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SCoT Markers Related to Effects of Gamma Irradiation on some Biochemical Parameters of Fenugreek (*Trigonella foenum-graecum* L.)

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.) warrants focused attention by researchers to enhance its value and products. The present article investigates how different gamma irradiation doses (25, 50, 75, 100, and 200 Gy) from cobalt 60 influence biochemical parameters of fenugreek and DNA fingerprinting following exposure to irradiation. Irradiated and unirradiated seeds were grown in the field for two successive seasons, and the impact of the applied treatments on photosynthetic pigments (chlorophyll a, b, and carotenoids) and biochemical parameters, particularly total protein, total amino acids, DPPH radical scavenging activity, total flavonoids, and total phenolics, was examined. Results revealed that the 75 Gy dose exhibited a stimulatory influence on chlorophyll content, carotenoids, and biochemicals, whereas the 200 Gy dose exhibited an inhibitory influence on the same traits. Eleven SCoT primers produced 131 markers, including 67 monomorphic, 48 polymorphic, and 16 unique markers with a mean of 46.91% polymorphism. Polymorphic information content (PIC) varied between 0.30 for the SCoT-1 primer and 0.06 for the SCoT-10 primer. In comparison with the control plants, the results exhibited substantial changes in SCoT profiling in the M2 generation due to γ-irradiation treatments and reveal the significant impacts of lowered gamma irradiation dosages, particularly enhancing chlorophyll content and biochemical parameters in fenugreek plants.

1. Introduction

Gamma radiation possesses the capacity to induce modifications in the physiological and biochemical responses exhibited by plants [1]. Ling et al. [1] examined the findings that low doses of ionizing radiation improves a wide range of biological processes, including cell growth, germination, enzyme activity, stress resistance, and crop yields. Plant growth and development can be adversely affected by exposure to elevated gamma radiation dosages, as indicated by De Micco et al. [2]. These include disturbances in hormonal balance, enzyme activity, and leaf exchange. The influence of radiation depends on the dose, since low doses stimulate plant development, while high doses can have harmful effects [3]. Hence, gamma radiation has the potential to serve as a valuable physical stimulus for enhancing agricultural productivity and inducing favorable characteristics in diverse crop species, both under optimal and challenging environmental settings [4].

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Gamma irradiation doses can activate a resistance mechanism that mitigates DNA damage [5]. Nevertheless, subjecting seeds to elevated levels of gamma radiation can result in adverse consequences for crucial constituents within plant cells through the interaction with molecules and atoms, inducing the generation of free radicals within cells. These free radicals subsequently inflict detrimental effects on diverse physiological, morphological, and anatomical elements of the plants [6]. Depending on the dose of radiation, these free radicals can disrupt various biological processes such as protein synthesis, hormone regulation, enzyme activity, water exchange, and leaf gas exchange [7,8]. The utilization of gamma irradiation is a well acknowledged and important technology for the creation of mutants possessing distinct agricultural characteristics [9].

Fenugreek (*Trigonella foenum-graecum* L.), belonging to the Fabaceae family, represents an herbaceous plant known for its significant medicinal and economic properties. Originating from Asia and Southern Europe, this plant possesses numerous phytochemicals that exhibit protective properties. These include the alkaloid trigonelline, the steroidal sapogenin diosgenin, and the polysaccharide mucilage [10–12]. Numerous research studies have provided evidence that fenugreek seeds and

leaves have therapeutic applications for treating certain diseases. Experimental trials involving human and animals subjects have shown that fenugreeks can effectively lower blood sugar and blood cholesterol levels. Basu et al. [13] indicating the potential implication of fenugreek leaves and seeds in treating microbial infections, diabetes, and cancer. The fenugreek rich therapeutic qualities can be attributed to the production of different phytochemicals, including 4-hydroxy isoleucine, galactomannan, trigonelline, and diosgenin [14]. Therefore, agricultural produce exhibits significant worldwide demand within the pharmaceutical, dietary supplement, and functional food industries. Fenugreek, recognized as a chemical crop, possesses extensive utilization within many industrial sectors. The seeds of this plant possess a dependable steroid diosgenin resource, a supplementary component within the pharmaceutical industry [15].

DNA markers have become increasingly popular for evaluating genetic diversity [16,17] and in investigating the genetic variations induced by mutagens, particularly gamma radiations [18–21]. The start codon targeted (SCoT) marker is established for specifically targeting the region adjacent to the ATG start codon, which is known to be a conserved region within the genetic makeup of various plants [22]. Due to its numerous advantageous characteristics, the targeted DNA fingerprinting marker known as SCoT polymorphism has garnered significant attention from researchers in the fields of plant genetics, molecular breeding, and genomics. The SCoT technique is described as involving a single 18-mer primer in a single primer polymerase chain reaction (PCR) with 50°C annealing temperature, then the amplicons obtained from PCR are separated by the utilization of conventional agarose gel. SCoT markers have several applications in quantitative trait loci mapping and genetic analysis, especially in agarose gel electrophoresis-preferring laboratories [23–25].

This work aims to explore the response of fenugreek plants to various gamma irradiation doses (25, 50, 75, 100, and 200 Gy). The induced variations will be recorded and evaluated in the M_1 and M_2 generations based on changes in chlorophyll content, biochemical parameters, and molecular polymorphism as indicated from the SCoT markers within the M_2 plants.

2. Materials and Methods

2.1. Plant materials

The fenugreek seeds (*Trigonella foenum-graecum* L.) have been acquired from the Field Crops Research Institute (Food Legumes Research Department), which is part of the Agricultural Research Center located in Giza, Egypt.

2.2. Seed treatment and seed sowing

At the Egyptian Atomic Energy Authority (National Center for Radiation Research and Technology), Nasr city, Cairo, Egypt, fenugreek dry seeds were irradiated with 25 Gy, 50 Gy, 75 Gy, 100 Gy, and 200 Gy from a cobalt-60 source. Irradiation was not applied to the seeds in the control samples. In a completely randomized block design (CRBD), M_1 and M_2 generations were produced from irradiated and unirradiated (control) seeds over two growing seasons (2018–2019 and 2019–2020) in the Botany Department's Botanical Garden, Faculty of Science, Suez Canal University in Ismailia, Egypt.

2.3. Determination of Physiological and biochemical parameters

Fresh leaves from the different treatments of M_1 and M_2 generations were collected to estimate photosynthetic pigments (chlorophyll a, b, and carotenoids) and total protein. For plant extract preparation, fresh leaves (1 gram) were soaked in 20 ml 70 % ethanol to be used for determining total amino acids, total flavonoids, total phenolics, and antioxidant activity using DPPH radical.

2.3.1. Determination of photosynthetic pigments

The quantification of photosynthetic pigments, comprising carotenoids, chlorophyll a, and chlorophyll b, was conducted using spectrophotometric analysis on plant leaves derived from both the M_1 and M_2 generations, according to the procedure of Lichtenthaler et al. [26]. The optical density was measured at 662, 644, and 440.5 nm. The chlorophylls a and b and carotenoids concentration were estimated with the subsequent formulae:

Chlorophyll (a) = (9.784×E662) – (0.99×E644) = mg $100g^{-1}$ FW.

Chlorophyll (b) = (21.426×E644) – (4.65×E662) = mg $100g^{-1}$ FW.

Carotenoids = (4.695×E440.5) – 0.268 (chlorophyll a + b) = mg $100g^{-1}$ FW.

E = optical density at three wavelengths.

2.3.2. Total protein determination

Total protein was measured as follows Urbanek et al. [27] and Bradford [28]. The process involves homogenizing approximately 0.2 g of fresh leaves in a pre-chilled mortar with 1 ml of 0.1 M phosphate buffer (pH 7) and centrifuging the obtained suspension at 1000 rpm for 15 minutes to collect the supernatant. The determination of soluble protein concentration is carried out in samples according to Bradford [28] using 2 ml Bradford reagent mixed with leaf extract (200 µl).

2.3.3. Total free amino acids determination

Ninhydrin reagent (4 g ninhydrine +300 ml acetone) was adopted in estimating the total free amino acids [29].

2.3.4. Total phenolics determination

The total phenolic compounds assessment involved utilizing a modified Folin-Ciocalteu method, and their measurements were recorded at a wavelength of 650 nm [30].

2.3.5. Determination of total flavonoids

To estimate total flavonoid contents the aluminum chloride colorimetric assay was adopted [31].

2.3.6. Determination of DPPH radical scavenging Activity

The method employed in determining the extracts' free radical scavenging activity involved mixing a sample (one ml) with 1 ml of 0.1 mm DPPH ethanolic solution. The

absorbance was measured at 517 nm at room temperature following 30-minute of darkness incubation [32].

2.4. Statistical analysis

A completely randomized block design (CRBD) was employed for the treated samples and the controls. An analysis of variances (ANOVA) of the considered factors was accomplished with IBM SPSS26 statistical software (SPSS Inc. Chicago, IL, USA). Data are represented by means of 3 replicates ± SE. The mean values were compared using the Least Significant Differences (LSD) and Duncan's multiple range test (DMRT) for post hoc analysis at 5% level of probability ($P \leq 0.05$).

2.5. SCoT-PCR of genomic DNA

2.5.1. Extraction and purification of genomic DNA

Liquid nitrogen was used to grind the leaf tissues of M_2 fenugreek plants into a fine powder. The DNA Easy Plant Mini Kit (Qiagen, Santa Clarita, CA) was then employed for DNA extraction following the manufacturer's guidelines. The DNA concentration was assessed by running 2 µl of the parent DNA samples on a 1% agarose gel and 10 µl of a DNA size marker (100 bp DNA ladder). Fluorescence comparisons between the DNA sample and bands in a DNA size marker were used to estimate the concentration of the DNA sample.

2.5.2. SCoT primers and PCR reaction

The M_2 genotypes obtained from M_1 plants developed from seeds subjected to the five applicable gamma irradiation doses were examined for polymorphism using eleven SCoT primers (Table 1). Following Ibrahim et al. [33], a 25-µl reaction container with 12.5 µl Master Mix (Sigma), 7 µl dH_2O, 3 µl template DNA (10ng), and 2.5 µl primer (10 pcmol) was used to conduct the amplification reaction. To do the PCR amplification, a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) was employed.

Table 1: List of eleven SCoT primer sequences utilized in M_2 generation of *Trigonella foenum-graecum* following the exposure of the parent seeds to varied g-radiation doses.

Primer	Sequence
SCoT-1	5'-ACGACATGGCGACCACGC-3'
SCoT-2	5'-ACCATGGCTACCACCGGC-3'
SCoT-3	5'-ACGACATGGCGACCCACA-3'
SCoT-4	5'-ACCATGGCTACCACCGCA-3'
SCoT-5	5'-CAATGGCTACCACTAGCG-3'
SCoT-6	5'-CAATGGCTACCACTACAG-3'
SCoT-7	5'-ACAATGGCTACCACTGAC-3'
SCoT-9	5'-ACAATGGCTACCACTGCC-3'
SCoT-10	5'-ACAATGGCTACCACCAGC-3'
SCoT-11	5'-ACAATGGCTACCACTACC-3'
SCoT-12	5'-CAACAATGGCTACCACCG-3'

It was set up to complete 40 cycles following a 5-minute denaturation cycle at 94 ºC. During each cycle, the amplification process involved a 45-second denaturation step at 94 ºC, followed by a 50-second annealing step at 50 ºC, and finally, a 1-minute elongation step at 72 ºC. In the last cycle, the primer extension section was prolonged to 7 minutes at 72 ºC.

2.5.3. Gel electrophoresis

The amplicons were run on 1.5% agarose gel encompassing ethidium bromide (0.5 ug/ml) in 1X TBE buffer at 95 volts. Gel Documentation System (BIO-RAD 2000) was employed to photograph PCR products within UV light. To determine the DNA bands size, a DNA size marker (a 100 bp DNA ladder) was utilized.

2.5.4. Data analysis

SCoT-PCR marker analysis banding patterns were employed to compare study treatments for genetic similarities. Both monomorphic and polymorphic bands were involved in the ultimate datasets. Only distinct and clear-cut bands were visually evaluated for their absence (0) or presence (1) in all samples during SCoT analysis. Subsequently, a data matrix consisting of binary values was developed. Utilizing the unweighted pair-group method with arithmetic averages (UPGMA), the Dice's similarity matrix coefficients were computed between the treatments. This matrix was then utilized to generate a dendrogram with the PAST software Version 1.91, to express the resemblance between the M2 genotypes based on SCoT polymorphism [34].

3. Results and discussion

3.1. Effect of irradiation on chlorophyll contents

Gamma irradiation could efficiently control the levels of chlorophyll content and phyto-assimilate formation [35–37]. Low doses of radiation have been found to increase the level of chlorophyll due to the activation of the enzyme system [38]. Singh et al. [39] stated that the presence of a considerable rise in total chlorophyll content in plants subjected to a radiation dose of 100 Gy suggests that gamma rays have a helpful impact on enhancing the overall growth and developmental processes of plants. Meanwhile, Gamma radiation has detrimental effects on the photosynthetic pigments due to the damage of thylakoid and chloroplast structures which leads to a disruption to the arrangement of thylakoid and grana membranes [1,40]. The radiation of 100–200 Gy causes an elevation in the chlorophyll a, b levels up to 64.5 % in wheat seedlings [41] whereas radiation at 100 Gy exhibited an increased chlorophyll a than chlorophyll b. Nevertheless, an increased concentrations (300 Gy) causes reduction in the total chlorophyll a and b contents [40]. Carotenoid pigments exhibit a rapid recovery response following irradiation due to their high radiosensitivity [42]. Comparable findings were documented by Alikamanoğlu et al. [43], Borzouei et al. [40], Ferreira-Castro et al. [38].

The effect of the applied gamma radiation doses on the chlorophyll content and carotenoids in the M_1 and M_2

generations of fenugreek is listed in Table 2 and the change (increase or reduction) in chlorophyll content compared to the control plants of fenugreek plants derived from seeds experiencing various gamma ray doses at M_1 generation & M_2 generation is illustrated in Fig. 1a and 1b.The levels of chlorophyll a, chlorophyll b, chlorophyll a + b, and carotenoids exhibited a notable increase with the increasing radiation dose from 25 Gy to 100 Gy. The highest chlorophyll a content (27.10 ± 0.49 mg/100g FW) in M_1 generation with an increase of 22% to the control plants was observed at 75 Gy. The maximum chlorophyll b content (15.80 ± 0.15 mg/100g FW) in the M_1 generation with an increase of 27% and (21.10 ± 0.49 mg/100g FW) in the M_2 generation with an increase of 63% compared to the control plants were also observed at 75 Gy. The chlorophyll a+b contents revealed significant increase, and a specifically elevated content (42.90 ± 0.37 mg/100g FW) in M_1 plants with an increase of 24% and (40.30 ± 0.40 mg/100g FW) in M_2 plants with an increase of 14% than the control plants were found at 75 Gy (Fig. 1).

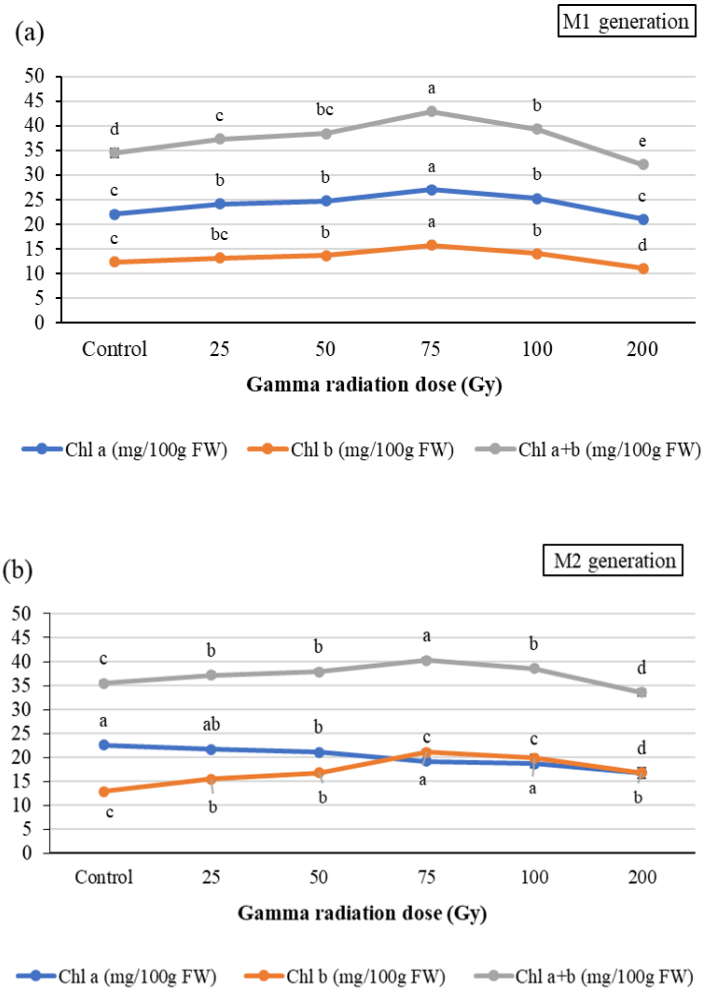

Fig. 1. Effect of gamma irradiation on chlorophyll content (mg/100 g FW) of fenugreek plants produced from seeds irradiated with different doses of gamma rays at M_1 & M_2 generations.

The highest carotenoids content of 10.10 ± 0.24 mg/100g FW in the M_1 generation (an 36% increase over control plants) and 10.60 ± 0.46 mg/100g FW in the M_2 generation (a 29% increase over control plants) was observed at the 75 Gy dose level. The total chlorophyll and carotenoids contents revealed significant increase upon irradiation from 25 Gy to 100 Gy in both M_1 and M_2 generations, while the 200 Gy dosage negatively affected the chlorophyll contents and carotenoids.

3.2. Effect of irradiation on biochemical parameters

Gamma irradiation reacts with various molecules and atoms within the cell, especially water molecules, and produces free radicals that have the potential to alter significant plant cells constituents based upon the irradiation dosage [44]. The formation of free radicals serves as stress signals and initiates stress reactions that have the potential to enhance the concentration of polyphenol acids, which are known for their significant antioxidant qualities [45]. Conversely, the lower phenolic content seen under elevated doses of radiation might potentially be ascribed to the phenolic compound's breakdown. It was noticed that the exposure of Chinese cabbage to a high dosage of gamma-irradiation resulted in a considerable reduction in phenolic levels [46]. Plants employ various defense mechanisms to mitigate damage, including the biosynthesis and subsequent accumulation of specific chemicals, like the increasing the soluble protein contents [47].

Several studies have indicated that the gamma irradiation enhances the biosynthesis and subsequent phytochemicals accumulation inside plant tissues [48] through the biosynthesizing flavonoid and phenolic within *Rosmarinus officinalis* L. Plants possess a defensive mechanism against irradiation by accumulating phenolic and flavonoid compounds, which exhibit antioxidant characteristics. Antioxidant defense characteristics are shown by phenolic substances, which may stabilize intermediate radicals and donate hydrogen atoms or electrons. The impact of gamma rays on augmenting phenolic contents was revealed in soybean plants that were exposed to γ-irradiation doses varying between 50 and 150 Gy [49]. Flavonoids, a type of subordinate metabolites found extensively throughout plants, have a significant role in justifying the harm resulting from radiation-induced stress. The leaves of *Curcuma alismatifolia* can accumulate bioactive chemicals, like flavonoid and phenolic compounds, when exposed to radiation doses of up to 20 Gy. This accumulation has been found to enhance the scavenging ability of these leaves [50]. Maity et al. [51] explored the impact of gamma radiation on significant proteins and found that the radiation influenced the total protein contents. This reduction results from exposure to an elevated dose (1 kGy) of gamma rays on *Oryza sativa*. In the current study, plants obtained from gamma-irradiated seeds exhibited the maximum vegetative growth, more reproductive performance, physiological and biochemical changes than the control plants. The potential positive impacts of gamma radiation on plant growth are

linked with enhancements in germination patterns and the acceleration of overall plant development.

The consequence of the applied gamma radiation doses on the examined biochemical parameters in the M_1 and M_2 generations of fenugreek is shown in Table 2 and the change (increase or reduction) in total flavonoid and phenolic contents compared to the control plants of fenugreek plants derived from seeds experiencing various gamma ray doses at M_1 generation & M_2 generation is illustrated in Fig. 2a and 2b.

Table 2: Effects of gamma irradiation on photosynthetic pigments (chlorophyll a, chlorophyll b, chlorophyll a + b, and carotenoids) of fenugreek plants grown from seeds exposed to various gamma ray doses. Control means plants produced from unirradiated seeds.

γ-radiation dose (Gy)	Chlorophyll a (mg/100g FW)	PC %	Chlorophyll b (mg/100g FW)	PC %	Chlorophyll a + b (mg/100g FW)	PC %	Carotenoids (mg/100g FW)	PC %
M_1 generation								
control	22.13 ± 0.47 ^{c}		12.40 ± 0.40 ^{c}		34.53 ± 0.85 ^{d}		7.47 ± 0.29 ^{b}	
25	24.17 ± 0.60 ^{b}	9%	13.20 ± 0.25 ^{bc}	6%	37.38 ± 0.35 ^{c}	8%	7.90 ± 0.38 ^{b}	6%
50	24.83 ± 0.46 ^{b}	12%	13.67 ± 0.24 ^{b}	10%	38.50 ± 0.38 ^{bc}	11%	8.40 ± 0.40 ^{b}	13%
75	27.10 ± 0.49 ^{a}	22%	15.80 ± 0.15 ^{a}	27%	42.90 ± 0.37 ^{a}	24%	10.10 ± 0.24^{a}	36%
100	25.33 ± 0.33 ^{b}	14%	14.07 ± 0.23 ^{b}	13%	39.40 ± 0.56 ^{b}	14%	9.62 ± 0.31^{a}	29%
200	21.10 ± 0.49 ^{c}	-5%	11.10 ± 0.38 ^{d}	-10%	32.20 ± 0.44 ^{e}	-7%	6.46 ± 0.29 ^{c}	-14%
LSD 0.05	1.48		0.89		1.59		0.99	
F-ratio	20.64		30.11		53.27		17.87	
P-Value	≤ 0.001***		≤ 0.001***		≤ 0.001***		≤ 0.001***	
M_2 generation								
control	22.57 ± 0.30 ^{a}		12.93 ± 0.18 ^{c}		35.50 ± 0.45 ^{c}		8.20 ± 0.12 ^{cd}	
25	21.67 ± 0.33 ^{ab}	-4%	15.53 ± 0.29 ^{b}	20%	37.20 ± 0.61 ^{b}	5%	9.10 ± 0.67 ^{bc}	11%
50	21.07 ± 0.07 ^{b}	-7%	16.82 ± 0.48 ^{b}	30%	37.89 ± 0.48 ^{b}	7%	9.80 ± 0.02 ^{ab}	20%
75	19.20 ± 0.42 ^{c}	-15%	21.10 ± 0.49 ^{a}	63%	40.30 ± 0.40 ^{a}	14%	10.60 ± 0.46 ^{a}	29%
100	18.67 ± 0.88 ^{c}	-17%	19.93 ± 0.46 ^{ab}	54%	38.60 ± 0.31 ^{b}	9%	10.05 ± 0.33 ^{ab}	23%
200	16.78± 0.40 ^{d}	-26%	16.83 ± 1.09 ^{b}	30%	33.61 ± 0.70 ^{d}	-5%	7.65 ± 0.35 ^{d}	-7%
LSD 0.05	1.44		1.86		1.57		1.29	
F-ratio	21.28		24.11		21.51		7.30	
P-Value	≤ 0.001***		≤ 0.001***		≤ 0.001***		0.0023**	

Data presented as means of 3 replicates ± SE, SE: Standard Error. Various superscripted letters (a, b, c, d, e, ab, bc, and cd) denote significant difference at P ≤ 0.05 between various treatments based on Duncan's multiple-range test. ***: significant at P ≤ 0.001 according to LSD test. PC % (Percentage change between each treatment and control plants).

In M_1 and M_2 generations, the total protein gradually increased with the gamma radiation till the 100 Gy dose. Then, a diminishing effect on total protein content was seen with the 200 Gy dose. The maximum gamma radiation dose (200 Gy) promoted a substantial reduction in the total protein contents relative to control plants in the M_1 and M_2 generations respectively. At a radiation dose of 75 Gy, the maximum total protein content (55.37 ± 0.68 mg/g FW) occurred in M_1 plants, with an increase of 7% than the controls, and (54.70 ± 0.56 mg/g FW) in M_2 plants, with an increase of 8 % than the control plants. However, the minimum amounts (46.32 ± 0.43 mg/g FW) for M_1 plants with a decrease of -11 % than the control plants and (45.85 ± 0.46 mg/g FW) for M_2 plants with a decrease of -9 % than the control plants were recorded at 200 Gy.

The highest total free amino acids (14.40 ± 0.31 mg/g FW) in M_1 plants with an increase of 39 % than the controls and (12.73 ± 0.23 mg/g FW) in M_2 plants with an increase of 26 % than the control plants were recorded at 75 Gy while the minimum value of total amino acids for both M_1 and M_2 generations was recorded at the dose of 200 Gy.

As shown in Table 3, gamma irradiation has significant consequences on the total phenolics of fenugreek plants in M_1 and M_2 generations relative to controls. The maximum value of total phenolics (6.97 ± 0.55 mg/g FW) in M_1 plants with an increase of 9 % than the controls and (7.37 ± 0.32 mg/g FW) in M_2 plants with an increase of 12 % than the control plants were recorded at 75 Gy while the minimum value of total phenolics for M_1 and M_2 generations was recorded at 200 Gy (Fig. 2).

In M_1 and M_2 generations, total flavonoids considerably improved by gamma radiation doses from 25 Gy to 100 Gy. The maximum value of total flavonoids (8.53 ± 0.29 mg/g FW) in M_1 plants with an increase of 39 % than the controls and (7.93 ± 0.41 mg/g FW) in M_2 plants with an increase of 25 % than the control plants were recorded at 75 Gy while the minimum value of total flavonoids for both M_1 and M_2 generations was recorded at 200 Gy (Fig. 2).

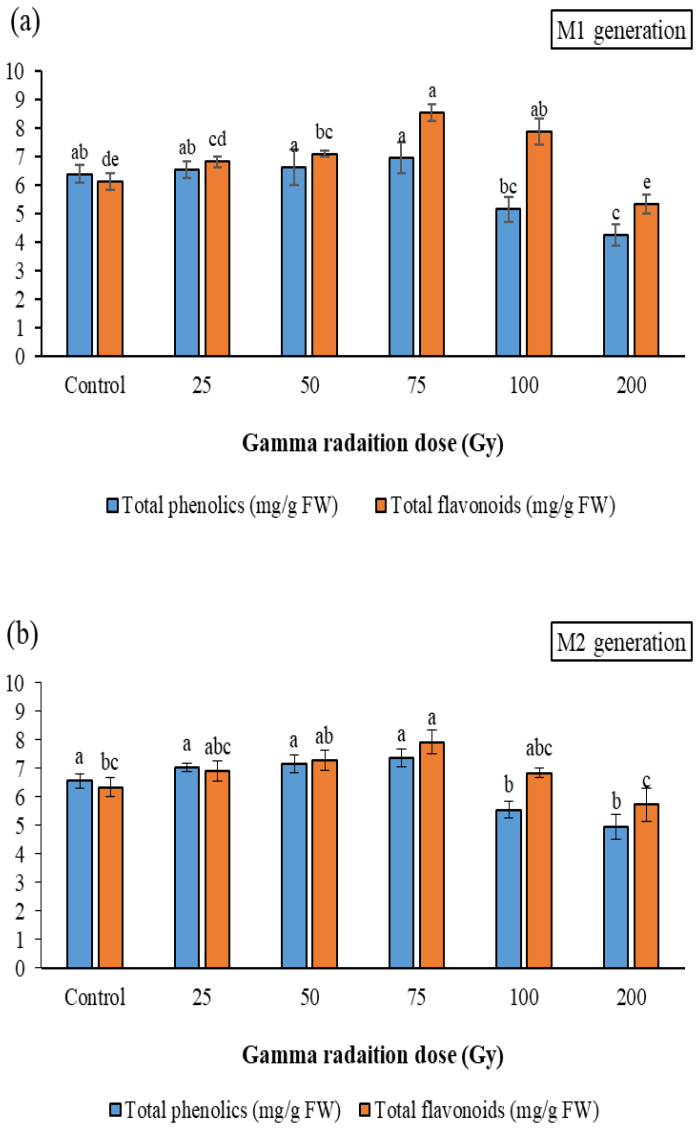

Fig. 2. Effect of gamma irradiation on total phenolic content (mg/g FW) and total flavonoid content (mg/g FW) of fenugreek plants produced from seeds irradiated with different doses of gamma rays at M1 & M2 generations.

The percentage of antioxidant Activity using DPPH radical after exposing the parent seeds to the applied gamma irradiation doses for M_1 and M_2 generations of fenugreek is illustrated in Table 3. In M_1 and M_2 generations, the dosages from 25 Gy to 100 Gy significantly increased DPPH radical scavenging activity percentage. The highest value of DPPH radical scavenging activity (72.83 ± 0.44 %) in M_1 plants with an increase of 21 % than the control plants, (65.67 ± 1.20 %) in M_2 plants with an increase of 13 % than the control plants.

3.3. Start codon-targeted (SCoT) marker analysis

Molecular markers play a crucial role in detecting genetic variability among many species, hence serving as valuable tools for the conservation of germplasm and the identification of cultivars. Molecular markers facilitate the utilization of improved breeding techniques, including marker-assisted backcross breeding and marker-assisted selection [52]. To effectively quantify genetic variation in plant species, a variety of DNA-based markers are now accessible. An innovative marker technique termed Start codon targeted (SCoT) was established, utilizing the conserved region adjacent to the ATG start codon within plant DNA. The utilization of SCoT markers is considered more effective relative to random markers, primarily because of their elevated annealing temperatures and prolonged primer distances [53]. The technique of designing SCoT markers necessitate no extensive knowledge of the genomic sequence, hence facilitating its application to plants without a reference genome [54].

The emergence of unique bands and absence of polymorphic bands commonly result from various structural modifications in DNA like deletion, transpositions, breaks, etc [55]. El-Khateeb et al. [56] found that gamma irradiation caused mutant individuals developing new genetic markers and losing some markers present in the controls. These findings indicate that the positive markers identified have the potential to act as effective tools for identifying genes for stress tolerance. Furthermore, these markers can aid in the process of marker-assisted breeding, specifically for enhancing radiation tolerance. Therefore, it can be inferred that the utilization of SCoT analysis to detect DNA polymorphism provides a valuable molecular indicator for detecting alterations in plants exposed to gamma radiation.

Eleven SCoT primers were employed in evaluating the genetic diversity changes in the M_2 generation of fenugreek genomes after the exposure of their parent seeds to the applied doses of gamma irradiation (Table 1 and S1). For SCoT-PCR analysis, a total of 131 DNA bands (67 monomorphic bands, 48 polymorphic bands, and 16 unique bands) were distinguished (Table 4 and Fig. 3). The percentage of polymorphism fluctuated between 17 % and 75 % with a mean of 46.91%. The size of the amplified DNA bands varied from 105 to 1499 bp. The polymorphic information content (PIC) was averaged around 0.18 with values varying between 0.06 and 0.30. The SCoT-1 primer produced the highest level of polymorphisms, detecting 12 distinct polymorphic amplification products. SCoT-6 and SCoT-10 produced the fewest amplified polymorphic fragments, 2 fragments each. The band frequency means ranged from 0.6 (SCoT-1 and SCoT-9 primers) to 0.9 (SCoT-6 and SCoT-10 primers) and have averaged around 0.76. The eleven primers gave sixteen molecular markers (fourteen positive and two negative) linked to radiation stress (Table 5 and S1), which might potentially be implicated for marker-assisted selection of genes

conferring stress tolerance and facilitate marker-assisted breeding programs aimed at improving radiation tolerance. The Dice's similarity coefficient along with the treatments ranged from 0.79 (between control plants and the treatment of 75 Gy) to 0.89 (between the treatment of 50 Gy and 75 Gy) as shown in Table 6. The dendrogram exhibited two primary clusters; the first one comprises the control plants and the treatments of 100 Gy and 200 Gy, and the second cluster includes the treatments of 25 Gy, 50 Gy, and 75 Gy (Fig. 4). The study results indicate that the positive markers found could be useful for finding genes that help the plant manage the harmful effects of radiation. In addition, these markers can help with marker-assisted breeding, especially when it comes to making plants more resistant to radiation. Because of this, it can be concluded that using SCoT analysis to find DNA polymorphism is a useful chemical way to find changes in plants that have been exposed to gamma radiation. Radiation mutation breeding has played an important role in the cultivation of new crop varieties [57].

Table 3: Influence of gamma irradiation on total protein, total amino acids, total phenolic and flavonoid contents, and DPPH radical scavenging activity of fenugreek plants grown from seeds exposed to various gamma ray doses. Control means plants produced from unirradiated seeds.

γ-radiation dose (Gy)	Total protein (mg/g FW)	PC %	Total amino acids (mg/g FW)	PC%	Total phenolics (mg/g FW)	PC %	Total flavonoids (mg/g FW)	PC%	DPPH (%)	PC%
M_1 generation										
control	51.84 ± 0.43 ^{b}		10.34 ± 0.33 ^{d}		6.38 ± 0.31 ^{ab}		6.13 ± 0.30 ^{de}		60.43 ± 0.43 ^{c}	
25	52.18 ± 0.42 ^{b}	1%	11.32 ± 0.32 ^{cd}	9%	6.53 ± 0.29 ^{ab}	2%	6.82 ± 0.18 ^{cd}	11%	61.60 ± 0.83 ^{c}	2%
50	54.51 ± 0.51 ^{a}	5%	11.78 ± 0.40 ^{bc}	14%	6.62 ± 0.51 ^{a}	4%	7.10 ± 0.10 ^{bc}	16%	66.74 ± 0.81 ^{b}	10%
75	55.37 ± 0.68 ^{a}	7%	14.40 ± 0.31 ^{a}	39%	6.97 ± 0.55 ^{a}	9%	8.53 ± 0.29 ^{a}	39%	72.83 ± 0.44 ^{a}	21%
100	54.67 ± 0.67 ^{a}	5%	12.58 ± 0.30 ^{b}	22%	5.16 ± 0.43 ^{bc}	-19%	7.87 ± 0.47 ^{ab}	28%	71.14 ± 0.46 ^{a}	18%
200	46.32 ± 0.43 ^{c}	-11%	8.27 ± 0.50 ^{e}	-20%	4.26 ± 0.38 ^{c}	-33%	5.34 ± 0.33 ^{e}	-13%	55.17 ± 0.60 ^{d}	-9%
LSD 0.05	1.65		1.13		1.37		0.93		1.91	
F-ratio	31.75		31.96		5.51		14.70		119.84	
P-Value	≤ 0.001 ***		≤ 0.001 ***		0.0073 **		≤ 0.001 ***		≤ 0.001 ***	
M_2 generation										
control	50.60 ± 0.75 ^{d}		10.13 ± 0.12 ^{c}		6.57 ± 0.26 ^{a}		6.35 ± 0.33 ^{bc}		58.33 ± 0.33 ^{c}	
25	51.77 ± 0.52 ^{cd}	2%	10.75 ± 0.38 ^{c}	6%	7.03 ± 0.15 ^{a}	7%	6.92 ± 0.36 ^{abc}	9%	61.17 ± 0.44 ^{b}	5%
50	52.43 ± 0.35 ^{bc}	4%	10.98 ± 0.56 ^{bc}	8%	7.15 ± 0.31 ^{a}	9%	7.29 ± 0.35 ^{ab}	15%	63.93 ± 0.07 ^{a}	10%
75	54.70 ± 0.56 ^{a}	8%	12.73 ±0.23 ^{a}	26%	7.37 ± 0.32 ^{a}	12%	7.93 ± 0.41 ^{a}	25%	65.67 ± 1.20 ^{a}	13%
100	53.67 ± 0.33 ^{ab}	6%	12.08 ± 0.36 ^{ab}	19%	5.55 ± 0.29 ^{b}	-15%	6.85 ± 0.15 ^{abc}	8%	64.40 ± 0.31 ^{a}	10%
200	45.85 ± 0.46 ^{e}	-9%	7.33 ± 0.33 ^{d}	-28%	4.97 ± 0.44 ^{b}	-24%	5.73 ± 0.59 ^{c}	-10%	56.80 ± 0.42 ^{c}	-3%
LSD 0.05	1.58		1.11		0.95		1.19		1.79	
F-ratio	36.72		27.49		9.92		3.78		37.78	
P-Value	≤ 0.001***		≤ 0.001***		≤ 0.001***		0.027 *		≤ 0.001***	

Data presented as means of 3 replicates ± SE, SE: Standard Error. Various superscripted letters (a, b, c, d, e, ab, bc, cd, de, and abc) signify significant difference at P ≤ 0.05 between various treatments based on Duncan's multiple-range test. *: significant at P ≤ 0.05, **: significant at P ≤ 0.01, ***: significant at P ≤ 0.001 according to LSD test, DPPH: 2, 2-diphenyl-1-picrylhydrazyl. PC % (percentage change between each treatment and control plants).

Table 4: The polymorphism generated by eleven SCoT primers used in M_2 plants of *Trigonella foenum-graecum* following exposure of parent seeds to the applied γ-radiation dosages.

Primer	Range of band size (bp)	M-bands	P-bands (without unique bands)	U-bands	P-bands (with unique bands)	Total number of bands	Mean of band frequency	PIC	P (%)
SCoT-1	135-1083	4	10	2	12	16	0.6	0.30	75
SCoT-2	172-1202	6	5	3	8	14	0.7	0.20	57
SCoT-3	182-710	4	6	1	7	11	0.8	0.24	64
SCoT-4	182-693	7	5	1	6	13	0.7	0.21	46
SCoT-5	173-1023	6	2	1	3	9	0.8	0.11	33
SCoT-6	165-828	6	2	0	2	8	0.9	0.12	25
SCoT-7	116-1120	6	5	1	6	12	0.8	0.21	50
SCoT-9	105-1499	5	4	4	8	13	0.6	0.20	62
SCoT-10	120-996	10	1	1	2	12	0.9	0.06	17
SCoT-11	115-911	8	4	2	6	14	0.8	0.16	43
SCoT-12	204-1126	5	4	0	4	9	0.8	0.22	44
Total		67	48	16	64	131	---	---	---
Mean per primer		6.09	4.36	1.45	5.82	11.91	0.76	0.18	46.91

M-bands (monomorphic bands), P-bands (polymorphic bands), U-bands (unique bands), P (%) polymorphism percentage.

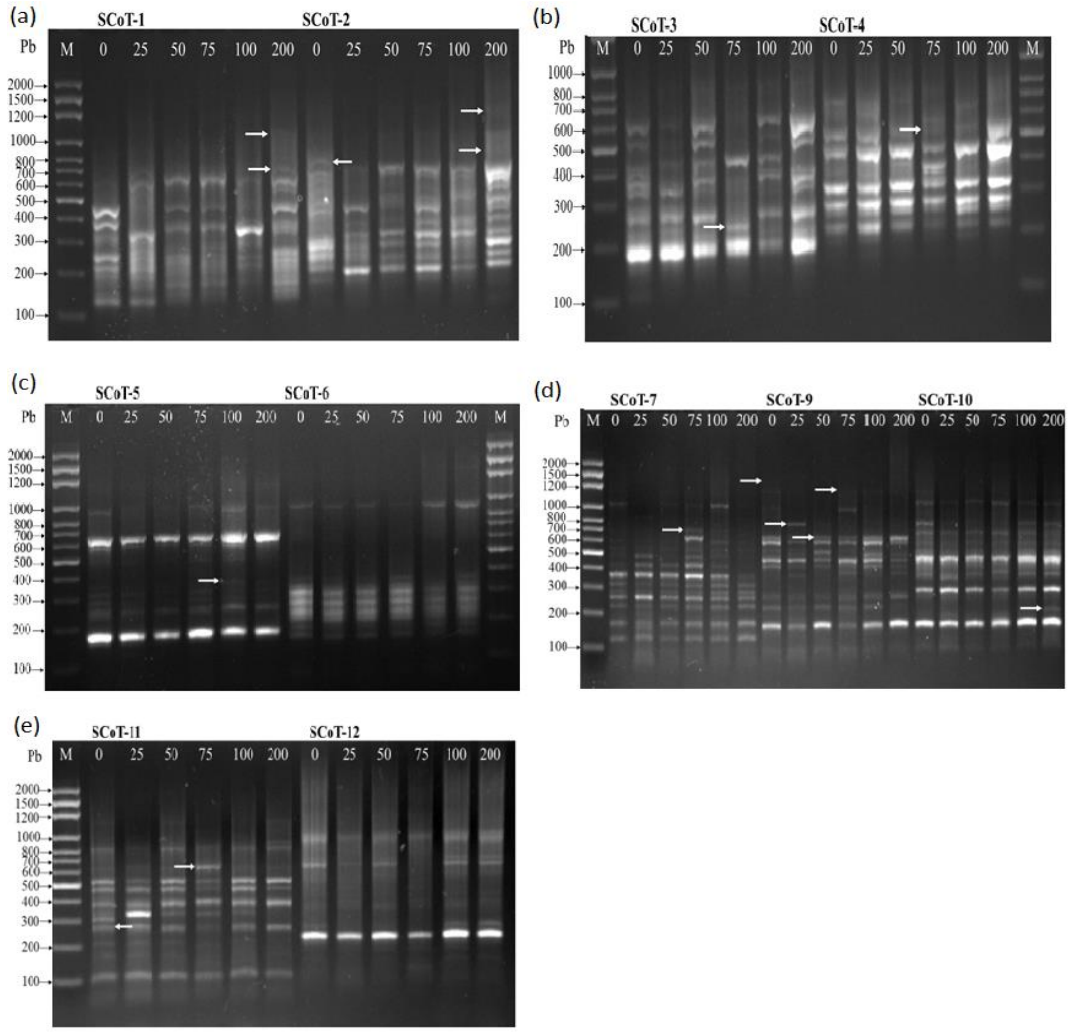

Fig. 3. SCoT amplification profiles patterns obtained using eleven primers in M_2 generation of *Trigonella foenum-graecum* following exposure of parent seeds to the applied doses of g-radiation, (M) referred to size marker 100 bp DNA ladder. (a) SCoT-1 and 2 primers, (b) SCoT-3 and 4 primers, (c) SCoT-5 and 6 primers, (d) SCoT-7, 9, 10 primers, (e) SCoT-11,12 primers.

Table 5: Positive unique markers (PUM) and negative unique markers (NUM) of the eleven SCoT primers used in M_2 generation of *Trigonella foenum-graecum* following the exposure of the parent seeds to varying γ-radiation dosages.

Primer	PUM (bp)	NUM (bp)
SCoT-1	742-1083	
SCoT-2	990-1202	858
SCoT-3	254	--
SCoT-4	534	--
SCoT-5	377	--
SCoT-6	--	--
SCoT-7	731	--
SCoT-9	626-918-1193	1499
SCoT-10	233	--
SCoT-11	295-689	--
SCoT-12	--	--
Total	14	2

Table 6: Similarity matrix among M_2 plants of *Trigonella foenum-graecum* following exposure of parent seeds to the applied doses of γ-radiation according to Dice's coefficient from SCoT pattern generated by eleven SCoT primers.

Treatment	Control	25 Gy	50 Gy	75 Gy	100 Gy	200 Gy
Control	**1.0**					
25 Gy	**0.85**	**1.0**				
50 Gy	**0.83**	**0.85**	**1.0**			
75 Gy	**0.79**	**0.85**	**0.89**	**1.0**		
100 Gy	**0.88**	**0.84**	**0.88**	**0.85**	**1.0**	
200 Gy	**0.86**	**0.80**	**0.86**	**0.80**	**0.86**	**1.0**

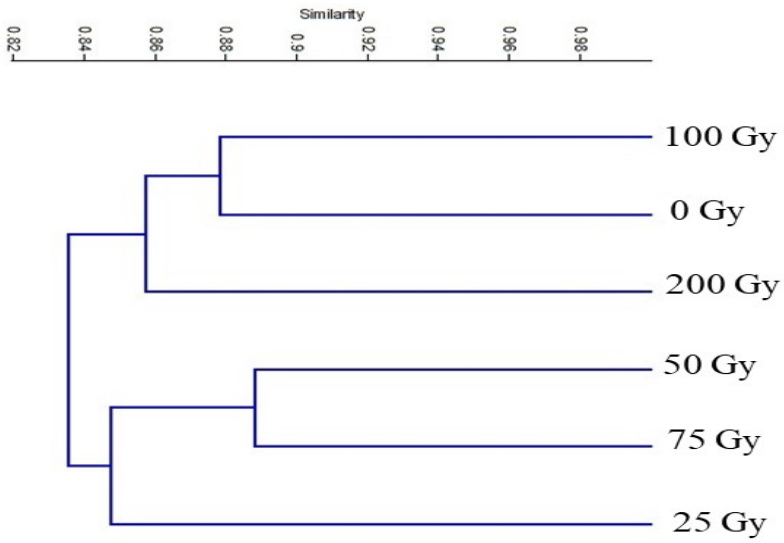

Fig. 4. Dendrogram for M_2 plants of *Trigonella foenum-graecum* following exposure of parent seeds to the applied doses of γ-radiation constructed from SCoT data using UPGMA and similarity matrix computed according to Dice's coefficient.

4. Conclusion

Our outcomes show that lower doses of gamma rays applied to fenugreek plants result in improved chlorophyll content and biochemical secondary metabolites across M1 and M2 generations, with the 75 Gy dose demonstrating the most positive effects. Meanwhile, the SCoT fingerprinting indicated genetic variability resulting from radiation-induced effects. Furthermore, the detection and analysis of positive and negative marker band sequences can facilitate the detection of different kinds of DNA damage and mutations within plants due to radiation exposure, thereby offering potential advantages for crop enhancement.

Conflicts of Interest: The authors declare no conflict of interest.

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