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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Enhancement of Androgenesis and Plant Regeneration From Wheat Anther Culture by Seed Pre-Sowing Gamma Irradiation

Ekim Öncesi Tohumların Gamma Işınlaması ile Buğday Anter Kültüründen Adnrogenesis ve Bitki Rejenerasyonunun Artırılması

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Abstract

Combination breeding and mutation breeding are widely used methods in plant breeding. Intensive studies are carried out on biotechnological methods that will allow obtaining homozygous lines in a short time in populations obtained with these two techniques. Numerous studies have been carried out by different researchers on mutation breeding in wheat. There are few studies on anther culture response in mutagen-treated genotypes. Two different bread wheat promising advanced lines were used as the material in the study, in which the possibilities of combining mutation breeding with anther culture through ionizing radiation in high quality wheat breeding were investigated. In the study, the responses of advanced bread wheat mutant lines to anther culture, to which eight different doses of gamma rays (0, 100, 150, 200, 250, 300, 350, 400 Gy) were applied, including the control, were investigated. There are significant differences between genotypes and irradiation doses for all traits studied. It has been shown that it is possible to decrease albinism and increase the response of anther culture with dose-dependent gamma irradiation depending on bread wheat varieties. In the multiple comparison test to classify the difference between doses; statistically, 150 gray dose 5.60 is in the first statistical class and in the first place with the number of transferred green plants. After that, it ranks second in the same class with the number of green plants transferred with a 300 gray dose of 5.21. In the total number of regenerated green plants excluding controls (888), 635 unit (71.5%) and 205 unit (23.1%) haploids and spontaneous double haploid plants were obtained, respectively. In the study integrated into the bread wheat breeding program, a total of 205 spontaneous double haploid mutant lines were produced. According to the data obtained, it was shown that the gamma ray doses of 150 and 200 Gy had a significant stimulation effect on all parameters studied and ultimately the success index of anther culture in bread wheat compared to control.

Keywords: Anther culture, Doubled haploid, Embryo-like structure, Gamma ray, Plantlets, Wheat

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Öz

Bitki ıslahında kombinasyon ıslahı ve mutasyon ıslahı yaygın olarak kullanılan yöntemlerdir. Bu iki teknikde elde edilen populasyonlardan homozigot hatların kısa sürede elde edilmesini olanak sağlayacak biyoteknoloik yöntemler üzerine yoğun çalışmalar yapılmaktadır. Buğdayda mutasyon ıslahı üzerine farklı araştırıcılar tarafından çok sayıda çalışmalar yapılmıştır. Buğdayda mutagen uygulanmış genotiplerde anter kültürü yanıtı üzerine ise az sayıda çalışmalar bulunmaktadır. Yüksek kaliteli buğday ıslahında iyonlaştırıcı radyasyon yoluyla mutasyon ıslahının anter kültürü ile etkin bir şekilde birleştirilebilme olanaklarının araştırıldığı çalışmada, iki farklı ekmeklik buğday ileri hattı materyal olarak kullanılmıştır. Kontrol dahil sekiz farklı gamma ışını dozu (0, 100, 150, 200, 250, 300, 350, 400 Gy) uygulanmış 2 farklı ileri ekmeklik buğday mutant hattının anter kültürüne yanıtları araştırılmıştır. İncelenen tüm özellikler için genotipler ve ışınlama dozları arasında önemli farklılıklar vardır. Bu çalışma, ekmeklik buğday çeşitlerine bağlı olarak doza bağlı gama ışınlaması ile albinizmin azaldığı ve anter kültürü yanıtın artırılabileceğinin mümkün olabileceğini göstermiştir. Dozlar arasındaki farkı sınıflandırmak için yapılan çoklu karşılaştırma testinde; istatistiki olarak 50 gray doz 5.60 adet aktarılan yeşil bitki sayısı ile ilk sırada yer almış, bunu yine aynı sınıfta 5.21 adet aktarılan bitki sayısı ile 300 gray doz uygulaması ikinci sırada izlemiştir. 150 ve 200 Gy gama ışını dozlarının, kontrole kıyasla ekmeklik buğdayda anter kültürünün incelenen tüm parametrelerinde ve nihai olarak başarı indeksi üzerinde önemli bir stimülasyon etkisi olduğu gösterülmüştür. Kontroller hariç toplam rejenere yeşil bitki sayısından (888), 635 adet (%71.5) ve 205 adet (%23.1) sırasıyla haploidler ve spontan double haploid bitkiler elde edilmiştir. Ekmeklik buğday ıslah programına entegre edilen çalışmada toplam 205 spontan double haploid mutant hattı üretilmiştir.

Anahtar Kelimeler: Anter kültürü, Double haploid, Embriyo benzeri yapı, Gamma ışını, Bitkicikler, Buğday

1. Introduction

The double haploid system is increasingly being used in many large crop main breeding programs (Kasha and Maluszynski, 2003). The first green plants from anter culture in hexaploid bread wheat (*Triticum aestivum* L.) was obtained by Ouyang et al. (1973), then protocol improvements have been made (Tuvesson et al., 2000; Barnabás et al., 2001; Zheng et al., 2001). These developments led to further understanding by growers of the importance of double haploid plants and resulted in the development of more than 280 varieties in various crops (Sadasivaiah et al., 2004; Szarejko and Forster, 2007; Weyen, 2009) in some other countries, mainly in Hungary. (Barnabás et al., 2000; Pauk et al., 2003). Low calli/embryoid formation frequency, high albino frequency, genotype-dependent response, poor regeneration of green planters and chimera plant formation are among the most important factors limiting double haploid production from AC (Larsen et al., 1991; Gosal et al., 1997; Szarejko, 2003; Wedzony et al., 2009; Parmar et al., 2012). One of the frequently encountered problems in androgenetic research is the emergence of mixed ploidy plants due to a fusion of haploid nuclei in the early stages of androgenesis or endopolyploidy (Sangwan-Norreel, 1983). The use of mutation breeding through gamma rays together with anther culture to overcome these problems can be considered as an "ideal system" for plant breeding programs (Vagera et al., 1976; Maluszynski et al., 1995; Szarejko, 2003; Ahloowalia et al., 2004; Vagera et al., 2004; Arabi et al., 2005; Xu et al., 2012). Also, the integration of DH technology with existing biotechnological tools will increase genetic gains and breeding efficiency and ultimately lead to the rapid development of hybrids (Patial et al., 2017). More effective results are expected to be obtained especially when applied to self-pollinating plants such as wheat, which is stated to have narrow genetic gains of 0-1% (Baenziger and Peterson, 1992).

The gamma rays are effective mutagenic agents that can be applied easily to any stage of anther culture (Nakamura and Hattori, 1997). Two different approaches to applying mutagenesis in combination with haploid *in vitro* culture are described. The first, called in vitro haploid mutagenesis, is the application of mutagens to the plant organs and haploid cells such as anthers, spikes, panicle, flower buds, embryos, calli or protoplasts. There is also an alternative approach in which gametes of M_1 plants originating from seeds treated with mutagen before sowing can be used as donor material for haploid culture (Maluszynski et al., 1996; Szarejko, 2011). In vitro haploid mutagenesis has been reported to have some disadvantages (Mkuya et al., 2005; Szarejko, 2011; Sharma et al., 2017). First, the irradiated media can produce some toxic substances that can be harmful to anthers or calli, so they have to be transferred to a fresh medium immediately after irradiation. Second, anther-derived calli show a mixture of haploid and doubled haploid cells; radiation-induced mutation can make the homozygous diploid calli revert to a heterozygous state, eliminating the advantage of anther culture for breeding. Third, microspores isolated in the uni-nucleate stage, which is the best target for in vitro mutagenesis, are very sensitive to mutagenic treatments and post-treatment manipulations. Fourth, the magnitude of the contribution of additive mutagenesis to somaclonal variation is uncertain.

In contrast, the alternative approach has been reported to have some important advantages over in vitro haploid mutagenesis. First, all mutant DH plants regenerated from gametes of M_1 plants are completely homozygous and do not segregate in the progeny. Second, it is possible to use much higher doses of mutagen for dormant seeds than using microspores, anthers or inflorescences. Generally, however, doses applied to produce M_1 donor plants that serve as a source of gametic cells for culture are within the range of recommended dosage in conventional mutagenesis. However, before a large-scale experiment, it would be useful to assess the viability of microspores produced by M_1 plants, as too high doses of mutagen can result in a large reduction in the survival of microspore (Szarejko, 2011).

Since the above explanations and considerations, seed irradiation has to be aforethought an effective and practical method to induce genetic variation in wheat anther culture, in vitro haploid mutagenesis is considered to have little practical value (Gao et al., 1988, Balkan, et al. 2019). One of the main objectives in breeding studies is the development of high-yielding advanced lines (Bilgin and Korkut, 2005). Although most studies on in vitro culture, particularly those combined with the induced mutation using gamma rays, have been performed with fresh explants, there are almost no reported studies on the anther culture response after gamma irradiation of the seed in wheat. We report here an investigation of the response of two bread wheat genotypes in anther

culture following their treatment with one of a range of doses of gamma radiation. This study also seeks to examine the mutual effects in terms of overcoming limitations of anther culture and mutation breeding of wheat anthers when anther culture and gamma irradiation are combined.

2. Materials and Methods

2.1. Plant material and Induced gamma irradiation

Two bread wheat (*Triticum aestivum* L.) advanced lines, BSB (Bezostaja/Saraybosna; tall, mid-early, awnless, superior in flour quality for bread making, but inferior in lodging resistance and yield capacity) and FA (Flamura80/Atilla12; tall, mid-early, awned and moderate in flour quality for bread making, but inferior in lodging and disease resistance and yield capacity) and their 16 M_1 combinations together with the un-irradiated (control) were selected as the plant material. The moisture contents of seeds of wheat genotypes (*Triticum aestivum* L.) used in the study were 12.1% for BSB and 12.5% for FA. For each genotype, grains were divided into eight groups, each containing 2000 grains. One group was kept free from irradiation (control), while other groups were irradiated with various levels of gamma rays (100, 150, 200, 250, 300, 350 and 400 Gy). Seeds were treated by gamma rays from ^{60}Cobalt, Ob-Servo Sanguis Co-60 Research Irradiator with isotope model, while the dose rate was 2.190 kGy h^{-1} before the 2011-12 growing season at the Turkish Atomic Energy Authority, Sarayköy Nuclear Research and Training Center, Ankara, Turkey. The unit for the absorbed dose of radiation energy is the gray (Gy), which is equivalent to 1 J Kg^{-1} and 100 rads.

2.2.Anther Culture

After irradiation, the donor seeds of the genotypes were sown in the experimental field of the Field Crops Department of the Faculty of Agriculture of Tekirdağ Namık Kemal University during the growing season of 2011-12. Sowing was done on Nov 11, 2011, by hand at the rate of 400 seeds per m^2. Nitrogen and $P_2 0_5$ at 140 and 70 kg ha^{-1}, respectively, were incorporated into the soil as compound fertilizer (20-20-0) before sowing, urea during tillering and ammonium nitrate before heading. Donor plants were protected with fungicides and pesticides. The crop was kept free of weeds by hand hoeing when necessary. The remaining seeds were sown in the greenhouse to guarantee the work.

The totally 35-40 appropriate main spikes per genotype with anthers containing microspores at the vacuolated, early-mid uninucleate stage (checked by Olympus inverted microscope), approximately at stage 38 according to Zadoks Scale (Zadoks et al., 1974), were collected, put in Erlenmeyer flasks with tap water, covered with PVC (polyvinyl chloride) bags and cold transferred to pre-treatment conditions (2-4 °C, two weeks). After pre-treatment, the selected donor spikes at the uni-nucleated microspores stage were surface sterilized in 250 mL 2% NaOCl solution containing a drop of Tween-80 for 20 min of a shaker and then rinsed three or four times with sterile distilled water. Anthers from the top of sterilized heads of donor genotypes were isolated in 90 mm diameter petri dishes containing induction media namely W_{14}mf medium (Ouyang et al., 1989; Lantos et al., 2014). In total, 25-30 cold pre-treated main spikes per genotype were used as donor spikes. Petri dishes in incubators were controlled and the evolving embryo-like structures were observed weekly. Transferred anthers were incubated at 32 °C for 3 days in the dark to increase microspore division. At the end of this period, cultures were kept in the dark at 28 °C for approximately 5-8 weeks to induce ELS (embryo-like structures). After 5 weeks cultivation period (in the dark incubator at 25^0C), the ELS with a size of 1-2 mm were transferred onto 90 mm diameter plastic petri dishes with 30 mL of a 190-2Cu regeneration medium (Zhuang and Jia, 1983) with 0.1 mg l^{-1} gibberellic acid (GA_3) plus 5 mg l^{-1} IBA and placed in the controlled climate room. After about 2-3 weeks, the green plantlets, 20 to 30 mm long, were transferred into test tubes with 3 cm diameter and 15 cm length, only one plantlet per tube was selected from ELS when the regenerates could be recovered into 50 mL glass tubes with the same medium, and kept in a growth room (25 °C, 16/8 h light/dark photoperiod, fluorescent light at 200 µmol/m^2/s) for entire plantlets regeneration. Regenerated green and albino plantlets were counted and the albino plantlets were discarded.

The green plantlets were transferred to test tubes containing MS medium (Murashige and Skoog, 1962) without growth regulators, and the well-rooted green plantlets were transplanted into small pots, covered with plastic bags to maintain high humidity and kept in a cold room (+4 ^{0}C) to vernalize. Finally, the plantlets about 10 cm tall with strong roots were transferred to the greenhouse for acclimatization (3-4 days). The acclimatized

plants were grown in the greenhouse. Haploid and spontaneous doubled haploid plants were identified based on seed set production, the spontaneous doubled-haploid plants were transferred to ground soil and grown with the necessary cultural practices, and each plant was harvested and threshed separately.

In the experiment, the data concerning ELS, the number of green and albino plantlets, transplanted plantlets were collected. To characterize anther culture response, following some additive parameters suggested by Bhaskaran et al. (1983), Redha et al. (1998), Barnabás (2003) and Pauk et al. (2003) was calculated. These are Embryo Like Structures (ELS = embryo-like structures regenerated from plated anthers); Embryo-Like Structures Initiation Rate (ELSIR = the ratio of embryo-like structures to plated anthers); Albino Regenerated Plantlets (ARP = the number of albino plantlets regenerated from embryo-like structures); Green Regenerated Plantlets (GRP = the number of green plantlets regenerated from embryo-like structures); Plant Regeneration Rate (PRR = the ratio of green and albino plants regenerated to embryo-like structures); Transplanted plantlets (TP = the total number of green plants regenerated from callus and transferred to greenhouse); Number of Haploid Plant (NHP = the total number of haploid plants from green plants transferred to greenhouse -the numeral in brackets are percentage per green plant regenerant); Number of Spontaneous Doubled-Haploid Plants (NSDHP = the total number of spontaneous doubled-haploid plants from green plants transferred to greenhouse-the numeral in brackets is doubling index = [green DHs divided by total number of green plants] x1); and Final Success Index (FSI = number of green plants/number of cultured anthers).

2.3. Statistical analysis

The study carried out a completely randomized split-plot design with 5 replicated. To evaluate the effect of genotype, mutation doses and their interaction, the data of androgenic parameters concerning ELS, number of green and albino plantlets, transplanted plantlets were analysed by two-way ANOVA using Microsoft Exel 2007 statistical software (Redmond, WA, USA).

3. Results

The effect of genotype, mutagen dose and their interaction was studied using two-way ANOVA for the androgenic parameters *(Table 1).* The statistical analysis revealed that the effects of genotype, mutagen doses and their interactions on all tested androgenic parameters were significant at P<0.01.

Table 1. The two-way ANOVA for the androgenic parameters in the mutated population of bread genotypes

Source of variance	*df*	No. of ELS	No. of GRP	No. of ARP	No. of TP
Genotype	1	1445.000**	732.050**	54.450**	400.512**
Mutagen dose	7	2186.393**	582.050**	74.600**	396.098**
Genotype x Mutagen dose interaction	7	268.086**	69.593**	5.821**	52.941**
Error	56	1.387	2.259	1.367	1.631

**The values significantly differ at P<0.01; ELS-embryo-like structures; GRP- Green regenerated plantlets; ARP-Albino regenerated plantlets; TP- Transplanted plantlets

3.1. Evaluation of androgenic response

Table 2 summarizes the significant differences in the genotypes and mutation doses for all examined characters. Although the BSB genotype gave higher averages for ELS and ARP than the FA genotype, the averages of the FA genotype for GRP and TP were higher.

To gather with genotype, mutagen doses influenced the number of ELS in AC, significant differences were observed among the mutagen doses *(Table 2).* The highest mean was 65.1 ELS in AC of 150 Gy gamma ray dose, while the lowest value was 15.1 ELS in AC of 400 Gy gamma ray dose. In the experiment, an average of 8 gamma doses was 35.5 ELS. The seven gamma ray doses except 400 Gy were able to produce more ELS than the control.

Table 2. The androgenic response of embryo-like structures (ELS), Green regenerated plantlets (GRP), albino regenerated plantlets (ARP) and Transplanted plantlets (TP) for genotypes and mutagen doses in anther culture

Genotypes	No. of ELS	No. of GRP	No. of ARP	No. of TP
BSB	39.7±0.639 a	10.7±0.090 b	9.6±0.188 a	9.4±0.174 b
FA	31.2±0.556 b	16.8±0.358 a	7.9±0.398 b	13.8±0.289 a
$LSD_{(0.01)}$	*2.184*	*1.282*	*1.355*	*1.601*
Mutagen doses				
0	26.3±1.086 e	5.1±0.526 f	13.6±0.542 a	4.0±0.394 f
100	34.4±0.542 c	9.0±1.106 e	11.2±0.512 b	7.4±1.035 e
150	65.1±4.270 a	28.0±0.650 a	9.1±0.879 c	23.2±0.772 a
200	46.0±0.789 b	21.2±2.190 b	9.3±0.300 c	17.4±1.284 b
250	35.0±0.943 c	15.4±2.377 c	8.1±0.482 cd	13.8±1.837 c
300	31.4±2.212 d	12.3±1.096 d	7.2±0.416 de	9.7±1.155 d
350	30.5±3.209 d	11.5±1.241 d	6.6±0.521 e	10.5±1.213 d
400	15.1±1.663 f	7.3±0.300 e	4.9±0.888 f	6.7±0.367 e
Mean	*35.5*	*13.7*	*8.8*	*11.6*
$LSD_{(0.01)}$	*1.411*	*1.801*	*1.401*	*1.531*

Table 3. The androgenic response of embryo-like structures (ELS), Green regenerated plantlets (GRP), Albino regenerated plantlets (ARP) and Transplanted plantlets (TP) for genotypes x mutagen dose interactions in anther culture

Genotypes x Mutagen Interaction	No. of ELS	No. of GRP	No. of ARP	No. of TP
BSB-0	29.2±0.735 i	3.6±0.245 g	14.0±0.548 a	3.0±0.316 h
BSB-100	33.2±0.490 h	6.0±0.548 fg	12.2±0.583 ab	4.4±0.245 gh
BSB-150	77.8±0.735 a	28.2±1.114 a	9.6±0.510 cd	25.0±0.775 a
BSB-200	47.4±1.123 c	14.8±0.860 c	9.2±0.374 cde	14.0±1.049 c
BSB-250	32.6±0.510 h	8.4±0.400 ef	8.8±0.490 cde	8.4±0.400 de
BSB-300	37.8±1.020 f	9.4±0.748 e	7.8±0.374 def	6.4±0.245 efg
BSB-350	40.0±0.837 e	8.0±0.548 ef	7.6±0.510 d-g	7.0±0.633 ef
BSB-400	19.8±1.068 k	7.2±0.374 ef	7.4±0.509 efg	6.6±0.510 efg
FA-0	23.4±0.748 j	6.6±0.245 f	13.2±0.970 a	5.0±0.316 fgh
FA-100	35.6±0.600 g	12.0±0.837 d	10.2±0.583 bc	10.4±0.510 d
FA-150	52.4±0.927 b	27.8±0.800 a	8.6±0.510 c-f	21.4±0.678 b
FA-200	44.6±0.748 d	27.6±0.600 a	9.4±0.509 cde	20.8±0.735 b
FA-250	37.4±0.927 fg	22.4±0.872 b	7.4±0.748 efg	19.2±0.663 b
FA-300	25.0±0.707 j	15.2±0.800 c	6.6±0.678 fg	13.0±0.707 c
FA-350	21.0±0.707 k	15.0±0.707 c	5.6±0.678 g	14.0±0.316 c
FA-400	10.4±0.510 l	7.4±0.510 ef	2.4±0.400 h	6.8±0.583 ef
Mean	*35.5*	*13.7*	*8.8*	*11.6*
$LSD_{(0.01)}$	2.140	2.521	1.999	2.199

The green plantlets was regenerated from AC-derived ELS of each gamma dose. The number of green plantlets ranged from 5.1 to 28.0. The gamma ray dose of 150 and 200 Gy showed high values of GRP (28.0 and 21.2, respectively) while 0 and 400 Gy were the lowest values (5.1 and 7.3, respectively). The overall mean of GRP was 13.7 *(Table 2).*

Albino plantlets were observed in each mutagen dose. However, the values varied from 4.9 (400 Gy) to 13.6 (0 Gy). The average of albino plantlets production was 8.8 in the experiment *(Table 2). Table 2* shows the range of the transplanted plantlets varied from 4.0 to 23.2. The dose with a high value was 150 Gy (23.2 TP) while the overall mean was 11.6.

The effect of genotype x mutagen dose interaction on ELS, GRP, ARP and TP was significant at P<0.01 *(Table 3).* The values of ELS ranged from 10.4 to FA (400 Gy) and 77.8 in BSB (150 Gy) while the overall mean was 35.5 ELS *(Table 3).* The highest values were counted for 150 Gy dose of each genotype. An average of GRP varied between 3.6 in BSB (0 Gy) and 28.2 in BSB (150 Gy). The other highest values were obtained in 150 and 200 Gy dose of FA (27.8 and 27.6, respectively). Regarding ARP, the means of interaction were varied between 2.4 in FA (400 Gy) and 14.0 in BSB (0 Gy) while the overall average was 8.8 ARP *(Table 3).* The values of TP ranged between 3.0 in BSB (0 Gy) and 25.0 in BSB (150 Gy), with an overall mean of 11.6.

3.2.Evaluation of anther culture response

In our experiment, to the characterization of anther culture response of the genotypes, the other calculated AC parameters such as embryo-like structure initiation rate, plantlet regeneration rate, final success index concerning data for ELS, the number of green and albino plantlets, transplanted plantlets are given *Table 4*.

The average of embryo-like structure initiation rate (ELSIR) for BSB and FA were 3.97% and 3.12%, respectively. The controls (0 Gy) were 2.92% and 2.34%, and that of irradiation treatments ranged from 1.98% (400 Gy) to 7.78% (150 Gy) for BSB and 1.04% (400 Gy) to 5.24% (150 Gy) for FA. *Table 4* shows that there are nonlinear increases in ELSIR up to 350 Gy gamma irradiation applications compared with the control, then decreases. The rate of total plantlet (albino and green) regeneration was calculated for and mutagen doses of both genotype *(Table 4).* The Green plantlet regeneration rate (GPRR) varied between 12.33% (0 Gy) and 36.36% (400 Gy) for the BSB genotype and between 49.25% (0 Gy) and 76.92% (100 Gy) for the FA genotype. The mean values for genotypes were 25.61 (BSB) and 62.80 (FA). *Table 4* also shows decreasing albino plantlets rate as the irradiation dose increases. The rates of albino plantlet regeneration for genotypes ranged between 27.55% in BSB and 27.31% in FA. The controls (0 Gy) were 47.95% (BSB) and 56.41% (FA), and that of irradiation treatments ranged from 12.34% (150 Gy) to 37.37% (400 Gy) for BSB and 16.41% (150 Gy) to 28.65% (100 Gy) for FA.

The plantlets transplanted into the greenhouse were harvested after maturation. Based on the seed set production, three types of spike among the AC-derived plants such as fertile, partial fertile and sterile. The plants with sterile spike are haploid. The mean of haploid plants ranged from 1.6 (0 Gy) to 13.2 (150 Gy) in BSB with an overall mean was 5.80 and ranged from 5.0 (0 Gy) to 16.8 (200 Gy) in FA with an overall mean was 11. The fertile and partially fertile plants produced a small amount of seeds due to spontaneous diploidization. The rate of DHP varied from 14% (FA-350 Gy) to 46% (BSB-100 Gy). The average spontaneous doubled- haploid rates of genotypes were 33% and 18% (BSB and FA, respectively). 205 spontaneous double haploid mutant lines were obtained in the experiment, which has been integrated into the winter wheat breeding program. In the experiment, the spontaneous doubled-haploid rate was 23.1%. Although the SDHP rates of the BSB genotype are higher than those of the FA genotype, it is seen that there is an inverse trend when final success indexes are compared. When compared with their controls, it is understood that the highest final success index was calculated for both genotypes at 150 and 200 Gy gamma ray doses *(Table 4).*

4. Discussion

In the anther culture of wheat, the production of a high percentage of green plants is necessary for the efficient and successful application of the doubled haploid approach in wheat breeding programs. In wheat, anther culture ability can be divided into three components that are inherited independently, such as embryogenesis, plant regeneration, and green plant formation (Larsen et al., 1991). Each of them is governed by more than one gene (Lazar et al., 1984; Szakacs et al., 1988; Chaudhary et al., 2003). The production of DH lines of wheat from anther culture is limited by a relatively low callus/embryoid induction frequency, genotype-dependent response, poor regeneration and a large number of albino plants (Jauhar et al., 2009; Parmar et al., 2012). It has been explained that these difficulties can be overcome by integrating mutagen with anther culture, especially gamma irradiation (Patial et al., 2017; Sharma et al., 2017).

In the present study, the data also showed a highly significant effect between the two tested wheat genotypes under different doses of gamma rays. There was a surpassing response for BSB which gave the highest percentage (3.97%) of ELSIR, but FA gave the lowest (3.12%) *(Table 4).* Therefore, it could be concluded that genotypes differed in their response to different doses of gamma rays depending on the genetic makeup of ELS.

The average ELSIR at 150 and 200 Gy showed a highly significant increase (62.47-38.40% for BSB; 55.34-47.53% for FA) for both genotypes, with the large reduction (-11.43% and -125.00%) for FA only obtained with gamma ray treatments at 350 and 400 Gy, respectively, compared to control. However, the increase of gamma irradiation had a significant simulation effect on ELSIR as compared to control, except 400 Gy. This reduction at 400 Gy is due to the inhibiting effect of high doses of gamma-ray (Ashraf and Foolad, 2005), while 150 Gy stimulated ELS in all studied wheat genotypes. It is reasonably conjectured that gamma rays could stimulate ELS regeneration by some mechanism such as activation of retrotransposons (Hirochika, 2001). It can be concluded that the low dose of gamma ray caused an increase in ELS induction, while higher doses reduced it as stated by Szarejko (2011). Higher culture ability makes the application of seed radiation possible both for breeding and mutagen induction. The seed irradiation, or more precisely, the mature embryo irradiation, seemed to play an active role in promoting ELS induction while keeping the same regeneration capacity as the untreated one. Furthermore, seed irradiation also helped to extend the duration of plant regeneration by about 10 days, leading to a 10% increase in plantlet production (Gao et al., 1988). It is another benefit for irradiation of wheat seed that any potential mutation induced by gamma rays will be homozygous in anther-derived plantlets, while the mutagen-induced in radiated calli or anther will make the regenerated plantlets heterozygous. Similar results were obtained by Abdrabou and Salam (1992), Rashed et al. (2000), Abdel-Hady and Abou-Deif (2001) and Abdel-Hady and Ali (2006).

Table 4. The anther culture response parameters for gamma-irradiated bread wheat genotypes

Genotype	ELS (mean)	ELSIR (%)		GRP (mean)	ARP (mean)	PRR (%)		TP (mean)	NHP (mean)	NSDHP (mean)	FSI
		Mean	Range			Green Plantlets	Albino Plantlets				
BSB-0	29.2	2.92	0.00	3.6	14.0	12.33	47.95	3.0	2.0 (67)	1.0 (33)	0.003
BSB-100	33.2	3.32	12.05	6.0	12.2	18.07	36.75	4.4	2.0 (46)	2.0 (46)	0.004
BSB-150	77.8	7.78	62.47	28.2	9.6	36.25	12.34	25.0	13.0 (52)	7.0 (28)	0.025
BSB-200	47.4	4.74	38.40	14.8	9.2	31.22	19.41	14.0	10.0 (71)	4.0 (29)	0.014
BSB-250	32.6	3.26	10.43	8.4	8.8	25.77	26.99	8.4	5.0 (60)	3.0 (36)	0.008
BSB-300	37.8	3.78	22.75	9.4	7.8	24.87	20.64	6.4	4.0 (63)	2.0 (31)	0.006
BSB-350	40.0	4.00	27.00	8.0	7.6	20.00	19.00	7.0	5.0 (71)	2.0 (29)	0.007
BSB-400	19.8	1.98	-47.48	7.2	7.4	36.36	37.37	6.6	5.0 (76)	2.0 (30)	0.007
Mean	*39.7*	*3.97*	*17.95*	*10.7*	*9.6*	*25.61*	*27.55*	*9.4*	*5.75 (61)*	*2.88 (33)*	*0.009*
FA-0	23.4	2.34	0.00	6.6	13.2	49.25	56.41	5.0	5.0 (100)	1.0 (20)	0.005
FA-100	35.6	3.56	34.27	12.0	10.2	76.92	28.65	10.4	9.0 (87)	2.0 (19)	0.010
FA-150	52.4	5.24	55.34	27.8	8.6	53.05	16.41	21.4	15.0 (70)	5.0 (23)	0.021
FA-200	44.6	4.46	47.53	27.6	9.4	61.88	21.08	20.8	17.0 (82)	4.0 (19)	0.021
FA-250	37.4	3.74	37.43	22.4	7.4	59.89	19.79	19.2	15.0 (78)	3.0 (16)	0.019
FA-300	25.0	2.50	6.40	15.2	6.6	60.80	26.40	13.0	11.0 (85)	2.0 (15)	0.013
FA-350	21.0	2.10	-11.43	15.0	5.6	71.43	26.67	14.0	10.0 (71)	2.0 (14)	0.014
FA-400	10.4	1.04	-125.00	7.4	2.4	69.16	23.08	6.8	6.0 (88)	1.0 (15)	0.007
Mean	*31.2*	*3.12*	*6.36*	*16.8*	*7.9*	*62.80*	*27.31*	*13.8*	*11.00 (83)*	*2.50 (18)*	*0.014*

Success in plant regeneration rate is measured by the relative numbers of albino and green regenerated plantlets, and the final number of successfully regenerated green plants in anther culture. Albinism is one of the most serious problems encountered in routine anther culture experiments due to the production of high rates of albino plants from pollen (Wedzony et al., 2009). This limits the application of doubled haploid technology in some breeding programs due to the low overall yield of regenerated green plants. It is generally considered as a recessive nuclear trait driven by one or two genes with low heritability, especially in cereals (Kumari et al., 2009). However, low heritability for green plant regeneration in wheat anther culture has recently been reported by Redha and Talaat (2008), suggesting that this may be more dependent on environmental conditions. On the contrary, in harmony with some publications (Tuvesson et al., 2000; Kondic-Spika et al., 2011), in this study wheat genotype significantly influenced the tested parameters (green plantlets, albino plantlets and well regenerated green plants). Genotypic differences were found for green vs. albino plantlets and green plants. The most striking promising results for these parameters were obtained for both genotypes at 150 and 200 Gy gamma irradiation doses *(Table 4).* This study gave no evidence that radiation doses which generally raise response in culture decrease the green and albino regeneration ratio. The absolute number of green plants was raised, while albinos decreased for both genotypes and gamma irradiation treatments. Therefore, the process determining the proportion of albino regenerants appears to be affected by different levels of

gamma radiation within this dose range. While the production of albino regenerants per se is undesirable, their frequency does assist in assessing the overall regeneration potential of any genotype.

Albinism is difficult to eradicate; however, altering the number of green and albino plantlets by appropriate means such as the use of cold pretreatments and/or irradiation (Ding et al., 1991) or the development of interspecies crosses and double haploids (Kumari et al., 2009) can contribute to the reduction (Kumari et al., 2009). Our results agree with findings by Vagera et al. (2004) and Lu et al. (1999) who stated that it is possible to enhance the frequency of *in vitro* pollen embryogenesis through decreasing albino plant formation by mutagenic treatment of seeds.

Haploids must be chromosome doubled to restore fertility for use in plant breeding. Chromosome doubling of microspore-derived plantlets and calli is a critical step in haploid breeding programs. Therefore, rather than having a high number of spontaneous haploid plants, it is desirable to obtain more spontaneous doubled-haploid plants. In some studies, rates of the spontaneous genome doubling are given as 10%-40% in Brassica napus, 70%–90% in barley, 50%-60% in rice, 50%-90% in the rye and 25%-70% in bread wheat (Castillo et al., 2009; Henry, 1998; Segui-Simarro and Nuez, 2008). Stober and Hess (1997) achieved 15-44% spontaneous doubling in German spring wheat varieties, while Barnabás (2003) reported a frequency of 25% to 68% in winter varieties in Central and Eastern Europe. Lantos and Pauk (2016) found that the spontaneous double haploid rate in 10 winter bread wheat F_1 combinations was 32.72%. In the study, spontaneous doubled-haploid plants via spontaneous genome doubling ranged between 7-80% for genotypes with varying gamma doses, with an overall mean was 26%. The genotype FA had a higher potential to develop haploid plants from green plants, whereas the genotype BSB had a lower response. Regarding spontaneously doubled haploid plants, the situation is the opposite of the haploid plant. The results are supported by the findings of Grauda et al. (2016) who showed that genotype significantly affected haploid plant and spontaneous doubled-haploid plants developed from green plants.

Due to independent inheritance and the frequently observed negative correlations between embryogenesis, regeneration and percentage of green plants (Chaudhary et al., 2003), the percentage of green regenerated plants was the determining factor in calculating the final success index of wheat anther culture (Redha et al., 1998). In other words, success in the routine application of double haploid in wheat breeding mainly depends on obtaining acceptable highly regenerated green plants from the targeted genotypes. In combining methods to increase the production of embryos with high regeneration ability, the optimal doubling procedure will result in an improved SI. The magnitude of the SI is very important to breeders in deciding whether and to what extent the production of DHs will play a role in breeding programs. Redha et al. (1998) reported that FSI ranged from 0 to 2.0 by different colchicine treatments. FSI was calculated higher with gamma irradiation treatment compared to controls. The highest FSI was with 150 Gy and followed by 200 Gy. In addition to this, the average effect of gamma rays on FSI was higher compared to control *(Table 4)*. These findings indicate that gamma irradiation treatments can be increased FSI.

5. Conclusion

This study demonstrated the importance of in vitro haploid induction via androgenesis through gamma irradiation in a bread wheat breeding program. In conclusion, it was noted that an increasing level of gamma rays up to 300 Gy positively affected androgenesis. Gamma irradiation on seeds of bread wheat could allow alleviating the albinism phenomenon and improve green plant frequency. The doses of 150-200 Gy gamma rays on seeds have an obvious stimulation effect on all AC parameters (embryogenesis, plant regeneration and green plant formation) and spontaneous genome doubling confirms that the technique of anther culture applied in connection with induced mutations by seed irradiation can be considered a speedy, cheap and safe method to induce haploid and spontaneous doubled-haploid formation, which causes genetic variation in response to bread wheat anther culture. As a result of this study, a total of 888 green plants were regenerated from mutated populations of genotypes. From the total number of regenerated green plants, 635 (71.5%) were haploids and 205 (23.1%) were spontaneous doubled haploids. The remaining green plants were unable to survive. Altogether, 205 spontaneous doubled-haploid mutant lines were produced in the experiment, which has been integrated into the bread wheat breeding program.

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