

Full Length Research Paper

Variation in quantitative characters of faba bean after seed irradiation and associated molecular changes

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The successful use of faba bean breeding for broomrape resistance requires the existence of genetic variation. Unfortunately, the desired variation is often lacking. However, radiation can be used to induce mutations and thereby generate genetic variation from which desired mutants may be selected. This investigation was carried out to study the effects of gamma radiation on various quantitative characters in faba bean. Micro-mutations were scored for percentage of germinated seeds, pod length and photosynthetic pigment contents. The variation in DNA profile in responses to gamma irradiation treatments was detected by ISSR-PCR technique. 15 ISSR primers were used on 22 samples of faba bean issued from irradiated samples; four primers produced clear bands, which were polymorphic and the (AG)₈YC was the best one. Nei's standard genetic distances test showed that the ISSR markers classification was statistically different. Conclusively, this study supported the suggestion that gamma irradiation induce a genetic diversity in faba bean germplasm. The studied samples are promising for the production of synthetic varieties resistant/tolerant to plant parasites.

Key words: Faba bean, gamma rays, genetic diversity, ISSR.

INTRODUCTION

Faba bean is widely used in the Mediterranean region as source of protein in both human and animal nutrition (Larralde, 1982). The nutritional value of field bean has been traditionally attributed to its high protein content (Cubero and Moreno, 1982). It is also a good source of sugars, minerals and vitamins. Thus, the chemical analysis of this legume reveals a 50 to 60% content of carbohydrate, which is mainly constituted by starch, while the proportion of lipids is relatively low at about 1 to 2.5% with oleic and linoleic acids representing about 75% of fats (Mataix and Salido, 1985). Faba bean also contributes to farmer's income and improves the soil fertility through biological nitrogen fixation.

Despite all these beneficial aspects, the area and the production of legumes in Tunisia have not increased in

the last years. Diseases and pests have been reported as recurrent problems in Tunisia (Kharrat et al., 1991). This was highlighted during many seasons, where the majority of faba bean crop was devastated by chocolate spot incited by *Botrytis fabae*. Nematode (*Ditylenchus dipsaci*), rust (*Uromyces fabae* virus diseases and root rot (*Rhizoctonia* spp.) were also present (Hooper, 1983; Kerkoud et al. 2007). Chocolate spot was identified in almost all the areas covered by the survey including the semi-arid and arid areas of the central and southern parts of the country where the climatic conditions are normally not conducive for disease development. Aphids and others insects such as *Sitona* spp. and stem borer (*Lixus algerus*) cause some damage to faba bean (Bardner, 1983). The presence of *Orobanch* spp. in some faba bean growing areas is considered as a limiting factor to the expansion of the crop (Stoddard et al., 2010).

Genetic resistance is considered as the most desirable control method since it is more cost effective and

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environment friendly than the use of chemicals. Gamma irradiation was found to increase plant productivity. In this connection, Jaywardena and Peiris (1988) stated that gamma rays represent one of the important physical agents used to improve the characters and productivity of many plants (for example rice, maize, bean, cowpea and potato). Gamma rays belong to ionizing radiation and interact with atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending on the irradiation level. These effects include changes in the plant cellular structure and metabolism; dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system and accumulation of phenolic compounds (Kim et al., 2004; Kovacs and Keresztes, 2002; Wi et al., 2005).

Radiation mediated *in vitro* mutagenesis and selection has been successfully used to improve agronomic traits such as salinity and drought tolerance in different crop plants (Foster, 2001; Biswas et al., 2002; Predieri and Gatti, 2004; Zhu et al., 2004), advocating that tissue culture selection is useful to select stress-tolerant clones. Several works have shown that mutagenesis with gamma rays can be successfully used to develop new lines useful for breeding, such as sweet potato, grass pea (Ochatt et al., 2004), cocoyam, *Xanthosoma sagittifolium* (Blaye et al., 2004). A major aim for any crop breeding program is the development of good quality lines with an adequate resistance/tolerance to yield-reducing stresses. The aim of this work was to investigate the response of one variety of faba bean to different doses of gamma irradiation (50, 100, 150 and 200 Gy). Seeds percentage germination, plant growth, morphological modifications and nutrients contents were recorded. Certain associated molecular changes were also studied. The results of this work could be used in the breeding programs of faba bean resistant to broomrape.

MATERIALS AND METHODS

Seeds irradiation and germination

The humidity level of the seeds of faba bean was determined before irradiation. It showed humidity percentages of 8% for the variety used. The seeds were irradiated with gamma rays at the Center for Nuclear Sciences and Technologies, Sidi Thabet, Tunisia, derived from Co⁶⁰ source. The dose rate was 19.234 Gy min⁻¹. A completely randomized design was used with different treatments (0, 50, 100, 150, 200, 300, 400, 500, 600 and 700 Gy of gamma rays) to determine LD₅₀.

The irradiated seeds were surface-sterilized with 10% calcium hypochlorite for 30 min, and then rinsed three times with sterile water. Seeds were placed in Petri dishes on a sterile filter paper, imbibed with distilled water and allowed to germinate at 28°C in the dark for 5 days. The seeds that were not subjected to gamma rays served as controls. Germination rate was calculated according to the following formula (Hegazi and Hamideldin, 2010):

$$\text{Germination rate} = \frac{(G1 \times N1) + (G2 \times N) + \dots + (Gn \times Nn)}{G1 + G2 + \dots + Gn}$$

Where G = number of germinated seeds in a particular day and n = number of this particular day.

Vegetative growth characters

The irradiated seed lots (M0) and the untreated seeds, which served as controls were sown in pots in order to study the development of established plants under greenhouse conditions at 25°C with a 16 h photoperiod. After 13 weeks from sowing, the following growth criteria were recorded, using six random plants from each treatment: plant height (cm), number of branches/plant, fresh weight/plant (g) and dry weight/plant (g). Also, photosynthetic pigments: Chlorophylls a, b, "a+b" in leaves was determined calorimetrically. It was recorded that chlorophyll a showed the maximum absorbance at 662 nm and chlorophyll b at 645 nm, the amount of these pigments was calculated according to the formulas of Lichtentaler and Wellburn (1985). The formulas were used in the calculation of chlorophyll a (C_a) and b (C_b) levels:

C_a = Chlorophyll a; C_b = chlorophyll b

C_a = 11.75 A₆₆₂ - 2.350A₆₄₅

C_b = 18.61A₆₄₅ - 3.960A₆₆₂

The nitrogen content was determined using the micro-Kjeldahl method as initially described by the Association of Official Analytical Chemist (AOAC 1975). The nitrogen percentage was then multiplied by 6.25 (N percentage in legume proteins) to obtain the protein percentage.

ISSR analysis

A thousand of faba bean seeds already irradiated with 150 Gy gamma ray dose were planted. Experiments were conducted in field naturally infested with *Orobanche foetida* seeds. Only twenty two from these plants which presents less parasite were selected for ISSR analysis. The ISSR markers were used in order to study faba bean genetic polymorphism after gamma irradiation.

DNA extraction and ISSR-PCR procedure

DNeasy TM plant mini kit (igenomic plant, Intron biotechnology, Inc) was used for DNA isolations from plants. Leaf tissue (100 mg) was ground under liquid nitrogen to a fine powder. Extraction and purification of samples were carried out with the use of DNeasy mini spin columns. ISSR-PCR reaction was conducted using fifteen primers with the sequences shown later. Amplification was carried out in a programmed PCR, the amplification program included a denaturing step at 94°C for 5 min, followed by 40 cycles with denaturing step at 94°C for 1 min, an annealing step at 53°C for 1 min and an extension step at 72°C for 2 min. The last cycle was closed by 72°C for 5 min. Agarose (1.5%) was used for analyzing the PCR products.

(AG)8YC, (GA)8T, (GACA)5, (GA)8CC, (AG)8T, (CA)8G, (CA)6RG, (CA)5RC, (GA)8TC, (CAC)5RC, (CA)8AC, (TG)6YA, (AC)8YG, (AG)8YA. R = A/T, Y = C/G.

Statistical analysis

Data were analysed by using SPSS software. Means were

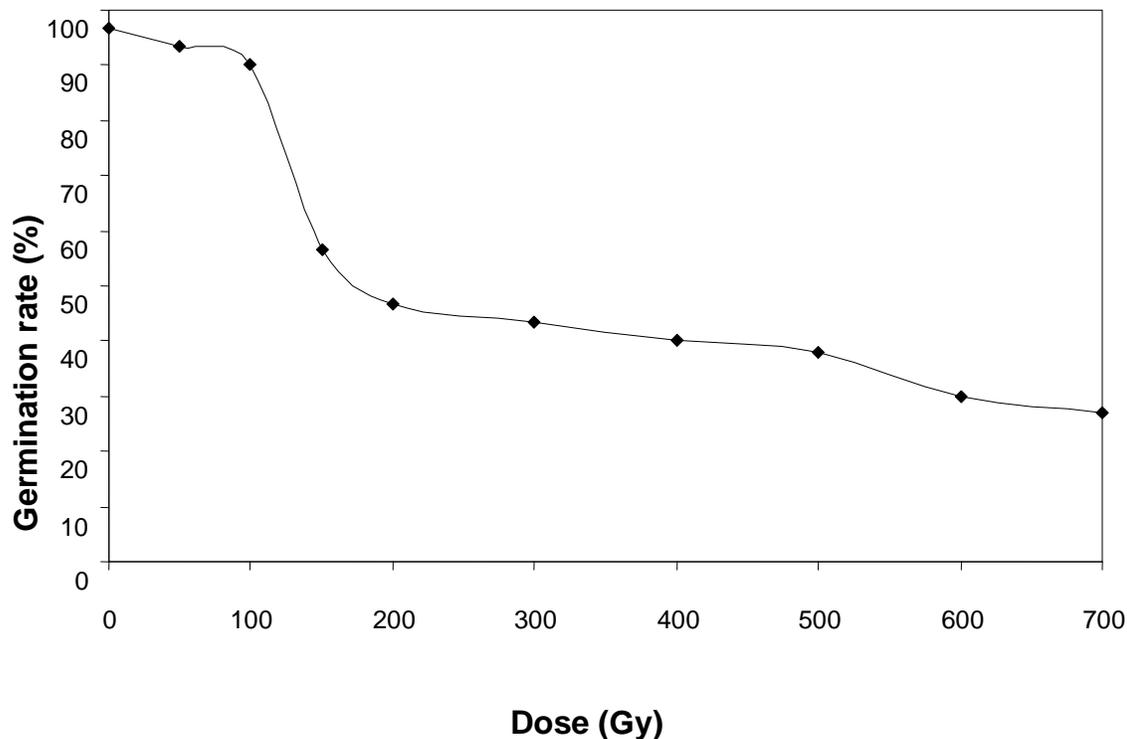


Figure 1. Effect of irradiation on seeds germination of faba bean (100 seeds were used for each dose).

compared using the Benferroni test at $P < 0.05$. In the tables, means followed by the same letter are not significantly different.

RESULTS AND DISCUSSION

Effect of irradiation on germination and survival rate

Treated seeds were evaluated for lethality from different doses of gamma irradiations. It was observed that seed germination was independent of dose of gamma rays and was mainly affected by the germination capability, which is in agreement with Ciftci et al. (2006). Gamma irradiations had an insignificant effect on germination. Irradiation with doses from 200 to 700 Gy, however, resulted in a germination score significantly lower than those of the unirradiated control (Figure 1). It was found that each increase in the dose of gamma irradiation was accompanied by a corresponding decrease in seeds germination. The LD_{50} for the genotype of faba bean used in this study was 150 Gy, causing 50% of reduction in seed germination.

After exposure to 200 Gy, seedlings exhibited a high rate of mortality (Figure 2). The highest exposure (700 Gy) caused death of all seedlings 10 days after germination. This result clearly shows that the significant biological damage for the growth of seeds is induced by the gamma irradiation. The results indicate that the seeds can repair damage produced by γ -irradiation below 100 Gy, whereas they are killed completely after

700 Gy irradiation. The same observations are reported by Chaudhuri (2002) in the case of irradiated rice and lentil seeds.

Effect of irradiation on dry weight and height

Different radiation exposures influenced plant dry weight during the course of the growth (Table 1). Except for 100 Gy, root length was significantly reduced as dosage increased (Table 1). With exception of 100 Gy, the seeds treated with 150 and 200 Gy significantly reduced shoot dry weight (Table 1). This observation suggests that dry matter production of faba bean reduces with increasing gamma ray radiation.

The height reduction caused by the doses was in agreement with the findings of Preussa and Britta (2003) who reported that reduction of plant heights is the most frequently arising type in mutation experiment. Zaka et al. (2002) also got dwarf or shorter plant height in their study on faba bean. The reduction in root length with increasing dosage also partly explains the reduction in plant height as these two variables are interrelated.

Other physical damages that occurred on the leaves of faba bean (Figure 3) seem to fit the description of Long and Sparrow (1954). Gamma irradiation is known to alter both morphological and physiological processes in crops (Long and Kersten, 1936; Sax, 1963; Sparrow, 1954). Kuzin et al. (1964) suggested that radiation at lower

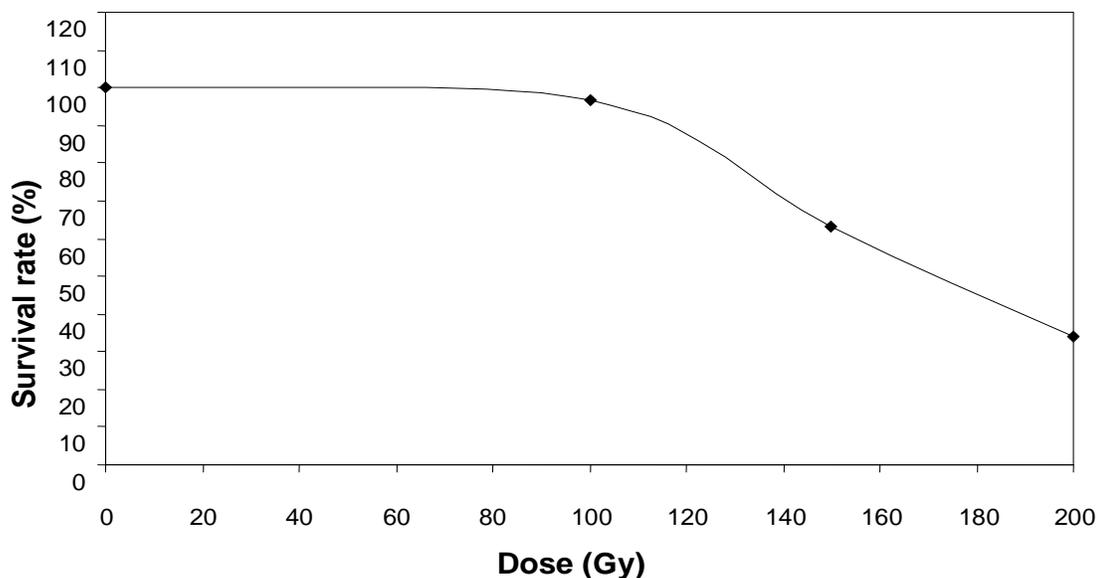


Figure 2. Effect of irradiation on survival of faba bean seedlings after 90 days seed germination (six seedlings were used for each dose).

Table 1. Effect of irradiation on dry weight and length of roots and shoot of faba bean 90 days after planting.

Parameter	Root		Shoot	
	FW (g)	Height (cm)	FW (g)	Height (cm)
Control	15.73 ± 5.15 ^a	55.5 ± 11.51 ^a	14.56 ± 3.28 ^a	26.16 ± 4.79 ^a
100 Gy	13.41 ± 4.40 ^b	52.66 ± 9.9 ^a	9.31 ± 4.61 ^b	28.83 ± 3.86 ^a
150 Gy	11.71 ± 2.62 ^c	45.66 ± 6.56 ^b	9.5 ± 1.32 ^b	25.5 ± 2.94 ^a
200 Gy	10.3 ± 2.30 ^d	43.5 ± 4.88 ^b	7.85 ± 3.72 ^c	24.5 ± 3.01 ^a

Values within the same column followed by the same letters were not significantly different using Benferroni test at 5% level.

Table 2. Nitrogen percentages (N%) of irradiated and wild-type samples at different localisation in the plant.

Parameter	Flowering time		Harvest time		
	Root	Shoot	Root	Shoot	Seed
Control	2.576 ± 0.103 ^a	3.652 ± 0.146 ^a	1.31 ± 0.052 ^a	1.79 ± 0.071 ^a	4.25 ± 0.17 ^a
100 Gy	1.178 ± 0.047 ^b	3.332 ± 0.133 ^b	1.93 ± 0.077 ^b	1.38 ± 0.055 ^b	4.36 ± 0.147 ^b
150 Gy	1.204 ± 0.048 ^c	3.108 ± 0.124 ^c	1.24 ± 0.049 ^a	1.176 ± 0.047 ^c	3.94 ± 0.157 ^c
200 Gy	1.26 ± 0.050 ^d	2.66 ± 0.106 ^d	1.156 ± 0.046 ^a	1.904 ± 0.076 ^a	4.17 ± 0.166 ^d

Values within the same column followed by the same letters are not significantly different, using Benferroni test at 5% level

doses often induced the production of small quantities of radiotoxins which resulted in a gross stimulation of growth and development. As mentioned earlier, gamma irradiation would induce noticeable morphological changes in plant tissues as well as a variety of biochemical responses at the cellular level (Blaye et al., 2004; Yang et al., 2004).

Chlorophyll and nitrogen content

Data presented in Figure 4 and Table 2 indicated that there was significant difference between plant from irradiated seeds and the control in chlorophyll and nitrogen contents. The results show that chlorophyll a and b content was significantly decreased as a result of gamma

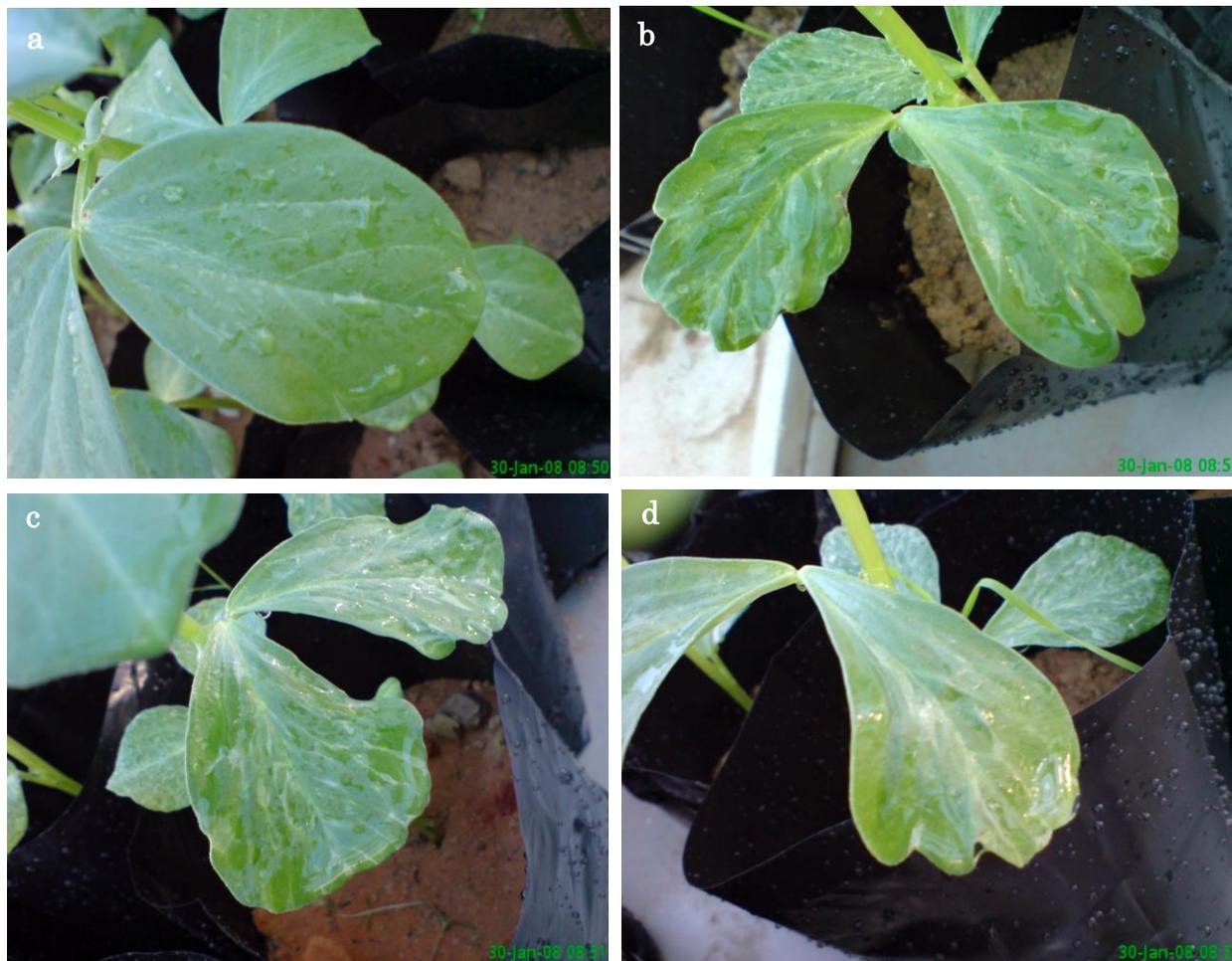


Figure 3. Morphological variance of faba bean leaves response to gamma irradiation. a, Wild type; b, c and d, irradiated samples (six seedlings were used for each dose).

irradiation treatments (Figure 4). Results suggest that seeds irradiation serve to modify plant metabolism and photosynthetic capacity. Pigments decrease gradually in leaves at increasing γ -ray irradiation doses. Chlorophyll concentration in faba bean leaves of the plant that emerged from treatment with 200 Gy, when compared with the wild type, decreased by 58.18 and 41.80% for Responses of plants to gamma rays and effects on chlorophyll were studied by Wada et al. (1998). They stated that gamma radiation with high doses of γ rays (100 to 500 Gy), caused plant growth inhibition, chlorophyll degradation and morphological aberration in *Nicotiana* plants. A dose dependent increase in the frequency of chlorophyll mutations was noticed. Other researches have shown that after gamma radiation with high doses of γ -rays, plants at the late developmental stage of seed maturation suffer leaf senescence and lose cellular components, for example proteins and chlorophylls (Crafts-Brandner and Egli, 1987; Abarca et al., 2001).

In contrast to our results, Rejili (2008) showed that exposure of two *Medicago sativa* populations (Mareth and Gannouch) to gamma irradiation (350 Gy), alone or in combination with salt stress, increased significantly chlorophyll b content, especially for the Gannouch population, while no change occurred for the Mareth population.

Nitrogen content measured at harvest time was close to 3.94, 1.24 and 1.176% N, respectively in seed, roots and shoot after 150 Gy irradiation dose. The protein levels were found to decrease upon gamma irradiation on faba bean seeds when compared with the wild-type. In contrast, these results show that the protein content in faba bean seeds was not affected by gamma irradiation treatment (Table 2).

ISSR-PCR of genomic DNA

ISSR-PCR was used for the detection of DNA profile

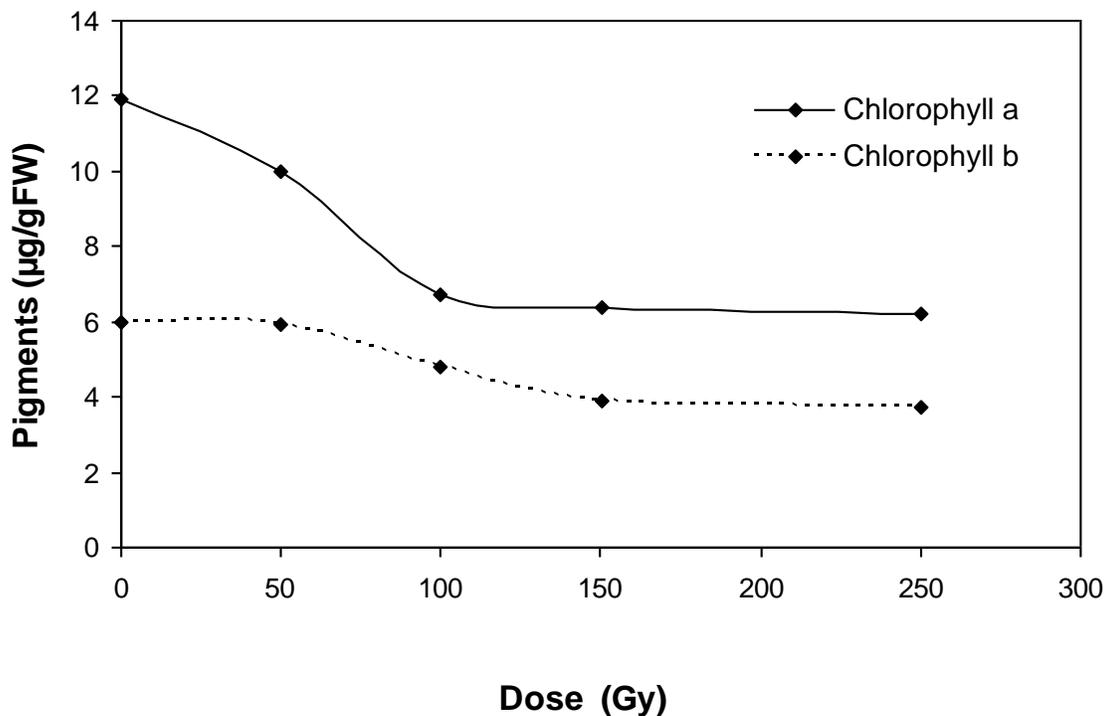


Figure 4. Effect of irradiation on pigments variance ($\mu\text{g/g}$ of fresh weight) in faba bean leaves (six seedlings were used for each dose).

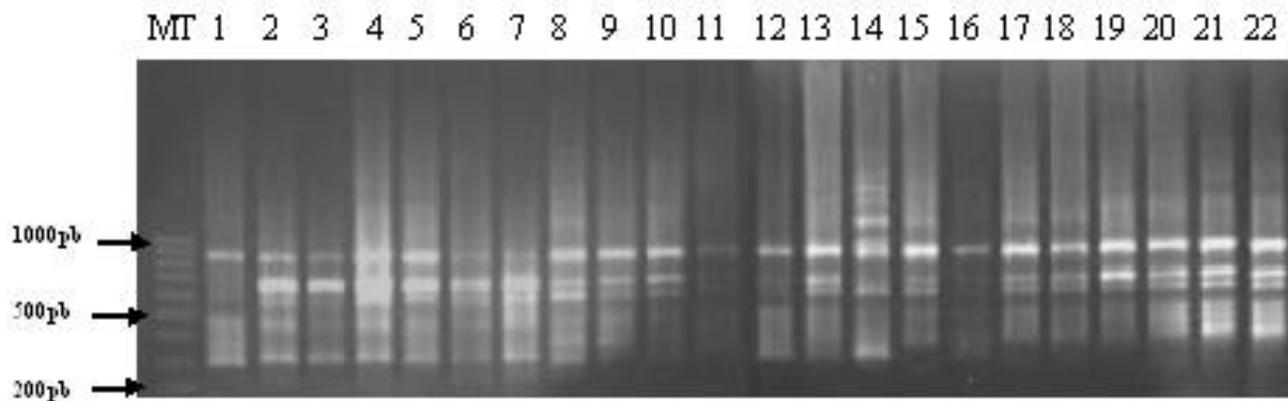


Figure 5. A representative ISSR amplification of faba bean population samples using primer $(\text{AG})_8\text{YC}$. Population code is on top of each lane, followed by a letter indicating the study sample. Samples 1 to 20 were issued from irradiated seeds culture and samples 21 and 22 are the wild types.

changes in mutants generated by gamma ray. 15 ISSR primers were evaluated in a preliminary experiment. Only four primers [$(\text{AG})_8\text{YC}$, $(\text{GA})_8\text{T}$, $(\text{GACA})_5$ and $(\text{GA})_8\text{CC}$] successfully amplified DNA fragments from faba bean DNA samples. As a result, they were used in this study. Genetic analyses of faba bean DNA showed a high degree of polymorphism in gamma rays irradiated seeds as compared to the controls. A high proportion of polymorphic bands were found using this ISSR marker

(Figure 5). Genetic analyses of faba bean's DNA showed a high degree of polymorphism in gamma rays irradiated seeds when compared with the controls. Among the 15 tested primers, the ISSR primer $(\text{AG})_8\text{YC}$ selected for this analysis generated a total of 187 fragments. The size of amplified products ranged from 900 to 300 bp. The number of scorable markers produced per primer ranged from 6 to 11. The total number of polymorphic markers and the percentage of polymorphism were 182 and

97.32%, respectively. ISSR marker profile produced by the primer (AG)₈YC in agarose gel is shown in Figure 5. ISSR primers generated six to 11 markers with an average of 8.5 per mutant. The main changes observed in the ISSR profiles are appearance or disappearance of different bands (Figure 5) with variation of their intensity as well. These effects might be connected with structural rearrangements in DNA caused by different types of DNA damages (breaks, transpositions, deletion, etc).

Conclusions

Exposing seeds of faba bean to higher gamma ray dosages, particularly 600 and 700 Gy cause reduction in seeds germination and survival rate. The lethal doses for these variables increase with increasing dosage of gamma ray. According to the germination seeds, the LD₅₀ was determined to be 150 Gy. Gamma irradiation also causes mutation of the leaflets and chlorophyll deficiency in faba bean. From this study, it can be generally concluded that irradiating seeds of faba bean with higher amounts of gamma rays greatly induce morphological changes. These modifications in growth traits and morphological changes were accompanied with a marked modulation in the DNA profile.

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