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Physiological and Molecular Changes in Fenugreek (*Trigonella foenum-graecum* L.) As A Response to Gamma Rays

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Abstract: Two pot experiments were carried out at the green house of National Research Centre, Dokki, Giza, Egypt, during the two successive seasons (2013-2014) and (2014-2015) to study the effect of gamma rays on growth, productivity and conduct genetic diversity analysis of fenugreek seeds. This study was performed by exposing the seeds of fenugreek (*Trigonella foenum-graecum* L.) to different gamma ray doses (0, 100, 150, 200, 250 and 300 Gy.). The study revealed that there was stimulation in germination percentage, plant survival percentage by increasing γ- ray doses up to 200 Gy, and inhibition appeared at higher doses. There were positive effect of γ- ray doses on morphological criteria; plant height, number of leaves/plant, stem and leaves fresh weight till the dose 200 Gy which recorded the highest values. Moreover, data recorded that γ- ray doses at 100,150 and 200 Gy increased all yield characters. The number of pods per plant was increased by increasing gamma ray doses up to 200 Gy which recorded the highest number (30.81 pods/plant) as compared to control (26.80 pods/plant). The same trend was found in other studied characters (pods yield/plant, seeds yield/plant and the weight of 1000 seed). Gamma ray also enhanced the percentage of protein and soluble carbohydrate content and decrease the percentage of oil in yielded seeds. The varied effect of gamma ray was cleared on content of phenolic content. There were many new protein bands in irradiated plants which can be used as markers for each dose. Using DNA-RAPD assay, there were three negative molecular markers which found only in control as compared to irradiated plants, with molecular sizes 751.687, 389.280 and 358.697 bp.
Key words: fenugreek, gamma ray, protein electrophoresis, DNA molecular markers, RAPD.

Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is one of the family *Fabaceae* [1], popular medicinal herb extensively used as a food [2]. Recent researches identified antioxidant, hepatoprotective, anticarcinogenic and other miscellaneous medicinal effects of fenugreek [3].

Gamma ray, X-ray, visible light and ultra violet are electromagnetic radiation that initiate or inhibit the growth and differentiation of plant cells and organs [4]. Gamma ray interact with cell internal components and release free radicals, these free radicals either damage or modify the differentiation process, morphology, physiology and bioactive components depending on applied dose [5].

Gamma ray are known to influence plant growth and development by inducing cytological, genetical, biochemical, physiological and morphogenetic changes in cells and tissues [6,7]. Whereas, low doses can enhance

the physiological activities of cells in plants by ameliorating germination and growth rates [8,7], increase stress resistance [9,10] and /or improving crop yields [11,12].

Exposing the dry seeds to low γ-irradiation doses resulted in the increasing yield of some plants such as sunflower [(13)] and *Ammi visnage* [14]. So, Gamma irradiation can be useful for the alteration of one or a few physiological characters [15,16].

Rashed *et al*. [17] stated that gamma rays induced appearance and/or disappearance of some protein bands causing modulation in protein patterns. Moreover, the effect of low and high gamma ray doses on the genomic DNA were studied and concluded that changes in Okra genomic DNA pattern due to high doses of gamma rays (400 and 500 Gy) was more pronounce than the low dose (300 Gy) [18]. Raisheed *et al.* [19] used RAPD method to detect the genetic variation induced by gamma rays. Different levels of DNA damage may be increased due to exposure to gamma rays and can be detected by changes in RAPD profiles [20.]

The objective of this study to investigate the effect of gamma ray on growth, productivity and conduct genetic diversity analysis of fenugreek seeds

Materials and Methods

The present study was carried out during two successive seasons 2013/2014 and 2014/2015 at the Greenhouse of Botany Department, National Research Centre, Giza, Egypt and Genetics Department, Zagazig University, Egypt. Fenugreek seeds (Giza33) were secured from Agricultural Research Centre, Ministry of Agriculture, Giza, Egypt.

Seeds of fenugreek were irradiated by six doses from gamma ray; 0, 100, 150, 200, 250 and 300 Gy. at National Center For Radiation Research and Technology, Cairo, Egypt. Irradiated seeds were sown directly in pots, filled with a mixture of peat-moss and sand, and arranged in a complete randomized block experimental design with three replicates. The percentage of germination and plant survival were determined. Random samples from irradiated plants were taken to study the effect of gamma ray on the following morphological characters at vegetative growth stage (60 days old); plant height (cm), number of leaves per plant, leaves fresh weight (g/plant), stem fresh weight (g/plant), leaves dry weight (g/plant), and stem dry weight (g/plant). At harvest, the following characters were determined; number of pods per plant, pods yield per plant (g), seed yield per plant (g) and 1000 seed weight (g).

Chemical analysis in yielded seeds

The yielded seeds were dried and grinned for chemical analysis. The percentage of protein content was determined by micro kjeldahl method according to AOAC, [21]. Total soluble carbohydrates were determined using the colorimetric method described by Dubois *et al.* [22]. The oil content of the seeds was determined according to the procedure reported by AOAC, [21]. Total phenolic compounds were determined calorimetrically according to the method defined by Snell and Snell [23] using Folin Ciocalteu phenol reagent.

Extraction of seed proteins and SDS-PAGE analysis

The protein extraction technique employed was similar to the extraction technique described by Saraswati *et al.* [24]. Protein profiling of seed samples was performed using SDS-PAGE as described by Laemmli [25].

Molecular assay

RAPD-PCR of genomic DNA

DNA Extraction

Total DNA was extracted from 1g of young leaves using Biospin plant genomic DNA extraction kit (Bio Basic Inc. Kit Leading Supplier and Manufactures of Life Science Products and services, Canada). DNA quality was checked using 1.0% agarose gel electrophoresis.

RAPD assay

RAPD assay performed with random decamer primers obtained from DNA amplification was done using 10 RAPD primers (Table **1**). Polymerase chain reaction (PCR) was carried out in a volume of 25 µL

containing Reaction buffer with $MgCl_2$, primer, (dNTPs), *Taq* polymerase and genomic DNA. The PCR mixture was subjected to 40 cycles in PCR with variable denaturation and annealing temperature. The products of amplification were stored at 4°C till further usage. Amplified products along with external size standard were stained with ethidium bromide and separated in a horizontal gel electrophoresis unit using 1.5 % agarose gel [(26)].

Statistical analysis

The data were statistically analyzed on complete randomized design system according toSnedecor and Cochran (1980). Combined analysis of the two growing seasons was carriedout. Means were compared by using least significant difference (LSD) at 5% levels of probability

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Table1. List of RAPD primers used in finger printing in irradiated *Trigonellafoenum-graecum* plant

S/N	Primer name	PrimerSequence (5`⟶3`)
1	A-**19**	CAA ACG TCG G
2	B-05	TGC GCC CTT C
3	B-10	CTG CTG GGA C
4	C-**17**	TTC CCC CCA G
5	A-**06**	GGT CCC TGA C
6	A-08	GTG ACG TAG G
7	C-**20**	ACT TCG CCA C
8	D-**03**	GTC GCC GTC A
9	D-05	TGA GCG GAC A
10	D-07	TTG GCA CGG G

Results and Discussion

The data presented in Table 2 show stimulation in seed germination and germinated plant survival with the increase in gamma ray doses up to 200 Gy. The maximum seed germination was noticed at 200 Gy (94%), whereas, the minimum seed germination was recorded at 300 Gy (50%).

Many workers have reported the adverse effects of gamma ray on various parameters depending on the dose [(28-29-30-31)]. The decrease in seed germination at high doses may be attributed to the damage of cell constituents at molecular level or due to altered enzyme activity [32,33]. Micco *et al.* [34] have correlated the decrease in seed germination with abnormalities in mitotic cycles and in metabolic pathways of the cells. The reduction in plant survival is attributed to cytogenetic damage and physiological disturbances [35]. Srivastava *et al.* [36] suggested that the reduction in wheat seedling survival due to the hindrance caused by the gamma ray on different metabolic pathways of the cells. Similar findings have also been reported in sunflower [37] and in *Trigonella foenum-graecum* L. [31]. On the other hand, low doses of gamma irradiation have profound effect on tomato and okra plants [38] and durum wheat [39].

Table 2. Effect of Gamma rays on seed germination and survival plants of irradiated*Trigonellafoenum-graecum*L.

Treatments	Germination %	Germinated Plant survival %
Cont	77	77.89
100 Gy	87	88.61
150 Gy	89	90.22
200 Gy	94	93.45
250 Gy	64	59.00
300 Gy	50	41.34
Mean	**76.83**	**74.59**

Highly significant effects were recorded between the effect of different doses of gamma ray and control (Table 3). Positive effects of low γ – ray doses were recorded on plant height, number of leaves/plant, fresh and dry weights of leaves and stem till 200 Gydose which recorded the highest values. Gama rays at dose of 300 Gy is considered the dangerous dose that caused the lowest values in all studied characters. These results are in a good agreement with Soliman *et al.* [40]; Nassar*et al.* [41]; Fayed *et al.*[42] and Khater *et al.* [43].

These results were confirmed with the principle concept of γ – ray effects as ionizing radiation, at low doses stimulated metabolism and subsequently increased performance of organism (Ashraf *et al.* [5] and Jan *et al.* [7]), while at high doses induce sever damage especially to enzymes and protein molecules (Fayed *et al.* [42]; Bashir *et al.* [31].

Data in Table (4) illustrated a markedly increase in all yield characters as a result of low doses of gamma ray. The number of pods per plant was increased by increasing gamma ray dose up to 200 Gy which recorded the highest number (30.81 pods/plant) as compared to control (un radiated) which recorded (26.80 pods/plant). Regarding, gamma ray dose higher than 200 Gy dose, it was noted that the all studied characters (number of pods and pods yield/plant, seeds yield/plant and the weight of 1000 seed) decreased gradually.

These results were confirmed earlier by Dubey *et al.*[44]; Mishra *et al.*[45]; Singh *et al.* [46] they stated that low dose of gamma irradiation lead to an increase in yield and yield components of different crops.

Low doses of γ-rays had a stimulatory effect on yield attributes of *Psoralea corylifolia* L, including number of pods per plant, number of flowers per plant, seed index, etc. and inhibition of the same attributes appeared at higher rates [(7)]. Moreover, the number of chickpea pods/plant and seeds/pod were reduced at high doses of gamma ray as shown by Karim *et al.* [47]

Table 3. Mean performance for morphological criteria in*Trigonella foenum-graecum*plants at vegetative stage under different doses of γ – ray at 60 days from sowing

Treatments	Plant height (cm)	Leaves number/ plant	Leaves fresh weight/ plant (g)	Stem fresh weight / plant (g)	Leaves fresh weight/plant (g)	Stem fresh weight/ plant (g)
Cont.	38.46	14.00	4.25	3.31	0.850	0.662
100 Gy	40.3	14.76	4.43	3.58	0.886	0.716
150 Gy	43.32	16.48	6.63	5.28	1.326	1.056
200 Gy	45.64	16.90	6.91	5.57	1.382	1.114
250 Gy	39.26	15.18	3.86	3.28	0.772	0.656
300 Gy	32.72	13.08	3.73	3.03	0.746	0.646
LSD 5%	**0.306**	**0.867**	**0.031**	**0.092**	**0.012**	**0.06**

Table 4. Mean performance of yield criteria in *Trigonella foenum-graecum*plants (at harvest stage) under different doses of γ – ray.

Treatments	Pods number/plant	Podsyield/plant (g)	Seeds yield/plant (g)	1000 seed weight (g)
Cont	26.80	4.98	6.26	31.74
100 Gy	28.85	5.65	6.81	32.37
150 Gy	27.20	5.92	7.48	35.42
200 Gy	30.81	6.29	8.57	38.24
250 Gy	27.21	4.32	5.72	34.24
300 Gy	21.64	3.14	4.12	30.14
LSD 5%	**1.118**	**0.024**	**0.241**	**0.035**

Irradiation of fenugreek seeds with gamma radiation caused a significant increase in protein percentage, soluble carbohydrate, and phenolic content (Table 5) in yield seeds. The highest level of protein and soluble carbohydrate was cleared at 200Gy of gamma ray. On other hand the high level of phenolic content reported at 250Gy. The 100Gy dose induced significant increase in oil percentage while all other treatment reduced the oil content, where the adverse effect was increased with increasing dose of gamma ray. Several studies reported the stimulatory effects of radiation on plant growth [48,49]. Nouri and Toofanian [50] stated that low doses of gamma ray caused a highly significant differences in the level of carbohydrate constituents in onion and potatoes. Kim *et al*. [48] attributed the effect of gamma ray on plant cell to the accumulation of phenolic contents. Anna *et al.* [51] reported that 30 Gy of gamma irradiation enhanced protein synthesis in *Citrus sinensis*.

Table 5. Effect of gamma ray on some chemical constituents of the yielded *Trigonella foenum-graecum*seed

Treatment	Protein%	Solublecarbohydrate mg/g DW	Oil %	Phenolic content mg/g DW
Cont.	17.77 e	29.78 c	5.760 ab	58.21 c
100 Gy	18.53 d	31.08 ab	6.15 a	55.70 c
150 Gy	21.79 b	30.23bc	5.57 ab	56.23 c
200 Gy	22.90 a	31.38 a	5.45 ab	65.11 b
250 Gy	18.95 c	31.66 a	4.99 ab	73.43 a
300 Gy	18.81 c	31.05 ab	4.63 b	65.46 b
L.S.D 5%	0.28	1.05	1.24	2.61

SDS-Protein electrophoresis

The electrophoretic banding patterns of total seed protein as revealed by SDS-PAGE were used to detect the genetic diversity among irradiated fenugreek plants. These results revealed a total of 36 polypeptide bands (Table 6) with different molecular weights ranging from 21.443 to124.474 kDa, of which 2 bands only were polymorphic (Table 6 and Figure 1). The maximum number of bands (8) was found in gamma ray doses 100 and 200 Gy of fenugreek with a polymorphism value of 22.22%. The minimum number of bands (4) was found in control and dose 150 Gy with a polymorphism value of 11.11%. On the other hand, data in (Table 6) illustrated that there were many new protein bands between irradiatedplantswhich can be used as markers for each dose. However, there were unique bands in each dose which varied in number (3, 3, 4 and 2) for (100, 200, 250 and 300 Gy.) respectively with sizes (64.540, 52.337 and 21.443 KDa) for 100 Gy, (65.382, 50.998 and 21.723 KDa) for 200 Gy, (84.551, 64.960, 51.999 and 24.623 KDa) for 250 Gy and (87.336 and 66.810 KDa) for 300 Gy, respectively. (Table 6).

Table 6. Summary of seed protein banding pattern of studied *Trigonella foenum-graecum*accessions using SDS-PAGE analysis.

MW	Cont.	100 Gy	150 Gy	200 Gy	250 Gy	300 Gy	Polymorphism
124.474	1	1	1	1	1	1	Monomorphic
87.336	0	0	0	0	0	**1**	Unique
84.551	0	0	0	0	**1**	0	Unique
81.855	1	1	1	1	0	0	Polymorphic
66.810	0	0	0	0	0	**1**	Unique
65.382	0	0	0	**1**	0	0	Unique
64.960	0	0	0	0	**1**	0	Unique
64.540	0	**1**	0	0	0	0	Unique
52.337	0	**1**	0	0	0	0	Unique
51.999	0	0	0	0	**1**	0	Unique
50.998	0	0	0	**1**	0	0	Unique
37.443	1	1	1	1	1	1	Monomorphic
28.397	1	1	1	1	1	1	Monomorphic
24.783	0	1	0	1	0	0	Polymorphic
24.623	0	0	0	0	**1**	0	Unique
21.723	0	0	0	**1**	0	0	Unique
21.443	0	**1**	0	0	0	0	Unique
Total	**4**	**8**	**4**	**8**	**7**	**5**	

Overall, SDS-PAGE analysis of seed storage proteins revealed three types of bands: monomorphic (bands appeared in all accessions), polymorphic (bands appeared in some accessions and not others), and unique (band appeared in only one accession). These bands varied quantitatively and qualitatively with respect to molecular weight, concentration, relative mobility, or fractionation. Consequently, these bands can be considered as biochemical markers to characterize each germplasm accession. In contrast, several protein bands disappeared in some accessions and appeared in others. The resulting profiles showed different patterns, indicating variability among accessions from different gamma ray doses (Figure 1)**.**

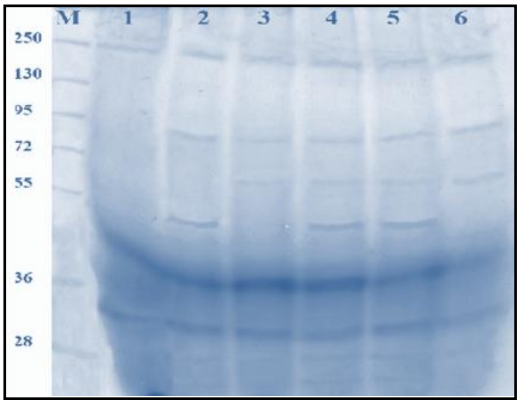

Fig 1. SDS-PAGE of protein extracts of *Trigonella foenum-gracum* irradiated seeds under different doses of gamma ray. Lane (M) = Marker. Lanes: 1=Control, 2= 100 Gy, 3= 150 Gy, 4=200 Gy, 5= 250 Gy, 6= 300 Gy.

Humera [54] stated that the stress reaction of plants often results in the alteration of protein metabolism. Several proteins are synthesized and accumulated in plant tissues under a range of stress conditions. Therefore, electrophoretic patterns of seed proteins as revealed by SDS-PAGE can be employed for various purposes, such as genetic diversity, biosystematic analysis, and determination of polygenetic relationships and evolutionary relationships of species collected from different natural habitats [52,53,54.]

Protein polymorphism helps in distinguishing plant germplasm at specific levels. Polymorphisms occurring within amino acid sequences may result due to specific environmental factors in different geographical regions [(55)]. Therefore, these polymorphisms may serve as genetic markers because they can be highly polymorphic and their variability is generally highly heritable. Additionally, protein polymorphisms resulting from insertions or deletions between mutated sites of protein bands are codominant, and these were found in agreement with Mondini*et al*. [56]. Moreover, appearance of new bands (unique) usually results from different DNA structural changes (e.g., breaks, transpositions, deletions), which leads to changes in amino acids, and consequently the protein formed [(57-54)]. Moreover, proteins might play a role in signal transduction, anti-oxidative defense, anti-freezing, heat shock, metal binding, anti-pathogenesis or osmolyte synthesis which were essential to a plant's function and growth [58.]

RAPD-PCR of genomic DNA

In the RAPD analysis, 10 primers were used for polymorphism screening, out of which only 5 primers were found polymorphic. A total of 35 bands in the size range of 272.573 to 3423.550bp were produced by examining across genotypes with 5 RAPD primers. The total number of bands was 35 ranged from 1 (Primer-D-03) to 14 (Primer- A-06). Moreover, with the five primers molecular markers, defined as positive molecular markers (28 molecular markers) which found in irradiated plants in comparable with control with numerous molecular sizes (Table 7).

On the other hand, there were two negative molecular markers, which found only in control as compared to irradiated plants, with molecular sizes 751.687, 389.280 and 358.697 bp. Moreover, there were three common molecular markers which found in both control and irradiated plants (Table 7).

The main changes in the RAPD profiles of banana under the effect of gamma irradiation were attributed to the appearance or disappearance of different bands with variation in their intensity [(59)].These changes may correlate with the level of photoproducts in DNA template after radiation which can reduce the number of binding sites for Taq polymerase. Danylchenko and Sorochinsky [60] reported that appearance of new bands is usually resulting from different DNA structural changes (Breaks, transpositions, deletion….etc).

Moreover, the obtained results indicated that RAPD marker can be used effectively in determination the variation among treated fenugreek, and these results is agree with previous results that obtained by Tomar *et al*. [61] using RAPD and ISSR molecular markers to determine the genetic relationship among 30 fenugreek genotypes.

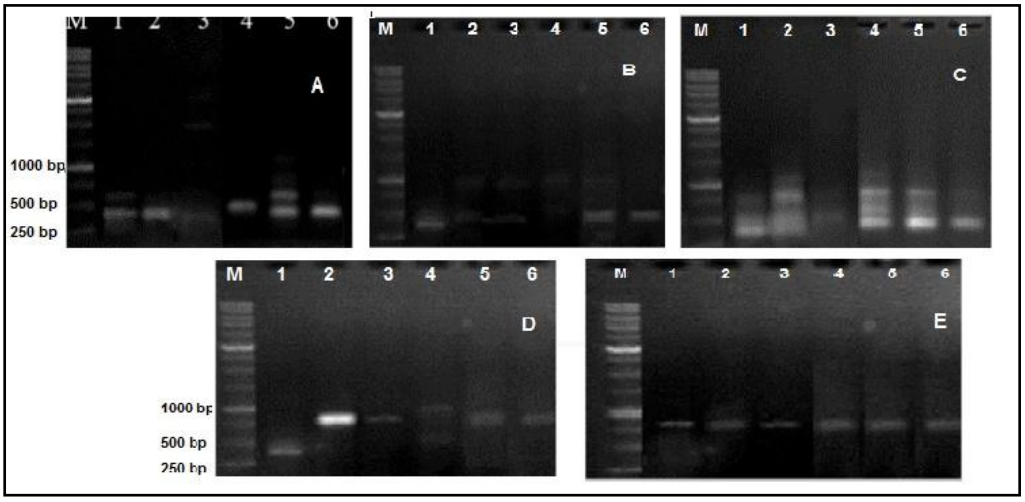

Fig. 2. RAPD-based PCR fragments of five primers in irradiated*Trigonella foenum-graecum* L, plants M= DNA standard marker, 1= Control, 2= 100 Gy, 3=150 Gy, 4= 200 Gy, 5= 250 Gy, 6= 300 Gy.

Table 7. RAPD- markers for for irradiated plants of of*Trigonella foenumgraecum* by five primers

Primer	M.S (bp)	Doses						Marker type
		Cont.	100 Gy	150 Gy	200 Gy	250 Gy	300 Gy	
A-**19**	**3423.550**	-	-	+	-	-	-	Positive
	2530.944	-	-	+	-	-	-	Positive
	1899.538	-	-	+	-	-	-	Positive
	1128.075	-	-	-	-	+	-	Positive
	906.196	-	-	+	-	-	-	Positive
	806.092	-	-	-	-	+	-	Positive
	586.990	+	+	-	+	+	+	Positive
	512.381	+	+	-	-	-	-	Positive
	419.447	-	-	-	-	+	+	Positive
	378.791	+	+	+	-	-	-	Positive
	480.523	-	-	-	+	-	-	Positive
C-**17**	**1344.010**	-	-	-	-	+	-	Positive
	949.898	-	+	+	+	+	-	Positive
	603.805	-	-	-	-	+	-	Positive
	437.157	+	+	+	+	+	+	Common
	272.573	-	+	+	-	+	-	Positive
A-**06**	**1244.538**	-	+	-	-	-	-	Positive
	1049.013	-	-	-	+	-	-	Positive
	872.944	-	-	+	-	-	-	Positive
	777.826	-	+	-	-	-	-	Positive
	751.687	+	-	-	-	-	-	Negative
	708.040	-	-	-	+	+	+	Positive
	516.104	-	-	-	-	-	+	Positive
	505.194	+	-	-	-	+	-	Positive
	496.633	+	+	+	+	+	+	Common
	488.217	-	-	-	+	-	-	Positive
	404.541	-	-	-	-	+	+	Positive
	395.990	+	+	-	+	+	+	Positive
	389.280	+	-	-	-	-	-	Negative
	382.683	-	+	-	-	-	-	Positive
C-**20**	**1064.419**	-	-	-	+	-	-	Positive
	794.204	+	+	+	-	+	+	Common
	480.738	+	+	-	+	-	-	Positive
	358.697	+	-	-	-	-	-	Negative
D-**03**	**850.905**	+	+	+	+	+	+	Common

\+ Present - Absent

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