ortamının, TDZ içeren MS ortamından daha uygun olduğu tespit edilmiştir. 50 µM BAP içeren MS ortamında ışınlanmamış tohumlardan gelişen sürgünlerde değişik oranda köklenme görülmüştür. Fakat Winner çeşidinin 60 Gy γ ışın ile muamele edilmış tohumlarında, Karina çeşidininin ise 140 Gy γ ışın ile muamele edilmiş tohumlarından 50 µM BAP içeren MS ortamda elde edilen sürgünlerde az miktarda köklenme görülmüştür.

Anahtar Sözcükler: Sitokininler, Gamma ışınları, mutasyon, bezelye, *Pisum sativum* L, tohum çimlenme.

Introduction

Pea (*Pisum sativum* L.) is among the 4 important cultivated legumes next to soybean, groundnut and beans in the world (1) and ranks fifth in terms of importance among food legumes in Turkey (2). It is a cheap source to meet the protein requirements of a large majority of the population. The role of plant breeding in increasing food production and sustainable nutrition is well recognized (3).

Over the past 50 years, the plant varieties coupled with improved management and agronomic inputs have made a significant increase in the yield of major crops (4). Besides conventional breeding techniques, mutation breeding is also used as an alternative for improvement of desired characters in agricultural crops. This is based on creation of variations, selection, evaluation and multiplication of desired genotypes. The use of nuclear techniques directed for inducing mutations is one of the most important ways to achieve the objective and their use has become an established technology for breeding of new varieties. Many crops with improved economic value have been obtained using induced mutation (5-7). Besides the economic benefits, some mutants also play an

important role in the study of genetics and plant development (8,9).

Although advantages are evident, there are surprisingly few reports describing induced mutations from seeds under in vitro conditions (3,10-16). Mutagens have been applied to suspension cultures, callus, and embryo cultures in many species including carrot, maize, rice, wheat, and tobacco.

The availability of efficient seed germination system after irradiation is crucial in achieving successful mutagenesis. The major advantage of inducing mutations under in vitro conditions is that many varieties can be exposed to mutagens for reliable screening in a relatively small space, which can save time, money and space compared to growing thousands of plants in the greenhouse or field. The study reports 3 different stages of optimization for creation of irradiation based mutagenesis in pea.

Materials and Methods

The humidity level of the seeds of 4 pea cultivars (commonly cultivated in Turkey) was determined before irradiation by the method described by Stanwood and McDonald (17). It showed humidity percentages of 5.1%, 4.7%, 5.0% and 6.6% for the cultivars (cv.) Winner, Karina, Sprinter and Bolero respectively. The seeds were irradiated with γ rays at the Türkiye Atom Enerjisi Kurumu, Ankara, derived from Co ^{60} source. The dosage was 2800 Gy h^{-1}. A completely randomized design was used with 5 treatments (60, 100, 140 and 180 Gy of γ rays) to determine LD_{50} at the first stage. The irradiated seeds were germinated in Jacobson trays at room temperature (18- 20 °C) using moist filter papers. The seeds that were not subjected to γ rays served as controls.

At the second stage, the seeds were germinated on MS medium (18) containing 5-10 µM TDZ (Thidiazuron) and 25-50 µM BAP (^{6} benzylaminopurine) to optimize the best concentration of TDZ and BAP for seed germination and shoot regeneration. They were sterilized with 50% H_2SO_4 for 1 min followed by treatment with 100% commercial bleach (Axion, Turkey, containing 5%-6% NaOCl) for 10 min 3 times, rinsing with sterile distilled water.

During the third stage selection, the extent of mutations created by 60 and 140 Gy γ rays treated seeds (optimized during the first stage) was evaluated by germinating these on MS medium containing 10 µM TDZ and 50 µM BAP (optimized during the second stage). The seeds that were not irradiated but cultured on BAP or TDZ served as controls.

Rooting

The pea shoots (0.5-1 cm) obtained during the second stage were rooted on MS medium containing different concentrations of 5-9.8 µM Indole 3 butyric acid (IBA) and 1-2.5 µM α Naphthalene acetic acid (NAA) to determine the best rooting media. Regenerated shoots from the third stage experiments were rooted on the MS medium containing 2.5 µM NAA optimized during the second stage. All rooting experiments were carried out in Magenta vessels or baby jars. The rooting observations were recorded 4 weeks after culture in each case.

For the classification of mutant phenotypes, they were subdivided into yellow, albino and variegated (mixture of albino and green). A seedling was considered mutant if it had one or more leaves that entered the above classification.

All cultures in the second and third stage selection including rooting were incubated under 16 h light photoperiod (42 µ Mol $m^{-2}s^{-1}$) provided by soft fluorescent light (Sylvania grolux™) at 24 ± 2 °C.

Statistical analysis

First, second and third stage selection experiments contained 4 replications with 5 explants in each replication and were repeated twice. However, the rooting experiment contained 4 replications with 4 explants in each treatment and was repeated twice. Phenotypic changes were recorded weekly and analysis was based on randomized complete block design, after 2 months of growth in vitro. Data given in percentages were subjected to transformation by arcsin (√X) method (19) prior to statistical analysis of variance (ANOVA) using SPSS for Windows (v. 11. SPSS Inc, USA). Post hoc tests were performed using Duncan's multiple range test.

Results and Discussion

First stage selection

Treated seeds were evaluated for lethality from different doses of gamma irradiations. It was observed

that seed germination was independent of dose of γ rays and was mainly affected by the germination capability of the genotypes, in agreement with Savaşkan and Toker (20). Gamma irradiations had an insignificant effect on germination of all genotypes having germination frequency of 63% to 97%. In the case of Winner and Karina, an increase in germination was recorded at 180 Gy γ irradiations compared to the control. In contrast, a decrease in germination was recorded from Bolero and Sprinter with all doses of irradiations compared to the control (Table 1).

It was not possible to determine LD_{50} on the basis of germination because of similar germination in all treatments; therefore, lethality due to morphological abnormalities like decreased seedling height and the ability of tissue (seed) to regenerate into visibly normal or mutant phenotypes was taken into account. It was found that each increase in the dose of γ irradiation was accompanied by a corresponding decrease in the plantlet height and root length (Table 1) in all genotypes compared to the control along with production of chlorophyll deficient mutants, which is a common phenomenon in monocots (21) and dicots (22-24). In this experiment, the appearance of yellow, albino (chlorophyll deficient) phenotypes or variegated was first observed at 140 Gy in all genotypes. The albino seedlings were able to grow photoautotrophically and survived ex vitro acclimatization, but the yellow ones were not able to survive under ex vitro conditions. It is speculated that the survival was more or less dependent on the absolute amounts of total chlorophyll a and chlorophyll b possessed by the plant, in agreement with earlier studies (25-27). Koh and Davies (27) further observed similar results when they irradiated *Tillandsia fasciculate* Swartz var. *fasciculate* (Bromeliaceae) with 120 Gy gamma rays. Our results are also in agreement with Savaşkan and Toker (20), who found that shoot and root length decreased considerably in rye (*Secale cereale* L.) subjected to gamma irradiations with sharp effects on the development of roots compared to shoots.

Second stage selection

Surface sterilization with 50% sulfuric acid followed by treatment with NaOCl resulted in tearing of the thin and delicate seed coats of Sprinter and Bolero, which

Table 1. Effect of gamma radiations on frequency (%) of seed germination, plantlet height (cm) and root length (cm) of 4 pea cultivars Jacobson tray.

Gamma irradiation (Gy)	Cultivars					
	Winner			Bolero		
	Frequency (%) of seed germination	plantlet height (cm)	Root length (cm)	Frequency (%) of seed germination	plantlet height (cm)	Root length (cm)
Control	80[1]	2.82 a*	4.46 a	93	5.55 a	10.52 a
60	80	1.63 b	4.10 ab	83	1.16 b	3.53 b
100	83	1.51 b	3.45 b	70	0.80 c	2.36 c
140	80	1.04 bc	2.90 c	63	0.70 c	1.98 d
180	90	0.90 c	2.94 c	73	0.54 c	1.77 d
Gamma irradiation (Gy)	Cultivars					
	Sprinter			Karina		
	Frequency (%) of seed germination	plantlet height (cm)	Root length (cm)	Frequency (%) of seed germination	plantlet height (cm)	Root length (cm)
Control	97	5.51 a	7.88 a	87	7.63 a	7.17 a
60	90	1.62 b	5.31 b	90	1.91 b	3.56 b
100	90	1.21 b	4.43 c	67	1.48 b	2.53 bc
140	90	0.71 c	3.42 d	83	1.10 b	1.85 c
180	87	0.47 c	2.77 d	93	1.13 b	1.89 c

*Values within a column followed by different letters are significantly different at 0.01 level of significance.

affected the seedling development negatively. No tearing of the seed coat was observed in Winner and Karina having a thick seed coat.

Seeds cultured on germination medium containing MS supplemented with 50 µM BAP (Figure 1a) were superior to those cultured on 10 µM TDZ in terms of germination, shoot regeneration, rooting and morphological appearance (Table 2). Seed germination on MS medium without growth regulators was not attractive and resulted in formation of single shoots. Multiple shoots were observed when the germination medium contained BAP or TDZ (with the best results on 50 µM BAP and 10 µM TDZ in respective cytokinins).

Moreover, 10 µM TDZ regenerated shoots of Winner and Karina were abnormally dark green and thick compared to those regenerated on 50 µM BAP. No germination was observed in Sprinter, and the germination potential of Bolero increased slightly when the seeds were germinated on 10 µM TDZ. In general, TDZ was inhibitory towards shoot growth and development. Development of shoots with normal leaves is of the greatest significance in tissue culture and mutagenesis.

The results are in agreement with Malik and Saxena (28), who found that medium without growth regulators resulted in the formation of single shoots. He was able to get only 1 shoot from 600 seeds except for 2 exceptions with 2 shoots.

Shoots regenerated on different cytokinins may have variable or negative effects on rooting (28,29). Therefore, to compare the effects of BAP and TDZ regenerated shoots on rooting, they were rooted on MS medium containing different concentrations of IBA and NAA. 2.5 µM NAA was found more effective to root the regenerated shoots compared to any concentration of IBA. The highest frequency of 40% rooting was recorded on shoots of seeds germinated on MS medium containing 50 µM BAP and 25% rooting on shoots of seeds germinated on 10 µM TDZ, after 4 weeks of culture (Table 2). Reduced rooting is in agreement with Malik and Saxena (28), who report that if the shoots remain growing on medium containing TDZ for more than 2-3 weeks they are difficult to root. Our study suggests better rooting on NAA compared to IBA with the best rooting on 2.5 µM NAA in line with the results reported by Polanco et al. (30) and Ahmad et al. (31) on lentils, and by Malik and Saxena (28), Lumsden et al. (32) and Griga et al. (33) on pea. Polanco et al. (30) also report that BAP is effective in shoot regeneration of lentils, but affects rooting negatively.

Özcan (34) found that 4.9 µM IBA was effective for the rooting of pea. Similarly, rooting was achieved by Barna and Wakhlu (35) on pea with 1 µM IBA, and by Khawar and Özcan (36) on lentils with MS medium containing 0.25 mg l^{-1} (1.225 µM) IBA.

Third stage selection

Analysis of variance showed that 60 and 140 Gy γ rays treated seeds of Winner, Karina, Sprinter and Bolero showed variability in terms of frequency of germination ($P < 0.01$) and similarity in terms of number of shoots per explant, shoot length and morphology of the leaves and aerial parts (Table 4).

The results further show that 60 Gy irradiation resulted in increased frequency of germination (Table 3), number of shoots per explant and shoot length (Table 4) of Winner and Bolero. They had a large majority (66.67%) of green leaves, whereas the remaining ones were either albino or variegated (a mixture of albino and green). Although germination was observed from 140 Gy γ rays treated seeds of Winner (Table 3), the developing shoots were rudimentary and deformed; therefore, they were counted as nil (Table 4). 60 Gy γ rays treated seeds of Karina and Bolero showed a partial increase in the frequency of seed germination when germinated on 10 µM TDZ (Table 3). No germination was observed in 140 Gy treated seeds of Bolero. Karina and Bolero showed a decrease in shoot regeneration and shoot length with each increase in the irradiation (compared to control) accompanied by number of albino (leaved) mutants (Table 4). No germination was recorded in 60 or 140 Gy treated seeds of Sprinter. There are many papers in the literature emphasizing shoot regeneration from different explants but only limited studies show direct shoot regeneration from the seed under in vitro conditions. Malik and Saxena (28), in their research on leguminous plants, emphasize the importance of seed explants in shoot regeneration. Similarly, Özgen et al. (37) found that mature embryos have a high capacity for shoot regeneration. At the same time, they emphasized that these could be used at any time of the year. Therefore, they used these embryos in their studies on the tissue culture of wheat. In brief,

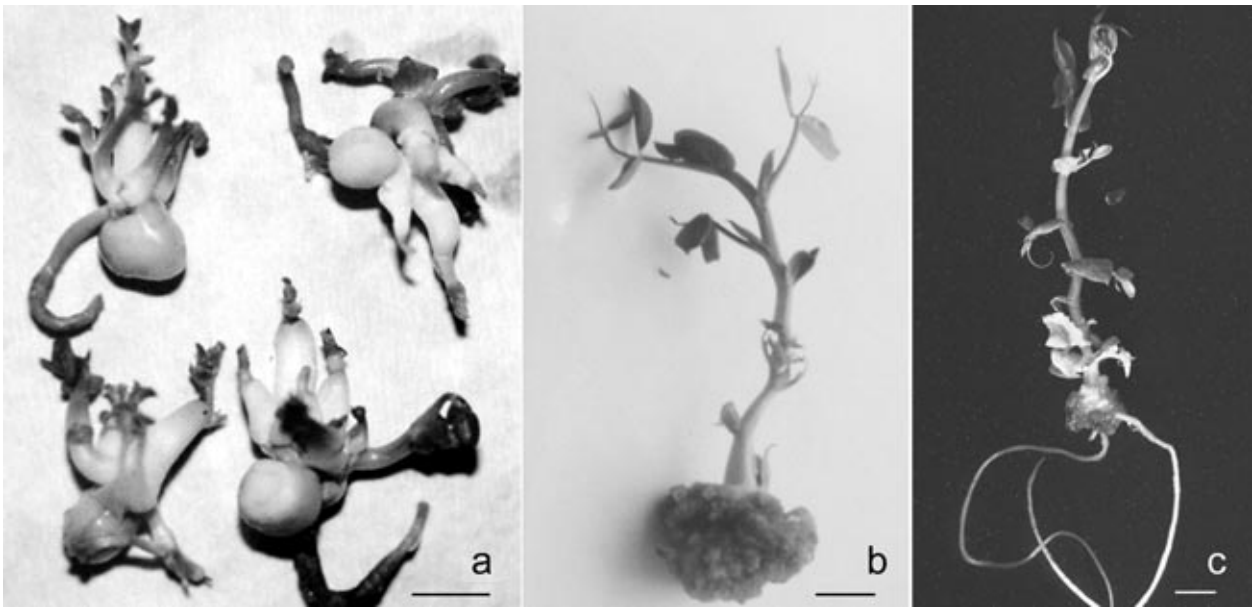

Figure 1. Effects of mutant gamma radiations on pea. (a) germination of irradiated seeds cultured on germination medium containing MS supplemented with 50 µM BAP, (b) no rooting and development of callus from TDZ regenerated shoots, (c) rooting of BAP treated shoots of Winner when subjected to 60 Gy γ radiations.

Table 2. Effect of 10 µM TDZ and 50 µM BAP on frequency of germination, number of shoots per explant, frequency of rooting and morphological abnormalities of 4 pea cultivars.

Frequency (%) of Germination					
Cultivars	Winner	Karina	Sprinter	Bolero	Average
Control	70.00 a*	60.00 a	30.00 a	30.00 a	47.50
10 µM TDZ	40.00 b	30.00 b	0.00 b	35.00 a	26.25
50 µM BAP	50.00 b	35.00 b	0.00 b	15.00 b	25.00
Number of shoots/seed					
Control	1.00 b	1.00 c	1.00 a	1.00 b	1.00
10 µM TDZ	2.75 b	1.90 b	0.00 b	1.25 b	1.73
50 µM BAP	4.39 a	3.30 a	0.00 b	2.75 a	2.61
Frequency (%) of Rooting					
Control	70.00 a	55.00 a	55.00 a	60.00 a	60.00
10 µM TDZ	40.00 b	30.00 b	0.00 b	40.00 b	25.00
50 µM BAP	60.00 ab	40.00 b	0.00 b	40.00 b	40.00
Morphological appearance (abnormally green and thick %)					
Control	0.00 b	0.00 b	0.00	00.00 b	0.00
10 µM TDZ	90.54 a	92.79 a	-	93.87 a	69.80
50 µM BAP	1.59 b	1.76 b	-	02.65 b	1.50

*Values within a column followed by different letters are significantly different at 0.01 level of significance.

Table 3. Effect of different cytokinins on frequency (%) of germination of 4 irradiated pea cultivars

Treatments	Winner		Karina		Sprinter		Bolero	
	10 µM TDZ	50 µM BAP	10 µM TDZ	50 µM BAP	10 µM TDZ	50 µM BAP	10 µM TDZ	50 µM BAP
Control	21.93 b*	30.79 b	21.93 a	38.86 a	16.92 a	8.86 a	10.73 a	13.39 a
60 Gy	73.08 a	63.44 a	26.57 a	8.86 b	0.00 b	0.00 b	13.08 a	6.57 b
140 Gy	8.86 c	39.23 b	30.00 a	0.00 b	0.00 b	0.00 b	0.00 b	0.00 c

*Values within a column followed by different letters are significantly different at 0.01 level of significance

Table 4. Effect of different doses of gamma rays on number of shoots per explant and morphology of 4 pea cultivars.

Treatments	Winner					Karina				
	Number of shoots per explant	Shoot length (cm)	Morphology of leaves and aerial parts			Number of shoots per explant	Shoot length (cm)	Morphology of leaves and aerial parts		
			Green	Albino	Variegated			Green	Albino	Variegated
Control	2.40 a*	2.82 a	100.0 a	0.00 b	5.55 a	3.80 a	7.63 a	100.0 a	0.00 c	1.00 a
60 Gy	2.73 a	1.63 b	66.67 b	30.54 a	2.79 b	1.03 b	1.91 b	83.33 ab	11.67 b	0.00 b
140 Gy	0.00 b	0.00 c	0.00 c	0.00 b	0.00 b	0.83 c	1.10 b	61.40 b	38.60 a	0.00 b
	Winner					Karina				
Treatments	Number of shoots per explant	Shoot length (cm)	Morphology of leaves and aerial parts			Number of shoots per explant	Shoot length (cm)	Morphology of leaves and aerial parts		
			Green	Albino	Variegated			Green	Albino	Variegated
Control	00.00	00.00	00.00	00.00	00.00	2.40 a	5.55 a	100.00 a	0.00 c	0.00
60 Gy	00.00	00.00	00.00	00.00	00.00	1.00 b	1.16 b	89.21 ab	10.79 b	0.00
140 Gy	00.00	00.00	00.00	00.00	00.00	0.30 c	0.70 b	61.40 b	38.60 a	0.00

*Values within a column followed by different letters are significantly different at 0.01 level of significance

Winner was the best, Karina and Bolero were better and Sprinter was the worst in terms of frequency (%) of germination from 60 Gy treated seeds. The results are in agreement with Pierik (38) and Bajaj (39), who found that lower doses of gamma radiation encourage and higher doses of gamma radiation discourage seed germination.

Chlorophyll deficient (albino) plantlets were considered to have multiplied if they produced 2 or more shoots. The seedlings that grew in BAP were largely normal; we speculate that they would grow normally to slight higher doses of γ radiations. To our knowledge, we find no literature on the effect of cytokinins on shoot regeneration from irradiated pea seeds. Novak et al. (40) found that under in vitro conditions, immature embryos of maize reduced their germination capacity after treatment with gamma radiations. Similarly, Cheng et al. (41) found that the appropriate dose for embryos of wheat was 5 Gy and higher doses of gamma radiations were adverse on shoot regeneration. Hell (42) found that treatment with gamma radiations results in an increase in buds but with considerably reduced life expectancy. Similarly, Moustafa et al. (43) and Bajaj (40) found that increased radiations result in reduced regeneration from the tissues of maize and common bean respectively. These results suggest carrying out of more studies under in vitro conditions to find out the effects of mutagens to get conclusive results.

Rooting

The seeds of Sprinter did not germinate after γ ray treatment on MS medium containing either 50 µM BAP or

10 µM TDZ, whereas the shoots obtained from 60 Gy γ rays irradiated seeds of Winner, cultured on MS medium containing 50 µM BAP and 14 Gy treated shoots of Karina cultured on 50M BAP, were rooted on MS medium containing 2.5 µM NAA (Table 5) optimized during the second stage of selection.

TDZ regenerated shoots from developed callus (Figure 1b), whereas shoots of Winner from 60 Gy γ ray treated seeds germinated on MS medium containing 50 µM BAP rooted easily (Table 5). The highest number of roots per shoot (1.11) and root length (4.42 cm) from treated seeds was observed from Winner (Figure 1c) treated with 60 Gy gamma radiations. In the case of Karina, number of roots/shoot decreased considerably with no rooting at 60 Gy and decreased (8.82%) rooting at 140 Gy irradiations. The rooting potential of shoots obtained from non-irradiated seeds (control) was better. This potential was completely lost in the case of germinating irradiated seeds on TDZ and partially on BAP. It takes a long time to breed varieties under field conditions. If the time and resources had been there, it would have been of interest to grow these seedlings to M_1 and then to maturity and observe physiological adaptations. The experiment is helpful for determining the care while preparing the explant and the effects of cytokinins and γ radiations on multiplication of pea seedlings.

Before the start of the experiment it was not possible to get an idea of enormous variations due to γ rays under the influence of varying doses of cytokinins. Although different doses of gamma rays did not affect the germination capability of seeds, they brought about considerable changes in growth and development pattern of respective pea cultivars based on internal biological responses of the seeds, which were sharply visible on seed germination, growth, leaf color and rooting when they were germinated on 2 different cytokinins - BAP and TDZ. This is important, since it allows the tracing of different modes of action of gamma rays against given cytokinins, if only the effects are to be determined by means of measuring quantitative and qualitative characters. This can be used to increase mutation efficiency under the influence of possible and expected mutation spectra. Pea was studied advantageously, since mutation could be detected easily based on the color (albino, variegated, yellow) of aerial parts and other physiological changes. The study positively evaluates screening of γ ray irradiated pea seed mutants on MS medium containing BAP. Moreover, the study presents a very simple protocol that could provide a useful way of measuring mutagenic effects on large material within a reasonable time independent of the inhibitions brought about by the varying climatic conditions when the experiments are carried out in fields or greenhouses.

Table 5. Effect of cytokinins and γ radiations on rooting of 3 pea cultivars using 2.5 µM NAA.

Treatments	Number of roots per shoot						Frequency (%) of root formation					
	Winner		Karina		Bolero		Winner		Karina		Bolero	
	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP
Control	0.11 a*	0.81 a	0.72 a	3.61 a	0.21 a	2.22 a	13.01 a	30.00 a	25.71 a	90.01 a	13.01 a	68.81 a
60 Gy	0.00 a	1.11 a	0.00 a	0.00 b	0.00 a	0.00 b	0.00 b	42.22 a	0.00 b	0.00 b	0.00 b	0.00 b
140 Gy	0.00 a	0.00 a	0.00 a	0.67 b	0.00 a	0.00 b	0.00 b	0.00 b	0.00 b	8.82 b	0.00 b	0.00 b

Treatments	Primary root length (cm)					
	Winner		Karina		Bolero	
	TDZ	BAP	TDZ	BAP	TDZ	BAP
Control	0.62 a	2.03 a	2.12 a	0.33 a	4.14 a	6.02 a
60 Gy	0.00 a	4.42 a	0.00 a	0.00 a	0.00 b	0.00 b
140 Gy	0.00 a	0.00 a	0.00 a	0.31 a	0.00 b	0.00 b

*Values within a column followed by different letters are significantly different at 0.01 level of significance.

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Corresponding author:
Khalid Mahmood KHAWAR
Central Laboratory, Institute of Biotechnology
Behind Rektorluk, University of Ankara
06000, Tandoğan, Ankara - TURKEY
E-mail: kmkhawar@gmail.com

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