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Micropropagation, Bioactive compounds, and Molecular diversity responses of *Gardenia jasminoides* Ellis using Gamma radiation

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Abstract

Mutation stimulation is a viable and well-established breeding approach for plants enhancement, induction bioactive compounds, and genetic variety generation to create novel plant mutants. The objective of this study was to mutate *Gardenia jasminoides* Ellis plant by gamma radiation (0, 15, 20, 25, 30, 35, and 40 Gy) as well as to determine the morphological and biochemical parameters of *in vitro* and *in vivo* treated plants. Consequently, the study has to detect the genetic diversity of the obtained mutants. The results indicated that gardenia plant irradiated with gamma at dose of 15 Gy showed the greatest growth rate. In addition, the maximum contents of chlorophyll (a, b) and carotenoids were detected in shootlets mutated with 15 Gy of gamma rays. Whereas, the highest total phenolic (TPC) and flavonoid contents (TFC), anthocyanin amounts, and antioxidant capacity were found in shootlets exposed to 40 Gy of gamma rays in *vitro* and *in vivo*. Furthermore, the suspected genetic variation in *Gardenia jasminoides* Ellis after treatment with γ-irradiation was evaluated using inter-simple sequence repeat (ISSR) analysis which produced a total of 98 bands, with percentage of polymorphic bands ranging from 30 to 60%. According to the similarity matrix, the dendrogram was constructed into three clusters. Consequently, the results concluded that γ-irradiation of *Gardenia jasminoides* Ellis has given a sufficient number of induced mutations. Subsequently, the ISSR analysis has provided a powerful molecular marker for identifying mutants. Therefore, the *in vitro* γ radiation-induced mutation could be a useful technique for assisting breeding programs for new Gardenia cultivars.

Key words: Gardenia jasminoides Ellis, gamma rays, in vitro, in vivo, biochemical, ISSR.

1. Introduction

Gardenia jasminoides Ellis is a member of the Rubiaceae family, and it was found originally in tropical and subtropical regions, particularly in Japan and China [1, 2]. *Gardenia jasminoides* Ellis is an ornamental plant, sometimes known as Cape jasmine or gardenia. It is an evergreen plant. It's commonly grown in a garden. It is also a medicinal plant because it exhibits a wide range of biological activity *in vitro* and *ex-vitro*. It has antioxidant, anti-inflammatory, antihypertensive, and antidepressant effects. Wounds, hepatitis, and fever are also treated with it [3, 1].

Application of micropropagation has been employed commercially over the world, even though the ability for plant regeneration varies significantly in several genotypes [4, 5]. In the last years, many agents regulating plant regeneration have been tested, for example, exogenously supplied phytohormones *in vitro*, physiological properties of the donor plants [6], mineral uptake, and their distribution patterns [7]. *In vitro*, culture techniques have been widely utilized to induce mutation in plants for various purposes, including enhancing plants by increasing genetic diversity and choosing mutants as potential sources for developing new cultivars [8]. *In vitro* breeding of mutation can solve challenges when developing superior new cultivars for sustainable ornamental plants production without disease spread. Since the discovery of this tool, great varieties for commercial cultivation have been generated [9].

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Genetic variation is vital for breeding and selecting the stronger genotypes to gain access to inventive genetic makeup and generate unique and superior cultivars [10]. Several energy rays, for example α, γ and β-rays, as well as protons and neutrons, are commonly utilized in mutation breeding. Gamma rays are the most effective ionizing radiation for producing mutants in plants, as they can cause large numbers of mutations. As well; Gama rays were chosen as a mutagenesis agent because they have a higher level of precision, a high absorption power for plant biological material, and can ionize the molecules they travel through [11]. In addition, plant breeders prefer to use gamma radiation as a physical mutagen. The species, cultivars, mostly determine radiation's impact age of the plant and its physiological, morphological, and genetic structure. Ionizing radiation produces constructer and functional alteration in DNA, which have implications at the cellular and systemic levels. Base changes, substitutions, and deletions, and chromosomal aberrations are all examples of DNA modification as Dianthus, and Tulip [12, 2]. They could significantly change physiological parameters to generate novel mutants with superior properties, such as those that can create greater levels of commercially important metabolites resulting in agriculture and economically valuable types with high productivity potential [13]. Also, radiation is one of the most effective mutagenic agents, having caused mutants breeding in a variety of ornamental plants and crops [12]. Additionally, it was a fantastic way to stimulate the expression of recessive genes, resulting in a novel genetic variety [14]. In addition, there is little information on new mutant variants with novel important parameters in gardenia cultivars. Therefore, the main purposes of this work were to investigate the effect of gamma irradiation on shooting, rooting behavior, and acclimatization. Furthermore, using gamma radiation as a mutagen to obtain novel cultivars variants and to estimate the secondary metabolism and genetic diversity of the obtained *Gardenia jasminoides* Ellis mutants.

2. Materials and Methods

This study was carried out for two years (2021 and 2022) on *Gardenia jasminoides* Ellis at Central laboratories Network, Tissue Culture Technique laboratory, Dep. of Ornamental Plants and Woody Trees National Research Centre (NRC), Egypt.

2.1. Plant material

The mother plants (2 years old) of gardenia were collected from the nursery of Central laboratories, Department of Ornamental Plants and Woody Trees, National Research Centre (NRC).

*2.1. 1.*Surface sterilization of explants

The explants (stem node) were excised from mother plants were used (1cm in length) and washed using soapy water for 30 minutes followed by one hour under running tap- water. They were rinsed then treated in ethyl alcohol 70% (v/v) for 30 sec., sodium hypochlorite 6% (Clorox) for seven min, then $HgCl_2$ 0.2g/l for five min., and then washed 3 times with sterilized distilled water under aseptic conditions.

2.1.2. Incubation conditions

All cultures of the different experiments were incubated at 25±2 °C and 16h day/ 8h dark photoperiod at the light intensity of 2000 lux from cooling white florescent lamps in a growth room [15].

2.1.3. Multiplication stage

Stem nodes of Gardenia were cultured on MS medium [16] added with1mg/l BA and 0.2 mg/l IBA. Developing shoots were obtained from culture medium at the end of 2 month on the same media treatments. Stem nodes (2-4 leaves, 4-5 cm in length) were exposed to gamma rays at different doses (0, 15, 20, 25, 30, 35 and 40 Gy), the irradiated shoots were sub-cultured on MS medium added with 1mg/l BA and 0.2 mg/l IBA for *in vitro* propagation. Each treatment of irradiation dose consisted of six jars containing three shoots in each jar. The collected data were determined monthly for three times by sub culturing: length of shoots, number of shoots, and number of leaves.

2.1.4. Rooting stage

Shoots of Gardenia (4-6 cm in length with 4-6 leaves) were exposed to gamma rays at different doses (0, 15, 20, 25, 30, 35 and 40 Gy) then cultured *in vitro* on MS medium supplemented with 1mg/l BA and 1.2 mg/l IBA to improve roots formation percentage. Number of roots, root length (cm), leaf area cm^2, was recorded after final sub-culture.

2.2. Acclimatization at the greenhouse

2.2.1. Pre- acclimatization

The obtained rooted plantlets were washed from agar medium and cleaned with running water, and soaked for 10min., in fungicide solution and bactericide of 1 gr/l each separately, then planted in an acclimatization medium consisting of peatmoss and perlite at a ratio of 1:1 (v:v) separately in one bottle. After that, the pots with clear transparent plastic sheets were covered for two weeks. Gradually, the plastic covers were removed to reduce the humidity and acclimating plantlets to greenhouse conditions, after 4 weeks the gardenia was then transferred to 15 cm plastic pots containing a medium filled with a mixture from peatmoss, clay, and perlite with 1:1:1 ratio for post acclimatization in the greenhouse of National Research Centre. the survival

percentage was determined in the stage of pre and post acclimatization.

2.2.2. **Post- acclimatization**

During this stage, the data include plant height (cm), number of branches/plant, number of leaves/plant, and leaf area (cm^2)/plant were recorded, after two months from starting the acclimatization.

2.3. **Gamma irradiation**

The irradiation capability was tested utilizing Cesium 137 as a gamma rays source at a rate of 0.658 rad secG1 at Atomic Energy Commission-united irradiation-Gamma, Nasr city, Egypt.

2.4. **Determination and extraction**

2.4. 1. **Photosynthetic pigments**

Chlorophyll a, b and total carotenoids were determined according to (Saric *et al*., [17]), were determined *in vitro* and *in vivo*.

2.4.2. **Anthocyanin content**

For anthocyanin pigment determination, the extraction was done with an ethanol hydrochloric acid solution (85 ml ethanol 95% + 15 ml 1.5 N HCl) according to the method of (Fuleki and Francis, [18]), were recorded *in vitro* and *in vivo*.

2.4.3. **Antioxidant activity, total free amino acids and total phenolic content**

Measured in terms of hydrogen-donating or radical- scavenging ability, using the stable radical DPPH according to Brand- Williams *et al*., [19]. Total free amino acids content in fresh shoot was determined by using ninhydrin reagent according to Moore and Stein [20]. Total phenolic content (mg /g F.W.) of each treatment were determined in the fresh plant samples according (Swain and Hillis,[21]), were determined *in vitro* and *in vivo*.

2.4.4. **Flavonoids content and total sugar (mg /g F.W.)**

Determined flavonoids content in the fresh plant samples according to Quettier *et al*., [22]. Total sugar of each treatment described by Dubois *et al*., [23], were recorded *in vitro* and *in vivo*.

2.5. Statistical analysis

The data were investigated using a randomized complete design with three replicates per each treatment and were showed using COSTATV-63 (Duncan [24]), one way ANOVA (analysis of variance) was used to estimate the significance by new multiple range tests at $p<0.05$.

2.6. ISSR-PCR analysis

Amplification and analysis of ISSR-PCR for Untreated and mutants gardenia plants, Kit cat no # 69104 (Qiagen Sciences, Maryland, USA) was used to extract genomic DNA from these plants according to the manufacturer's instructions. To detect polymorphism between untreated and treated plants, ten common ISSR-PCR primers were employed as described Table (1).The amplification reaction was performed in 25 µl reaction volume containing 12.5 µl Master Mix (sigma), 2.5 µl primer (10pcmol), 3 µl template DNA (10ng) and 7 µl dH_2O, according to (Ibrahim *et al*., [25]2019). PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (*PE* Applied Biosystems) programmed to accomplish 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 45ºC for 1 min, and an elongation step at 72ºC for 1.5 min. The primer extension segment was extended to 7min at 72ºC in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000).For ISSR analysis, only clear and unambiguous bands were visually scored as either present (1) or absent (0) for all samples and final data sets included both polymorphic and monomorphic bands. Then, a binary statistic matrix was constructed. Dice's similarity matrix coefficients were then calculated between genotypes using the unweighted pair group method with arithmetic averages (UPGMA). This matrix was used to construct a phylogenetic tree (dendrogram) was performed according to Euclidean similarity index using the PAST software Version 1.91 (Hammer *et al*., [26]).

Table 1. ISSR primers were used in the detection of polymorphism

Primer Name	Sequence
ISSR-1	5'-ACGACGACGACGACGACC-3'
ISSR-2	5'-AGAGAGAGAGAGAGAGYG-3'
ISSR-4	5'-ACACACACACACACACYG-3'
ISSR-5	5'-GTGTGTGTGTGTGTGTYG-3'
ISSR-6	5'-CGCGATAGATAGATAGATA-3'
ISSR-7	5'-GACGATAGATAGATAGATA-3'
ISSR-9	5'-GATAGATAGATAGATAGC-3'
ISSR-10	5'-GACAGACAGACAGACAAT-3'
ISSR- 11	5'-ACACACACACACACACYA-3'
	5'-ACACACACACACACACYC-3'

3. Results

Effect of gamma radiation on regeneration *in vitro* and plant development

3.1. **Shooting ability *in vitro***

The explants of tested *Gardenia jasminoides* Ellis responded variously to the utilize γ irradiation concentration with the maximum explant survival rate 100% at 15 Gy and control in all sub-cultures. In contrast, the 40 Gy doses decreased the explant survival level (rate) by 50, 66.6, and 50% in the first,

second and third sub-cultures compared with untreated shootlets and other doses (Table 2). Similarly, the 40 Gy dose reduced the shoot number and leave number in all sub-cultures. Whereas, the 15 Gy doses increased the shoot length, shoot number, and leave number in all sub-culture. Overall, the *Gardenia jasminoides* Ellis treated with 15 Gy was considered more tolerant to (GR) treatments than other tested doses. A high explant survival level is important to establish optimized mutagenesis platform application gamma rays. All doses of gamma rays in all sub-culture induced moderate impact on phenotypic principles as comparable with untreated treatment (Table 2 and Figure1).

3.2. Induction of rooting *in vitro*

Data presented in Table 3 , Figure 1and 3., tabulated that exposure of explants to gamma rays at the concentration 15 Gy resulted in the highest root percentage (88.87%),the most extended root length (3.51cm),and the most significant values for the number of roots (4.00). While, the largest leaf area was obtained from the explants treated with (15Gy) of GR (4.77 cm^2) comparable to other doses tested and control explants .In contrast, the minuscule root percentage was gradually exposed with shootlets to gamma rays (35 and 40Gy) (50, 33.3%). In addition, the lowest root length was obtained from explants tested with 30Gy (1.03mm) compared to other tested doses. However, we observed changes in the leaf area and the colour of leaves of gardenia plants with treated with 25, 30, 35, and 40Gy doses (Figure 1).

Table 2. Effect of different doses of gamma radiation on shooting ability in *vitro* of *Gardenia jasminoides*.

Parameters / γ-ray (Gy)	Subculture 1			
	Survival (%)	**Shootlet length (mm)**	**Shootlet number/explant**	**Leaves number/shootlet**
Control(0 γ-ray (Gy))	100.0± 5.0^{a}	40.00 ±2.00^{c}	4.67±0.34 ^{d}	29.60±2.08 ^{c}
15 γ-ray (Gy)	100.0± 1.03^{a}	62.00±2.00 ^{a}	6.33±0.18 ^{a}	43.67±1.97 ^{a}
20 γ-ray (Gy)	86.0 ±1.00^{b}	50.00±3.00 ^{b}	5.67±0.09 ^{b}	42.50 ±2.12^{ab}
25 γ-ray (Gy)	83.0 ±1.05^{b}	33.67±3.00 ^{d}	5.50 ±0.09^{bc}	41.67±2.12 ^{ab}
30 γ-ray (Gy)	66.6±2.01^{c}	29.67±1.92 ^{e}	5.30±0.06 ^{c}	39.00±4.018 ^{b}
35 γ-ray (Gy)	66.6±1.05^{c}	43.00 ±1.00^{c}	4.33±0.09 ^{e}	30.33±4.22 ^{e}
40 γ-ray (Gy)	50.0±5.00^{d}	41.00±1.00 ^{c}	4.00±0.13 ^{f}	24.00±3.81 ^{d}
Parameters / γ-ray (Gy)	**Subculture 2**			
	Survival (%)	**Shootlet length (mm)**	**Shootlet number/explant**	**Leaves number/ shootlet**
Control(0 γ-ray Gy)	100.0±1.00^{a}	47.00±2.00^{d}	5.4±0.07 ^{c}	49.00±1.89 ^{c}
15 γ-ray (Gy)	100.0±1.00^{a}	66.67 ±1.08^{a}	6.6 ±0.03^{a}	61.00±1.97 ^{a}
20 γ-ray (Gy)	88.5±2.00^{b}	57.00± 1.00^{b}	6.5±0.075 ^{a}	56.00±3.07 ^{b}
25 γ-ray (Gy)	86.0±2.00^{b}	45.00 ±1.00^{d}	6.00 ±0.11^{b}	50.00±2.74 ^{c}
30 γ-ray (Gy)	82.0±2.00^{c}	35.50 ±2.00^{c}	5.91±0.085^{b}	50.50±3.97 ^{c}
35 γ-ray (Gy)	80.0±0.97^{c}	55.67±2.00 ^{bc}	4.60 ±0.26^{d}	43.50±1.44 ^{d}
40 γ-ray (Gy)	66.6±2.05^{d}	53.33±1.00 ^{c}	4.50 ±0.105^{d}	41.00±5.07 ^{e}
Parameters / γ-ray (Gy)	**Subculture 3**			
	Survival (%)	**Shootlet length (mm)**	**Shootlet number/explant**	**Leaves number/ shootlet**
Control(0 γ-ray Gy)	100.0± 1.01^{a}	51.33±2.00 ^{d}	5.88±0.23 ^{d}	50.00±3.60 ^{e}
15 γ-ray (Gy)	100.0± 1.00^{a}	84.00±1.00 ^{a}	8.33±0.19 ^{a}	69.00±2.00 ^{a}
20 γ-ray (Gy)	88.5 ±2.00^{b}	80.67±0.88 ^{b}	7.00±0.09 ^{b}	63.67±1.45 ^{b}
25 γ-ray (Gy)	86.0±1.00^{c}	43.00±2.15 ^{e}	6.50±0.21 ^{c}	61.33±1.63 ^{c}
30 γ-ray (Gy)	86.0±2.00^{c}	36.67± 2.16^{f}	6.25±0.1 ^{cd}	54.00±1.73 ^{d}
35 γ-ray (Gy)	66.6±2.00^{d}	71.00±2.17 ^{c}	4.88±0.1 ^{e}	45.33±1.73 ^{f}
40 γ-ray (Gy)	50.0± 1.00^{e}	71.00±0.99 ^{c}	4.84±0.45 ^{e}	43.33±1.20 ^{f}

Averages (means) having the different letter(s) within the same column are significantly different among the treatments according to Duncan's multiple range tests at 5% level of probability. Data are mean of three replications ±SD. γ-ray (Gy): gamma radiation doses.

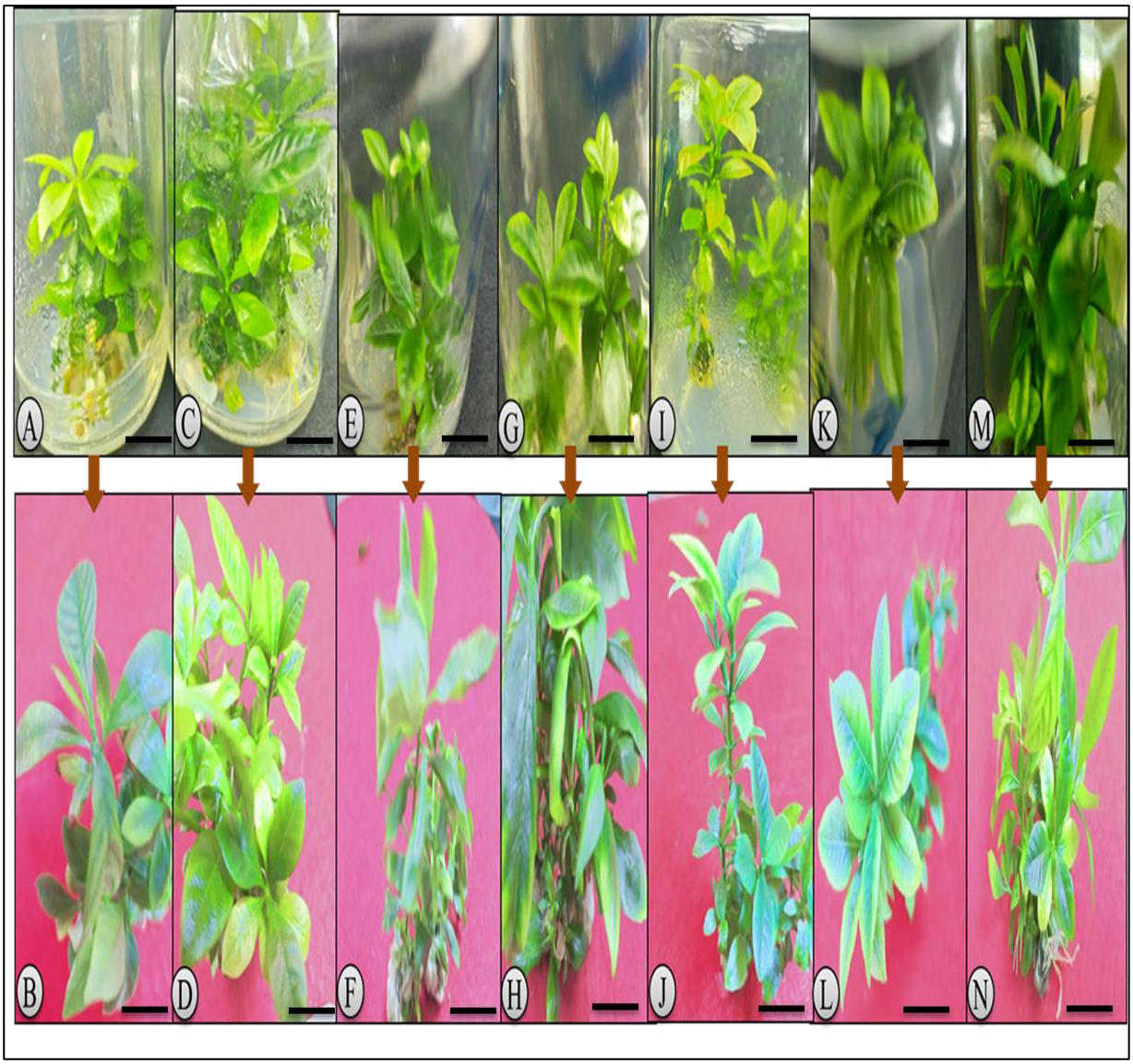

Figure 1. Effect of different levels of gamma radiation on shooting ability *in vitro* of *Gardenia jasminoides* Ellis**.(A,B)** control plantlets**,(C,D)** plantlets radiation with 15Gy**,(E,F)** plantlets radiation with 20Gy **(G,H)** plantlets radiation with 25Gy **(I, J),** plantlets radiation with 30Gy, **(K,L),** plantlets radiation with 35Gy, **(M,N),** plantlets radiation with 40Gy.

Table 3. **Effect of gamma radiation on rooting induction and leaf area (cm^2) after the final sub-culture on *in vitro.***

Parameters / γ-ray (Gy)	Final sub- culture			
	Root (%)	**Number of roots/shootlet**	**Root length(mm)**	**Leaf area (cm^2)**
control 0 **γ-ray (Gy)**	66.6±1.91^{c}	1.03±0.069^{c}	0.83±0.062^{f}	2.46±0.514^{c}
15 γ-ray (Gy)	88.87±0.87^{a}	4.00±1.79^{a}	3.51±0.11^{a}	4.77 ±1.087^{a}
20 γ-ray (Gy)	81.66±2.46^{b}	3.58±0.12^{a}	2.53±0.060^{c}	4.60± 1.341^{ab}
25 γ-ray (Gy)	80.20±0.97^{b}	2.83±0.62^{ab}	1.98±0.075^{d}	3.99± 0.291^{ab}
30 γ-ray (Gy)	66.66±1.125^{c}	2.0 ±0.101^{bc}	1.03±0.017^{e}	3.80 ±0.241^{ab}
35 γ-ray (Gy)	50.0±1.058^{d}	1.83±0.072^{bc}	2.85±0.055^{b}	3.72 ±1.674^{ab}
40 γ-ray (Gy)	33.33±0.815^{e}	1.33± 0.026^{c}	2.58±0.03^{c}	2.48 ±0.102^{c}

Averages (means) having the different letter(s) within the same column are significantly different among the treatments according Duncan's multiple range tests at 5% level of probability. Data are mean of three replication ± SD. γ-ray (Gy): gamma radiation doses.

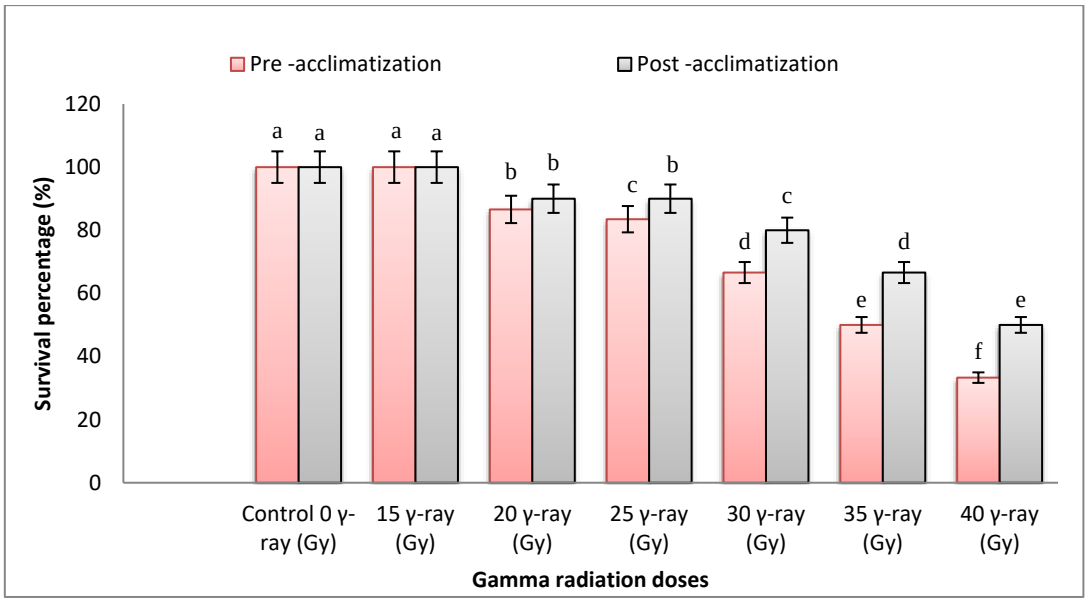

Figure 2. Effect of gamma radiation on survival percentage during pre-acclimatization and post-acclimatization of *Gardenia jasminoides* Ellis. Bars data with different letter/s differ significantly ($p \geq 0.05$). Letters (a–f) are comparison of individual treatment means. Data are mean of three replication ± SD.

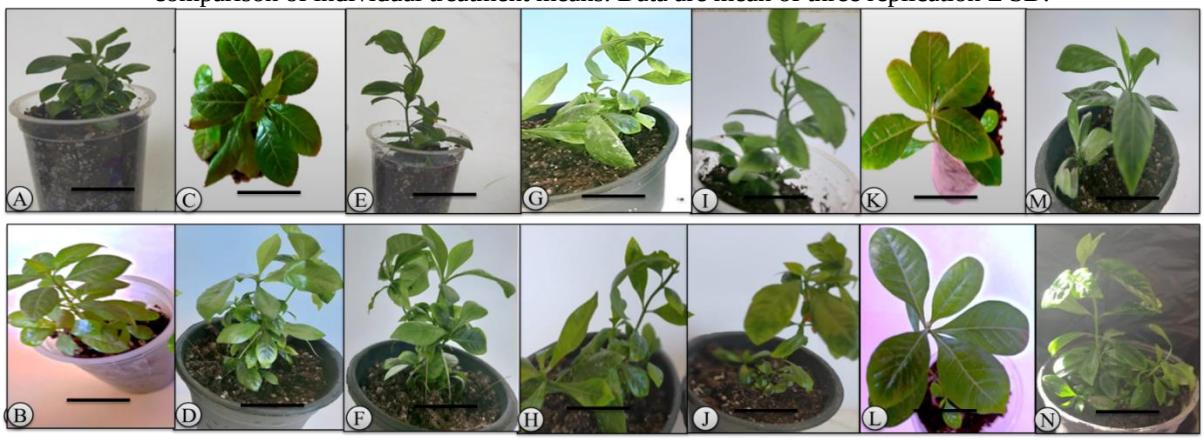

Figure 3. Successfully pre and post acclimization stage of *Gardenia jasminoides* Ellis. control plants **(A,B)**, plants radiation with gamma rays at 15 Gy **(C,D)**,20 Gy **(E,F)**, 25 Gy **(G,H)**,30 Gy **(I,J)**,35Gy **(K,L)**, and 40Gy **(M,N)**.

3.3. Acclimatization at the greenhouse

3.3.1. Survival percentage during pre and post acclimization

Acclimatization of *Gardenia jasminoides* Ellis was carried out after the third sub-culture when the plants rooted. There were two stages of acclimatization; pre- acclimization and post-acclimatization. Through pre-acclimatization, the highest growth survival the treated plants was recorded for the plant irradiated with a dose of 15 Gy (GR) and control by 100%. Alternatively, the 40 Gy-irradiated gardenia plants showed the lowest survival growth rate (33.33 %) among all treated plants (Figure 2). The achievement of pre-acclimatization at a greenhouse in untreated plants reached 100%. However, during post- acclimatization observed that the live percentage was increased than pre-acclimatization in 30, 35, and 40 Gy compared with pre-acclimatization stage. The maximum percentage 100% was obtained for plants exposure to 15 Gy (GR) and control.

3.3.2. Post- acclimatization

Data in (Table 4 and Figure 3) revealed that the exposed for gamma radiation at different doses (0, 15, 20,25,30,35, and 40Gy) resulted in a positive effect on acclimatization by showing a healthy and vigorously appearance of plantlets grown in peatmoss with perlite (2:1 v/v).The utilization of gamma radiation at the 15Gy resulted in the longest plantlets (15.83 cm), and the greatest number of branches (4.00), and the greatest number of leaves (52.00) compared to untreated plantlets and other tested treatments. On the other hand, the shortest plantlets were obtained from treatment with 30 Gy (GR) (8.00 cm). However, the minimum number of branches and

leaves were found with 40 Gy of gamma rays treatments by (1.67, 22.00), respectively. In addition, No differences were observed in leaf area in most doses tested from gamma rays. The largest leaf area was obtained for plants treated with both 15Gy (GR) (7.59 cm^2).

3.4. Chemical composition of shoots *in vitro*

3.4.1. Photosynthetic pigments and anthocyanin

Data presented in Table 5 showed that shootlets exposed to irradiation with 15Gy were recorded the greatest values for chlorophyll contents as compared to other tested shootlets and untreated shoots. The highest contents of chlorophyll (a, b) and total carotenoids (0.705, 0.304 and 0.340 mg/g F.W., respectively) were obtained from shootlets applied with 15Gy. However, the lowest values for chlorophyll content was obtained from shootlets treated with (35 and 40 Gy) comparable with other doss and untreated shoots. (Figure 1) . In addition, the highest amounts of anthocyanin recorded for shootlets exposed to 40 Gy gamma rays compared to other doses.

3.4.2. Total phenol and total Flavonoid content mg/g tissue F.W.

In Table (6), the plants exposed to 40Gy showed the highest contents of total phenolics and flavonoids compared with untreated and other treated plants. These changes in phenolic content would play functional roles in the irradiation stress response of gardenia plants.

3.4.3. DPPH Free Radical Scavenging Activity

The results showed the highest antioxidant activity (DPPH) for plants irradiated with 40 and 35 Gy gamma radiation by 51.8 and 50.03% compared to untreated and other treated plants Table (6).

3.4. 4.Total sugar

Results in Table (6) indicated the influence of gamma irradiation on the total sugar (mg/g F.W.) in shoot parts of gardenia plant. The dose of 15Gy increased the total sugar while decreasing by increasing the dose level. While the lowest content of total sugar is indicated to 40Gy dose irradiated plants.

3.4.5. Total free amino acid

Data in Table (6) found that the highest content of total free amino acid was recoded for plants applied with 15Gy followed by the control plants (without any irradiation), but indicated that the content of amino acid decreased with increasing doses of radiation.

3.5. Chemical composition in acclimization plants

3.5.1. Photosynthetic pigments and anthocyanin

The influence of various levels of gamma doses of *Gardenia jasminoides* Ellis, chlorophyll a, b and total carotenoids were released at all treatments expect (35 and 40 Gy groups) which decreased these values compared to the untreated plants and other treatments. The greatest values (2.287, 0.900 and 0.971 mg/g F.W., respectively) were obtained from shootlets irradiated with 15Gy .In contrast, the lowest values were found for 35 and 40Gy gamma rays comparable to untreated and other treatments in (Table 7).

Regarding the impact of various gamma rays doses, leaves anthocyanin contents in (Table 7) showed that anthocyanin content of Gardenia leaves was significantly increased with increasing the doses of gamma rays and reached up to greatest values (49.51 and 55.97and mg/100g F.W.) with both doses (35Gy and 40Gy) as compared with untreated plants (25.93 mg/100 g F.W.).

3.5.2 Total phenol and total Flavonoid content mg/g tissue F.W.

Table (8) indicated that the highest phenol and flavonoid content of plantlets leaves (2.28 and 0.86 mg /g F.W.) was detected for plantlets applied with high doses of irradiation (40Gy), but the lowest content was indicated with control plants.

3.5.3. DPPH Free Radical Scavenging Activity

Different doses of gamma applied showed significantly differences of DPPH (Table 8).Where found the highest value (62.78%) with 40 Gy doses, but the lowest value recorded with untreated plants (47.83%).

3.5.4. Total sugar

In Table (8) the lowest content of total sugar obtained with high doses of gamma treatment, but the highest content of sugar found with treated by 15 Gy dose.

3.5.5. Total free amino acid

Our results in Table (8) revealed the doses of irradiation at 15 Gy increased the content of total free amino acid in shoot of plant sample relative to control (unexposed plants).

Table 4. Effect of gamma radiation treatments during post- acclimatization stage of *Gardenia jasminoides* Ellis plants.

0	Plant height (cm)	Number of branches/plant	Number of leaves/plant	Leaf area (cm^2)
Control 0 γ-ray (Gy)	10.50±0.643^{d}	2.70±0.529^{c}	31.22±1.65^{d}	3.00±0.67^{c}
15 γ-ray (Gy)	15.83±1.217^{a}	4.00 ± 1.21^{a}	52.00±1.59^{a}	7.59±0.902^{a}
20 γ-ray (Gy)	14.00±1.47^{b}	3.67±0.61^{ab}	45.67±3.00^{b}	6.19±0.910^{ab}
25 γ-ray (Gy)	9.67±1.601^{d}	3.00 ±0.18^{bc}	39.00±1.69^{c}	6.06±0.910^{ab}
30 γ-ray (Gy)	8.00±1.27^{e}	2.83±0.072^{bc}	29.50±1.21^{de}	5.99±1.06^{ab}
35 γ-ray (Gy)	10.77±1.14^{d}	2.67±0.13^{c}	26.50±1.97^{e}	5.95±0.970^{ab}
40 γ-ray (Gy)	12.67±1.11^{c}	1.67±0.13^{c}	22.00±2.03^{f}	5.27±1.066 ^{ab}

Averages (means) having the different letter(s) within the same column are significantly different among the treatments according Duncan's multiple range tests at 5% level of probability. Data are mean of three replication ±SD. γ-ray (Gy):gamma radiation doses.

Table 5 . Effect of gamma radiation on shootlets Chlorophyll a, b and carotenoids and anthocyanin *in vitro.*

Determinations γ-ray (Gy)	Chlorophyll a (mg/ g F.W.)	Chlorophyll b (mg/ g F.W.)	Total Carotenoids (mg/ g F.W.)	Anthocyanin (mg/100 g F.W.)
Control 0 γ-ray (Gy)	0.429± 0.008^{d}	0.211±0.009^{b}	0.204±0.012 ^{e}	6.14±0.0.36^{f}
15 γ-ray (Gy)	0.705±0.005^{a}	0.304±0.011^{a}	0.3401±0.009^{a}	15.68±0.036^{e}
20 γ-ray (Gy)	0.588± 0.016^{b}	0.172±0.003^{d}	0.2458±0.006^{d}	15.60±1.243^{e}
25 γ-ray (Gy)	0.553± 0.008^{b}	0.19±0.011^{cd}	0.292±0.009^{b}	21.73±0.396^{d}
30 γ-ray (Gy)	0.527±0.009^{c}	0.207±0.007^{bc}	0.267±0.006^{c}	25.3±0.694^{c}
35 γ-ray (Gy)	0.332±0.204^{e}	0.149±0.001^{e}	0.1703±0.005^{f}	26.89±1.481^{b}
40 γ-ray (Gy)	0.303±0.012^{f}	0.1103±0.020^{f}	0.1588±0.011^{g}	29.44±0.646^{a}

Averages (means) having the different letter(s) within the same column are significantly different among the treatments according Duncan's multiple range tests at 5% level of probability .Data are mean of three replication ±SD. γ-ray (Gy):gamma radiation doses.

Table 6 . Effect of gamma irradiation treatments in some chemical composition of *Gardenia jasminoides* Ellis *in vitro.*

0	Total phenol mg /g F.W.	Total flavonoid mg /g F.W.	DDPH%	Total free Amino acid mg\g F.W. in vitro	Total sugar mg\g F.W.
Control 0 γ-ray (Gy)	0.616±0.02^{f}	0.24±0.06^{e}	33.25±2.91^{e}	7.364±0.078^{b}	7.796±0.304 ^{b}
15 γ-ray (Gy)	0.694±0.03^{ef}	0.27±0.04^{e}	40.31±1.37^{d}	8.01±0.155^{a}	9.552±0.633^{a}
20 γ-ray (Gy)	0.766±0.03^{e}	0.34±0.03^{d}	42.73± 2.27^{cd}	6.45±0.141^{c}	5.215±0.124^{c}
25 γ-ray (Gy)	0.945±0.04^{d}	0.44±0.06^{c}	45.41±4.11^{c}	5.965±0.134^{d}	4.497±0.339^{d}
30 γ-ray (Gy)	1.044±0.07^{c}	0.48±0.03^{bc}	48.58±2.47^{b}	4.53±0.296^{e}	3.903±0.159^{e}
35 γ-ray (Gy)	1.123±0.01^{b}	0.50±0.01^{b}	50.03±1.37^{ab}	3.42±0.254^{f}	3.192±0.443^{f}
40 γ-ray (Gy)	1.224±0.13^{a}	0.56±0.03^{a}	51.80±0.72^{a}	2.81±0.282^{g}	2.88±0.169^{f}

Averages (means) having the different letter(s) within the same column are significantly different among the treatments according Duncan's multiple range tests at 5% level of probability. Data are mean of three replication ±SD. γ-ray (Gy): gamma radiation doses.

Table 7. Effect of gamma radiation on chlorophyll a, b and carotenoids and anthocyanin in acclimization of *Gardenia jasminoides* Ellis plants.

Determinations γ-ray (Gy)	Chlorophyll a (mg/ g F.W.)	Chlorophyll b (mg/ g F.W.)	Total carotenoids (mg/ g F.W.)	Anthocyanin (mg/100 g F.W.)
Control 0 γ-ray (Gy)	0.851±0.024^{d}	0.449±0.010^{c}	0.594±0. 104^{c}	25.931± 1.750^{d}
15 γ-ray (Gy)	2.287±0.071^{a}	0.900±0.015^{a}	0.971± 0.220^{a}	27.273 ± 1.576^{cd}
20 γ-ray (Gy)	2.102±0.017^{b}	0.890± 0.012^{a}	1.002 ±0.119^{a}	28.095 ±0.955^{c}
25 γ-ray (Gy)	1.201±0.042^{c}	0.498 ±0.004^{b}	0.786±0.027^{b}	28.201 ±0.792^{c}
30 γ-ray (Gy)	0.868±0.009^{d}	0.291±0.006^{e}	0.481±0.004^{d}	49.223± 0.904^{b}
35 γ-ray (Gy)	0.675±0.020^{e}	0.367 ±0.012^{d}	0.293± 0.013^{e}	49.510 ±1.789^{b}
40 γ-ray (Gy)	0.386±0.035^{f}	0.178 ±0.019^{f}	0.299±0.011^{e}	55.982±1.090 ^{a}

Averages (means) having the different letter(s) within the same column are significantly different according Duncan's multiple range tests at 5% level of probability. Data are mean of three replications ±SD. γ-ray (Gy): gamma radiation doses.

Table 8 . Effect of gamma radiation on some chemical composition of *Gardenia jasminoides* Ellis during acclimization of *Gardenia jasminoides* Ellis plants.

Determinations / γ-ray (Gy)	Total phenol mg /g F.W.	Total Flavonoid mg /g F.W.	DDPH%	Amino acid mg\g F.W.	Total sugar mg\g F.W.
Control 0 γ-ray (Gy)	0.8735±0.04 ^{f}	0.37±0.06^{f}	47.83±3.51^{e}	8.277±0.21^{b}	12.848±0.73^{b}
15 γ-ray (Gy)	1.1317± 0.04^{e}	0.4± 0.04^{f}	49.25 ± 2.64^{de}	9.314±0.33^{a}	14.126±1.26^{a}
20 γ-ray (Gy)	1.3346± 0.03^{de}	0.57± 0.03^{e}	51.56±2.18 ^{cd}	6.8865±0.48^{c}	11.757±1.18^{b}
25 γ-ray (Gy)	1.4241± 0.03^{cd}	0.62± 0.06^{d}	53.22± 0.14^{c}	5.416 ±0.27^{d}	10.202±1.48^{c}
30 γ-ray (Gy)	1.57335±0.04 ^{c}	0.68±0.03 ^{c}	57.49 ±3.04^{b}	4.512± 0.71^{e}	8.776 ±0.78^{d}
35 γ-ray (Gy)	1.83782±0.04 ^{b}	0.75± 0.01^{b}	59.69±1.32 ^{b}	4.111±0.15^{e}	7.025± 0.84^{e}
40 γ-ray (Gy)	2.2834±0.46 ^{a}	0.86± 0.03 ^{a}	62.78 ±1.03^{a}	3.439±0.58^{f}	6.458± 0.35^{e}

Averages (means) having the different letter(s) within the same column are significantly different among the treatments according Duncan's multiple range tests at 5% level of probability. Data are mean of three replication ± SD. γ-ray (Gy): gamma radiation doses.

3.6. Analysis of ISSR polymorphisms in *Gardenia jasminoides* Ellis and their mutants

Ten ISSR PCR primers were employed to detect the genetic diversity between the control plant (No. 1) and the mutants (Nos. 2-6) which, were inducted *via* γ-irradiation. In this research, 10 ISSR primers produced scoreable and reliable banding patterns (descriptions of these PCR products are visualized in Figure). The amplified products produced 98 clear bands, ranging from 7 (in ISSR-7 and ISSR-11) to (in ISSR-10).The percentage of polymorphism was ranged from (30 to 60%). Polymorphic bands can help identify mutants through two ways, first is by the release of distinct banding patterns to individual species, and second is through the presence or absence of distinctive band(s) (marker bands) that distinguish an individual from its population, so it is critical to determine its DNA fingerprint. Primer ISSR-2 and ISSR-9 amplify the maximum number of unique bands, recording two, while ISSR-1, ISSR-6, ISSR-7, ISSR-10, and ISSR 12 no unique bands were amplified. ISSR primers were robust and informative, indicating that they might be a better tool for genetic diversity and phylogenetic analysis. ISSR-2 and ISSR-9 primers were found to be the most effective in distinguishing between wild and mutant types, as well as in generating large numbers of non-unique and unique polymorphic bands, as shown in Table 9 and Figure 4. These variations in ISSR banding patterns might be connected with structural rearrangements in DNA caused by different types of DNA damages caused by treatment with Gamma irradiation. As shown in Table10 and Figure 5 a phylogenetic tree illustrated three distinct groups. The first group) included one cluster with mutant (1) at 25Gy, with a low genetic similarity ratio 86% with wild-type (No. 1). On the other hand, the second group included wild type and their mutants (No.2, 5, and 6) 15, 30, and 35 Gy respectively with different distances between them. The third group included two mutants (No.3 and 7).

Table 9.The statistical analysis of ISSR primers used to discriminate between.

No	Name of primer	Monomorphic bands	polymorphic bands	Number of unique bands	Total bands	Polymorphism (%)	MW range (bp)	Mean of frequency
1	ISSR-1	6	6	0	12	50	191-1208	0.7
2	ISSR-2	4	4	2	10	60	161-860	0.7
3	ISSR-4	7	2	1	10	30	228-609	0.8
4	ISSR-5	5	2	1	8	38	207-809	0.7
5	ISSR-6	4	4	0	8	50	211-418	0.8
6	ISSR-7	3	4	0	7	57	240-731	0.7
7	ISSR-9	9	2	2	13	31	187-881	0.8
8	ISSR-10	12	2	0	14	14	187-955	0.9
9	ISSR-11	4	2	1	7	43	251-694	0.8
10	ISSR12	6	3	0	9	33	222-587	0.9
Total		60	31	7	98			7.8
Average		6	3.1	0.7	9.8			0.78

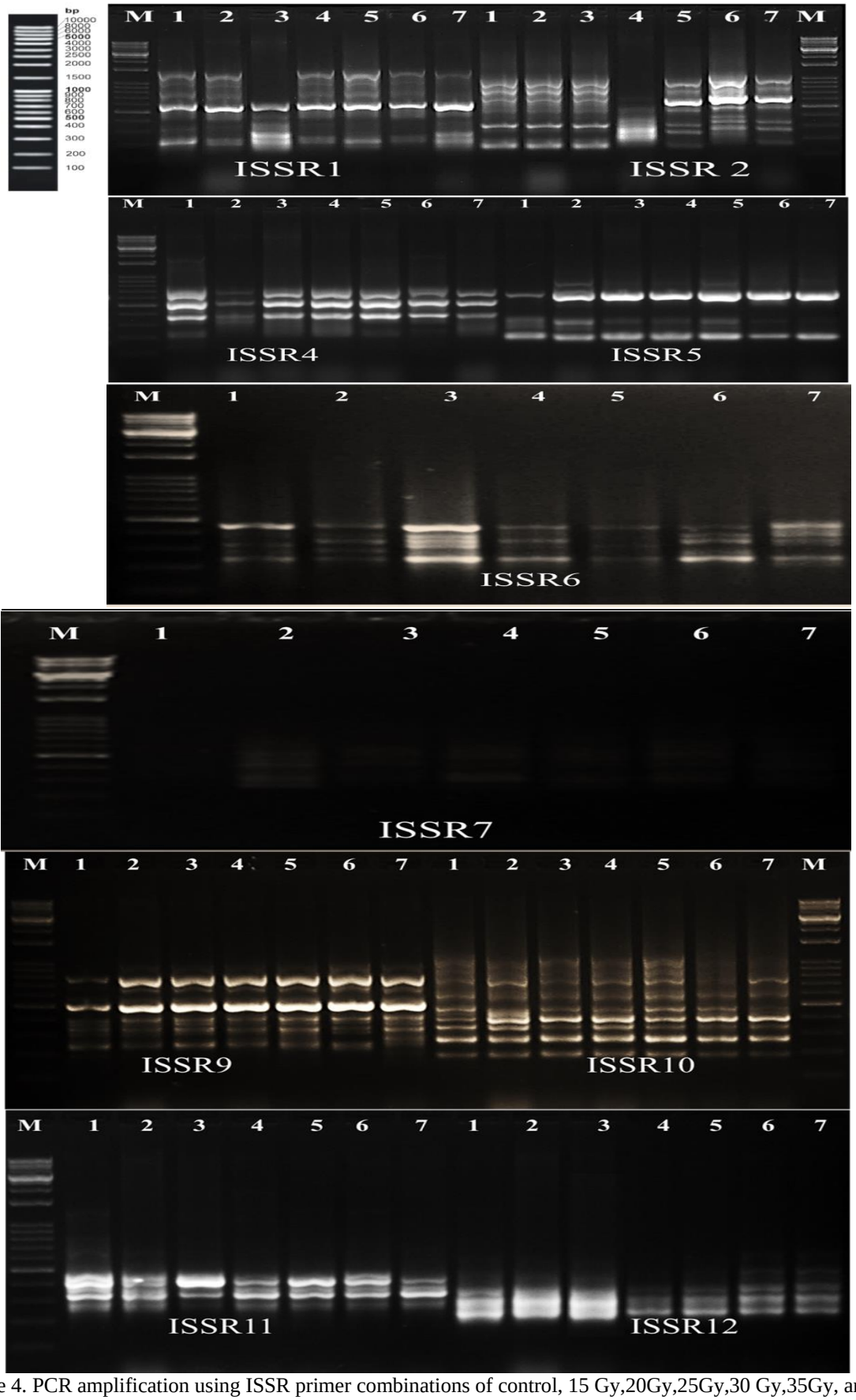

Figure 4. PCR amplification using ISSR primer combinations of control, 15 Gy,20Gy,25Gy,30 Gy,35Gy, and 40 Gy (1–7), M (DNA ladder 100 bp) of *Gardenia jasminoides* Ellis plant.

Table 10 .The similarity matrix between control and different gamma-irradiated treatments of *Gardenia jasminoides* Ellis plant.

Similarity matrix between .١							
Name	**Control**	**15 Gy**	**20 Gy**	**25 Gy**	**30 Gy**	**35 Gy**	**40 Gy**
Control	1						
15 Gy	0.93	1					
20 Gy	0.89	0.88	1				
25 Gy	0.86	0.88	0.88	1			
30 Gy	0.90	0.93	0.88	0.89	1		
35 Gy	0.89	0.94	0.90	0.91	0.88	1	
40 Gy	0.84	0.87	0.92	0.87	0.87	0.89	1

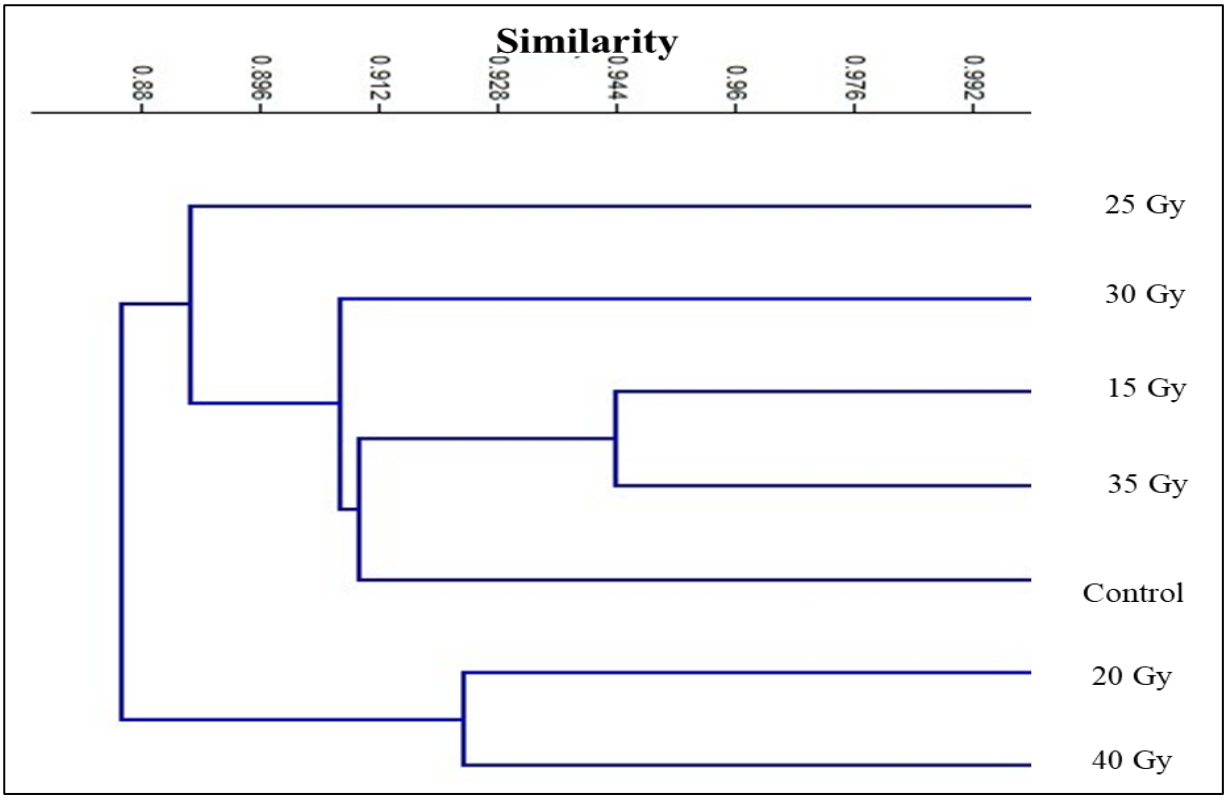

Figure 5. Dendrogram showing the relationships between *Gardenia jasminoides* (control) and their treatments after exposure to gamma radiation generated from ISSR data following the analysis of control and six treatments of *Gardenia jasminoides* Ellis plant. **1**: Control (without any treatment); **2**: plantlets treated with 15 Gy; **3**; plantlets treated with 20 Gy: **4**: plantlets treated with 25 Gy, **5**: plantlets treated with 30 Gy, **6**: plantlets treated with 35 Gy, **7**: plantlets treated with 40 Gy.

Dissection

Gamma irradiation has different effects on plants, in this study the low doses of gamma irradiation showed positive effect on gardenia plants *in vitro* especially 15 Gy. While, the high doses had inverse effects on different parameters. This result agreed with research indicated by Abdulhafiz *et al*., [27], who reported a decline in shoot length at maximum doses of gamma rays based on a decrease in the mitotic activity of plant tissues. Billore *et al*., [28] on irradiated shoots of *Dendrobium Sonia* orchid, reduced in shoot length, leaf area, fresh weight, and survival rate with increased doses of gamma irradiation, and the same result indicated with Limtiyayatin *et al*., [29] on *Exacum affine* plant. Gamma radiation can cause an improvement of enzyme accumulation and stimulation endogens content of gibberellic acid, which can resulting in elongation of cell wall [27]. Also, the dose of irradiation affected on the hormone's efficacy, which altered the production of shoots. Auxin activities may be inhibited by gamma irradiation, which could alter morphogenetic responses [30].

The favourable response of low doses to gamma irradiation on adventitious root cultures is consistent that low doses of irradiation have stimulatory effects on the growth of plant tissues [31]. Similarly, Ngoenngam *et al*., [32] confirmed that the low dose of GR has positive effect of consequent growth on *Stylosanthes hamate*. We

observed the gradual increase in percentage of live plants with the plants exposed to low doses of gamma radiation. These findings are in agreement with Sianipar *et al*.,[33] and Choi *et al*., [34] who reported that mutagen expression may be changed the plantlets transitory steady-state physiology. Additionally, the survival rate during acclimatization can be used to determine the success of plant *in vitro* propagation [35]. The success of pre-acclimatization in untreated plants may be attributable to genetic feature do not change in untreated plants genetic features. However, with the other treatments, genetic alterations occur that make it impossible to acclimate and control stomata conductivity *in vitro.* Also, plantlets roots have not been able to absorb nutrients and water yet. The stomata conductivity is still low in the acclimatization stage, which requires a gradually reducing humidity environment to allow transpiration which latterly may causes the wilt and death plantlets quickly [33]. These results are in agreement with similar studies on banana [36] and on *Curcuma heyneana* plants [37].

More than any other radiation, gamma rays induce different types of variations, and effective to inducing chlorophyll mutation and some morphological changes [38]. In this study, different contents of chlorophyll were indicated to be effective with different doses of gamma rays. These findings agree with Kumar *et al*., [39] showed that ch a, ch b, and total chlorophyll were improved in shootlets treated with 100 Gy gamma radiation of *Euryale ferox* compared with untreated plantlets *in vitro*. Previous reports have demonstrated that application of gamma rays can impact on physiology and biochemistry of treated plants [40, 2]. This destruction could be related to chloroplasts' high gamma radiation sensitivity compared to other cell organelles [41]. Previous reports have demonstrated that application of gamma irradiation can impact on physiology and biochemistry of irradiated plants [42, 2]. In other words, when radiation levels are above the plant's concentrated dose limit, the photosynthetic apparatus' capabilities are reduced due to photosystem damage, resulting in reduction in pigments content [2].

The importance of phenol evaluation is that the phenolic compounds perform significant physiological and ecological roles; participating in resistance to various types of stresses. Additionally, increases in total phenolic and flavonoids content are considered as a common response for high radiation doses [43]. The phenols paramount for plant is due to a fair correlation among antioxidant and free- radical scavenging activity and its phenolic content, and protects the plant against irradiation - induced oxidative stress [43]. It has been reported that total flavonoids as a group of phenolic responded significantly to gamma radiation doses particularly 100 Gy in *Cosmos caudatus* plant [44].The increase in flavonoids contents in irradiated plants, may be resulting from in degradation of large phenolic compound into smaller compound or release compounds of phenolic from glycosidic components of irradiated leaves [45]. These findings are confirmed with several workers in different plants [43, 40].

These results in study harmony with Aly *et al*., [45] reported an increase in total phenol on gamma irradiated eggplant. However, Del Valle *et al*., [46] on *Silene littorea* plant. Alternatively, Munim *et al*., [47] found the gamma irradiation of *Peperomia pellucida* plant *in vitro* decreased total flavonoid content. Flavonoid synthesis responds to irradiation (gamma and UV-B stress) whereas, the flavonoids alleviate the damage induced by the irradiation stress [2].

DPPH Free Radical Scavenging Activity, the gene expression may be altered as a result of obviates the oxidative damage induced by exposure of plants to high doses of gamma radiation [48]. This results harmony with Dmour *et al*., [48] on *Teucrium polium* plant, with gamma irradiation, applied showed increased of DPPH, over expression production of antioxidant under irradiation can be enhancement of the antioxidative defense. On black gram plant Yasmin *et al*., [49] suggested that the applied gamma irradiation produced ROS as well as demonstrate to counteract by antioxidant enzyme.

The total sugar reduction might be representing a regulatory response in the photosynthesis process and adjustment homeostasis in gamma irritated plants [50]. These results are in agreement with Patil *et al*., [51] who reported that the gamma irradiation decreased amino acids and sugar in callus but enhanced the antioxidant defense enzymes on *Artemisia annua* and Naz *et al*., [52] of *Allium sativum* plant. The results regularity with Jan *et al*., [53] when seeds of *Cullen corylifolium* plants utilized irradiation at (0,2.5,5,10, 15 and 20kGy dose rate) found the maximum values of sugar and total chlorophyll at 10 Gy . Conversely, Hussein, [54] indicated the 5 Gy gamma dose decreased sugar content and proline of barley plants. Besides, Mohajer *et al*., [55] suggested the applied of gamma did not stimulate significant increment in water-soluble components such as minerals, nitrogenous constituents and sugars.

In gamma irradiated plants, the accumulation of total free amino acid might be due to maintain osmotic potential in the cytoplasm and vacuoles at an optimal level of cell metabolism, hence cell structure protecting from free radicals. In addition, suggested that low doses of gamma radiation can act as an activator for amino acid biosynthesis, which change the content of protein that has essential roles in plant growth [54]. Ionizing radiation, including gamma rays, generates fragment deletions or insertions that eventually lead to changes in amino acids and a

modification of stem pigmentation [37]. These results accordance with Taha *et al*., [36], amino acids play diverse roles in growth, development and defense processes. Furthermore, under stress condition changes amount of amino acids modifying metabolism of plant [56, 57, 58]. Furthermore, According to ISSR analysis, these results could be due treatment with γ-irradiation leading nucleotide deletions or addition in the DNA sequence, resulting in a reading frame shift mutation. On the other hand, since direct damage and alterations to DNA are heritable, γ-rays are the primary cause of single-stranded and double-stranded breaks in DNA, and changes in DNA bases. Our results have shown that γ-irradiation, a key to mutation, is an effective way to enlarge the mutation map. Wu *et al*., [59] reported that using ISSR markers to analyze the mutants further may offer a useful molecular marker for the detection of mutants in the plant, which provides some valuable information for future breeding of ornamental plants. Li *et al.* [2] used ISSR-PCR molecular marker technique to identify the mutants of phenotypic variation plants in *Tulipa gesneriana L*. after exposure to different doses of γ-irradiation. Polymorphic genetic markers are widely used in plant improvement programs and can identify varieties and parents. They are extremely reproducible, polymorphic, instructive, and simple to use Chaudhary *et al*., [60] ISSR has been widely implemented to identify DNA polymorphisms in *Pimpinella anisum L*. [61], *Populus alba* and *Solidago canadensis* cv, Tara plants [62,1].

Conclusion

In brief, the utilization of gamma rays as a method for genetic variations had a positive impact on both the *in vitro* and *in vivo* growth rate and secondary metabolism. Hence, it can be concluded that gamma irradiation treatment is an effective tool for mutation stimulation in *Gardenia jasminoides* Ellis plant. Additionally, ISSR analysis proved to be an effective molecular marker for identifying mutant plants. This work is beneficial not only for producing new *Gardenia jasminoides* Ellis variants, but also for studying mutations in other ornamental plants. Further studies should be done of gamma radiation on adaptation plants until flowering stage.

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