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THE EFFECT OF GAMMA RADIATION ON *AGROBACTERIUM TUMEFACIENS*–MEDIATED GENE TRANSFER IN DURUM WHEAT (*TRITICUM DURUM* DESF.)

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ABSTRACT

The current study aimed to develop a new *Agrobacterium tumefaciens*-mediated transformation protocol for wheat by using Cobalt 60 (Co-60) originated gamma radiation. Mature embryos of durum wheat (*Triticum durum* Desf.) cultivar 'Cakmak 79' were irradiated by Cobalt-60 originated gamma rays at 0 (control), 20, 40, 60 and 80 Gy (Gray) doses. Then mature embryos were inoculated in 50 ml sterile distilled water having 50 µl bacterial solution of *Agrobacterium tumefaciens* strain GV2260 carrying p35S GUS-INT plasmid for 24 h in a rotary shaker at 180 rpm. In all the examined characteristics, the highest results were obtained from the embryos to which 80 Gy gamma dose was applied. From the results of the research, in the gene transformation to wheat through *Agrobacterium tumefaciens*, it was observed that 80 Gy gamma dose significantly increased the transgenic plant frequency compared to control implementation on which no gamma was used.

KEYWORDS:
Gene transfer, durum wheat, gamma radiation, *Agrobacterium tumefaciens*

INTRODUCTION

Considering that world population is increasing day by day on one hand, and that the fields used for agriculture have reached its limits on the other hand, it is coming to light that increase of yield is required to continue also in the future. In order to reach the required yield level, it is obligatory to improve the genetic structures of the plants. The scarcity of number of species among which cross-breeding is able to be performed, being unable to prevent the transition of undesired characteristics along with the desired ones in realized cross-breeds, and elimination of undesired characteristics through back cross-breeding taking a long period are among the significant negativities of conventional plant breeding [1]. For this reason, the use of environmental friendly biotechnological methods which complement and support the conventional plant breeding has become obligatory in order to ensure yield increase in the future. By the implementation of biotechnological methods, direct transfer of any isolated gene to the desired organism is possible. In these new techniques, permanent insertion of a DNA piece to the chromosomes of cells -having the ability to form a complete plant- forms the basis of gene transfer systems.

Wheat, among the plants used in nutrition of human, ranks the first order in the world in respect of sowing and production. Wheat kernel is in the status of basic nutrition of about 50 countries due to its proper nutritive value, and ease of its storage and processing. Wheat is being used in food and industry sectors as bakery products being in the first place. For this reason, the development of new wheat genotypes through both conventional and modern biotechnological techniques and bringing them to the use of people are extremely significant and valuable. The possibility of obtaining a transgenic (where a foreign gene is inserted in the genome) wheat as the result of gene transfer studies is very low. And this shows how difficult it is to integrate a foreign gene to the wheat genome. When the place of wheat in nutrition of human is considered, it is coming to light how important any study to be performed for placing an agricultural gene –which will increase resistance to biotic (disease and pests) and abiotic (high and low temperature, drought, salinity) stress factors that are decreasing the unit area yield of this plant- to the plant's genome is.

The current study aimed to develop a new *Agrobacterium tumefaciens*-mediated transformation protocol for wheat by using Cobalt 60 (Co-60) originated gamma radiation. Newly described transformation protocol was based on obtaining transgenic wheat plants directly from inoculated mature embryos by *Agrobacterium tumefaciens* bypassing the stages of 'co-cultivation', 'culturing explants on selection medium' and 'recovery of transgenic shoots on selection medium' as reported by Yildiz et al. [2].

MATERIALS AND METHODS

Plant and explant materials. In this study, durum wheat (*Triticum durum* Desf.) cultivar 'Cakmak 79' and their mature embryos – as explant material - were used.

Radiation material and gamma radiation doses. In this study, Cobalt 60 (Co-60) gamma radiation source – being present at Saraykoy Nuclear Research and Training Center (SANAEM) of Turkish Atomic Energy Authority (TAEK) – was used. In the study, the gamma doses of 0 (control), 20, 40, 60 and 80 Gy (Gray) were used in the irradiation of seeds of cultivar 'Cakmak 79'.

***Agrobacterium tumefaciens* strain.** *Agrobacterium tumefaciens* strain GV2260 carrying p35S GUS-INT plasmid was used for inoculation. In the T-DNA region of the plasmid, there is neomycin phosphotransferase II (*npt-II*) gene being controlled by nopaline synthase (NOS) promoter and enabling the selection of plant cells to which gene transfer is performed, in medium including kanamycin. The β-glucuronidase (GUS) gene is being controlled by cauliflower mosaic virus (CaMV) 35S promoter. *Agrobacterium tumefaciens* used in the gene transformation studies - was taken from glycerol stocks stored at -86°C, and was grown in NB (Nutrient Broth) medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin at 28°C in a rotary shaker (180 rpm) (OD_{600nm} = 0.6). Then, 100 µl of this culture was added to 10 ml NB having antibiotics and incubated overnight at 28°C in a rotary shaker (180 rpm) (OD_{600nm} = 0.6) and used for transformation.

Culture Conditions. Growth medium including MS mineral salt and vitamins [3], 3% sucrose and 0.7% agar (Type A) were used. In the preparation of medium, after adjusting the pH of the medium to 5.8 by using 1 N NaOH or 1 N HCl, the sterilization was achieved by keeping for 20 min. under 1.2 atmospheric pressure at 120°C in autoclave. All cultures were incubated at 24±1°C under photoperiod of 16 hours white fluorescence light (27 µmol m^{-2} s^{-1}) and 8 hours total darkness.

Seed surface sterilization and embryo isolation. For surface sterilization, seeds were immersed in ethyl alcohol (70%) for 5 min. and washed with sterile distilled water for 3 times. Seeds were then agitated with commercial bleach (including 5% sodium hypochlorite) for 25 minutes, and were rinsed with sterile distilled water for 6-7 times. The sterile seeds were kept for about 2 hours at 33°C in sterile distilled water to ensure easy separation of the embryos from endosperm.

Transformation procedure. GV2260 strain carrying p35S GUS-INT plasmid was grown overnight in a liquid NB (Nutrient Broth) medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin at 28°C in a rotary shaker (180 rpm) (OD = 0.6) and used for transformation. Isolated mature embryos were inoculated in 50 ml sterile distilled water having 50 µl bacterial solution for 24 h in a rotary shaker at 180 rpm.

Germination of inoculated embryos and seedling growth. The inoculated embryos were transferred to Magenta vessels in MS medium including 100 mg l^{-1} kanamycin and 500 mg l^{-1} duocid for germination and seedling growth. Duocid prevented the development of *Agrobacterium tumefaciens* in selection medium while kanamycin was used for the selection of plantlets to which gene transfer were performed. They were cultured for 3 weeks under 16 hours light/8 hours dark photoperiod at 24±1°C. Non-transformed plantlets turned white by losing their chlorophyll content gradually. The plantlets in dark green color were taken to pots including peat in growth chamber where temperature and humidity were being controlled, and thin nylon bags were applied on pots to ensure the humidity at high rate. Thus, the plants were prevented – which were not used to the outer environment conditions yet - from dying by suddenly losing water. Holes at specific intervals (4-5 days) were opened by scissors on the nylon bags placed on the pots, and the humidity rate was decreased gradually, and finally the nylon bags were completely removed. By this way, the plants were acclimated to outer environment conditions, and then the growth of the plants were ensured by taking the pots to greenhouse. Plants were irrigated with 50 ml water containing 100 mg l^{-1} kanamycin at 2-day-intervals during 14 days.

Genomic DNA extraction. Genomic DNA was isolated from leaves of putative transgenic plants and from control (non-transformed) plants by the modification of the protocol described by Dellaporta et al. [4].

Polymerase chain reaction (PCR). The confirmation of gene transfer was performed by PCR method. The percentage of the transgene was determined by PCR amplification and agarose gel electrophoresis. The PCR primers were chosen to amplify the coding sequence of the transgenes: 5'-ACAAGATGGATT GCACGAACG -3' and 5'-AACTCGTCAAGAAGGCGATAG-3' for a 0.75 kb portion of the *npt-II* gene.

PCR reaction mixtures were prepared using 200 ng/µl DNA, 2 mmol $MgCl_2$, 0.25 mmol dNTP, 0.5 pmol forward (sense) primer, 0.5 pmol reverse (antisense) primers and 0.625 U *Taq* DNA Polymerase (No. EP0402; Thermo Fisher Scientific, USA).

Reactions were incubated in a programmable thermocycler (Techne-Prime Thermal Cycler) for 36 cycles. Each PCR cycle consisted of denaturation at 95°C for 1 min, annealing at 56°C (*npt-II* gene) for 1 min, and extension at 72°C for 1 min. The PCR products were separated by electrophoresis in 1.5% agarose in TAE (tris-acetate EDTA) buffer followed by ethidium bromide staining. The bands were observed under UV after electrophoresis.

Experimental design and statistical analysis. Five replicates were tested. Petri dishes (100 x 10 mm) containing 20 embryos were considered the units of replication. All experiments were repeated two times. One-way Analysis of Variance (ANOVA) was used to test the effect of gamma radiation on transformation efficiency. All experiments were repeated two times. Data were statistically analyzed by "IBM SPSS Statistics 22" computer program. Duncan's multiple range test was used to compare the means [5].

RESULTS AND DISCUSSION

As the ionizing radiation is increasing the variation in population, it is being encountered as a technique used in both classic and modern breeding in obtaining the genotypes of interest. Ionizing radiation is defined as radiation forming ions in the mediums it passes through. When ionizing radiation interacts with biological materials, it is affecting the critical points in the cell. Radiation enters in mutual interaction with the other atoms and molecules and especially with water in the cell, and forms the free radicals that will reach and cause damage to significant elements. Radiation is causing significant deterioration of pectins. The softening in the tissues and breaking of bond among the cells are based on the increase of pectins that are water-soluble. The deterioration of membrane is arising as the result of non-esterification of phospholipids by the free radical forming by the effect of radiation [6]. Calcium plays a significant role in the protection of cell wall structure by interacting with the pectic acids on the cell wall in order to form calcium pectate. Calcium ions are effective in preventing the changes in cell wall structure through preservation of the bond among cells [7].

Different tissues of an organism show different degrees of sensitivity against radiation. One of the most distinct effects of high dose of radiation on the cell level is inhibition of cell growth. Especially during cell division, growth becomes interrupted in cells being exposed to radiation. The nucleus and especially the chromosomes are highly sensitive against radiation. Radiation may cause the chromosomes to break, to attach to each other, to interlock and bend. Chromosome breakages may be reorganized, may remain the same or may join with another chromosome. Due to all these reasons, mutation may arise or cell death may realize [8].

High doses of ionizing radiation is causing physiological changes such as increase of respiration of plants, increase in ethylene production, initiation of enzyme activity and accumulation of some protein types. Cellular macromolecules such as the cell walls, membranes and DNA are physically and biochemically significantly affected from ionizing radiation [9]. High dose of radiation is negatively affecting the plant hormones and their synthesis, and it is decreasing the sensitivity of radiated cells against substances stimulating growth [10].

Against high doses, it was reported by many researchers that the low dose of gamma radiation was encouraging *in vivo* and *in vitro* plant growth. Early ripening of fruits and increase in the weight of fruit and germination of seed may be listed among the encouraging effects of low dose of radiation. The encouraging effect of low dose of gamma radiation is also being observed in the tissue culture. It was noted that low dose gamma radiation increased the development of bean tissue culture, encouraged cell differentiation in tobacco tissue culture, accelerated the carrot's regeneration and increased the formation of micro bumps in potato [11].

When *Agrobacterium tumefaciens* – a plant pathogen - realizes an attack, the plant is operating defense mechanism and trying to withstand such attack and to overcome the attack with the least damage. Due to the defense mechanism of the plant, the regeneration ability of the plant's tissue is significantly decreased after the *Agrobacterium tumefaciens* infection. This condition is the most significant problem before gene transfer by *Agrobacterium tumefaciens*. For this reason, increasing the frequency of gene transition even by 1% is a great success. In order to increase the frequency of gene transformation, changes on parameters such as bacterium density and inoculation period were performed. Vacuum infiltration was another method being used in increasing the transgenic shoot frequency, and due to the negative atmospheric pressure formed on the tissue, reaching of more bacteria to the cell is being ensured, and by this way it is being intended to increase the gene transformation frequency. And the different implementations in inoculation method are able to significantly increase the gene transfer and transgenic shoot frequency.

TABLE 1
Results of PCR analysis in seedlings of durum wheat cultivar 'Çakmak 79' inoculated by *Agrobacterium tumefaciens* after irradiated with different gamma doses

Gamma Dose (Gy)	Number of Inoculated Embryos	Putative transgenic plants developed in selection medium	Number of putative transgenic plants transferred to soil	The number of PCR (+) plants	PCR (+) plant number after *chv* gene analysis	Transfor. efficiency* (%)
0	200	24 d	24 c	0 d	0 d	0.00 d
20	200	25 d	25 c	0 d	0 d	0.00 d
40	200	42 c	48 b	1 c	1 c	2.38 c
60	200	55 b	55 b	4 b	4 b	7.27 b
80	200	64 a	64 a	9 a	9 a	14.06 a

Values in a column followed by different letters are significantly different at the 0.01 level
* Transformation efficiency = (PCR (+) plant number after *chv* gene analysis/Number of putative transgenic plants transferred to soil) x 100

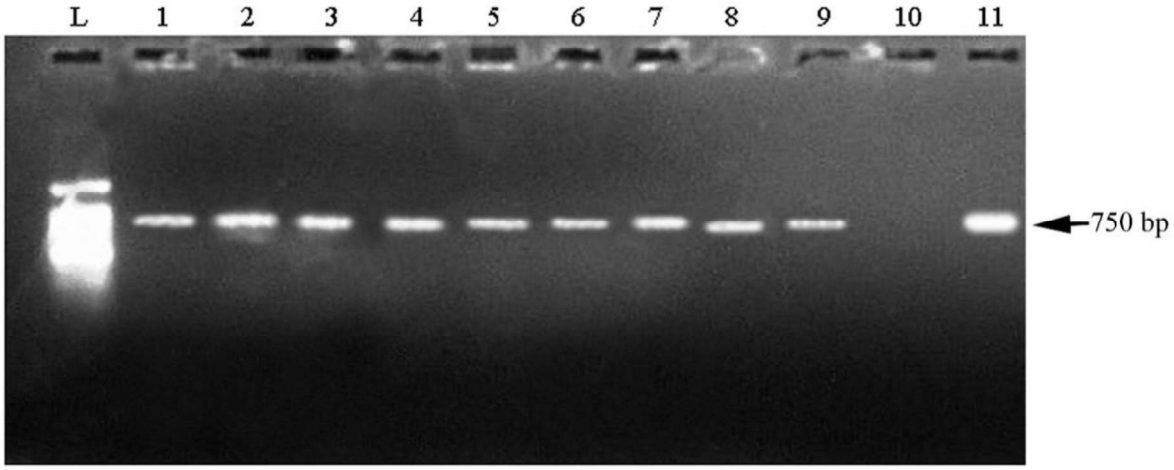

FIGURE 1
PCR analysis of genomic DNA of transgenic plants cultivar 'Çakmak 79' durum wheat grown from irradiated seeds with 80 Gy gamma dose for the amplification of 750 bp *npt-II* gene. L – DNA ladder, 1-9 – transgenic plants, 10 – negative control, 11 – positive control (plasmid DNA)

There are many studies in which gene transformation was achieved in monocots via *Agrobacterium tumefaciens*. There were many studies reporting that gene could be inserted to wheat by *Agrobacterium tumefaciens* [12, 13, 14, 15, 16, 17, 18, 19, 20]. But in these studies in which gene transformation to wheat was realized, the gene transition frequency was very low.

In the current study, after the surface sterilization of the mature wheat seeds, inoculation was performed through *Agrobacterium tumefaciens* to 200 embryos at each of the gamma doses (0-control, 20, 40, 60 and 80 Gy). Only 24 (12.00%) of the 200 embryos –taken to germination- were grown in the control dose on which the gamma radiation was not applied. As the result of the GUS analysis performed on the leaves of these plants, positive results were obtained only in 2 of them, but as the result of the PCR analysis, band of *npt-II* gene was been encountered in none of these 24 plants (Table 1, Figure 1).

In the increasing gamma radiation doses, increases were observed in the number of plants that grow as being germinated in selection medium, in the number of PCR positive plants (Figure 1). In all the examined characteristics, the highest results were obtained from the embryos to which 80 Gy gamma dose was applied. At 80 Gy gamma dose, 64 (32.00%) of 200 inoculated embryos formed plant in selection medium. Sixteen out of 64 putative transgenic plants were found GUS (+). However, only 9 plants was confirmed as transgenic after PCR analysis. In Figure 2, growth of putative transgenic plants in Magenta vessels (A-C) in selection medium and pots (a-c) in soil were shown. The highest number of transgenic plants were recorded in 80 Gy gamma application as 9.00.

The presence of *chv* gene was checked through the analysis performed to determine bacterial contamination, and accordingly, it was confirmed that

bacterial contamination didn't exist in all plants, all plants were transgenic.

In the research using low doses of gamma radiation which will not cause mutation, plant regeneration and gene transformation via particle bombardment technique were studied in wheat (*Triticum* sp.) [21]. In the research, in all the characteristics examined in both wheat cultivars ('Cakmak 79' and 'Bezostaja-1') used in the study performed on plant regeneration through callus culture from mature embryos, the highest results were obtained from the control (0 Gy) application, and gamma dose application of 15 Gy was followed that. In parallel to increase in gamma radiation doses, significant decreases were observed in the results recorded. Even

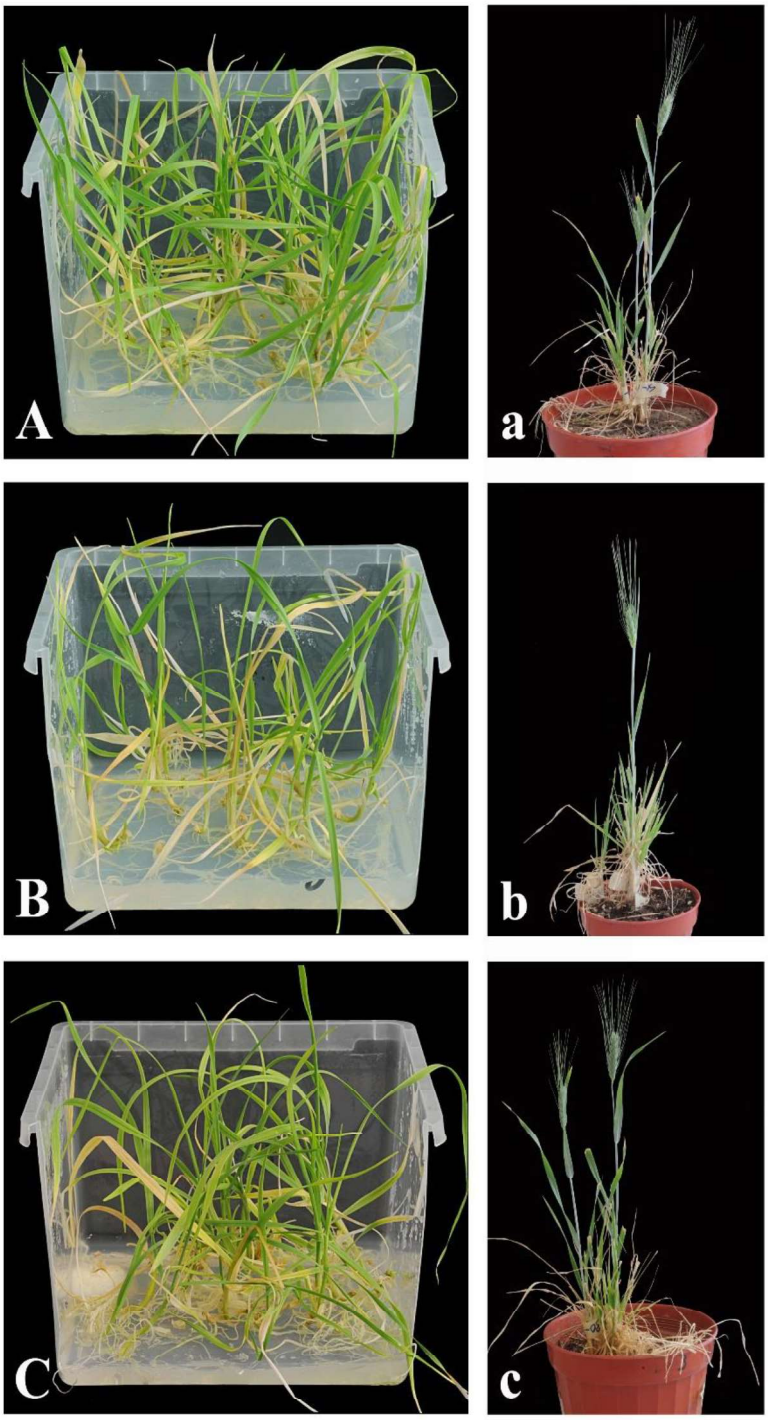

FIGURE 2
Seedlings grown in selection medium from embryos of durum wheat cultivar 'Çakmak 79' irradiated with different gamma doses (A-C) and development of plants in pots which were confirmed as transgenic by PCR analysis (a-c). A-a. 40 Gy gamma, B-b. 60 Gy gamma and C-c. 80 Gy gamma

if shoot regeneration was observed at gamma doses of over 15 Gy, no plant growth occurred. In both cultivars used in the research, the highest results were obtained from the implementations in which Cobalt 60 gamma source was used. When the callus and embryo explants were compared in the gamma ray application of 15 Gy, it was determined that the highest results in all the characteristics were obtained from callus. In transformation studies performed through particle bombardment, higher results were recorded in all the examined characteristics in the radiations performed by Cobalt 60 sourced 15 Gy gamma dose on both cultivars ('Cakmak 79' and 'Bezostaja-1'). Consequently, in the gene transfer to wheat studies performed by the particle bombardment method, the Cobalt 60 sourced 15 Gy gamma dose applied to embryo explant was significantly increased the gene transformation frequency.

From the results of the current research, in the gene transformation to wheat through *Agrobacterium tumefaciens*, it was observed that 80 Gy gamma dose significantly increased the transgenic plant frequency compared to control implementation on which no gamma was used.

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