In vitro Mutagenesis for Increasing Drought Tolerance and Molecular Characterization in Grape (*Vitis vinifera* L.) cv. "Black Matrouh"

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Abstract:

This study was conducted at the National Gene Bank and Atomic Energy Commission, Egypt started in 2012 to 2014; to produce grape (*Vitis vinifera* L.) plantlets more tolerant to drought. Shoot tip explants of grape cv. "Black Matrouh" were irradiated with various doses (0-50 Gray) of gamma rays and three vegetative generations (from M_1V_1 to M_1V_3) were obtained. To induce drought stress, different concentrations (0, 5 and 10 gl^{-1}) of polyethylene glycol (PEG 6000) were added to the media. The sensitivity of shoot tip explants to radiation increased gradually with increasing gamma rays dose, and LD_{50} was found at 50 Gray. Elevated shoot number and length were observed with increasing the subculture number. Under drought stress, vegetative and rooting parameters gradually decreased at the same radiation dose with increasing PEG concentration. Explants could tolerate the hard effect of drought when irradiated with 20 Gray of gamma rays. Out of the 20 Inter Simple Sequence Repeat primers used, 6 primers (17898-B, 852, ISSR-35, 834, 889 and HB-9) generated 8 unique bands in plants irradiated with 20 Gray that could be considered as potential markers for initial estimation and selection of drought tolerance in grape.

Keywords: Grape, Gamma rays, LD_{50}, Drought tolerance, PEG, ISSR analysis.

Introduction:

Water is becoming increasingly limited in many areas of agricultural production and water deficit is among the most important environmental stresses that limit crop productivity (Rajashekar *et al.*, 1995). One possible way to ensure future food needs of the increasing world population should involve a better use of water by the development of crop varieties which need less amount of water and more tolerant to drought (Shao *et al.*, 2006).

Genetic improvement of drought tolerance has traditionally been a problematic issue in plant breeding for a variety of reasons, among which is the lack of clearly defined selection criteria for tolerance. Moreover, plant breeding using conventional procedures is time consuming and sometimes impossible for a number of plant species (Ehsanpour and Razavizadeh, 2005).

During the last two decades, many scientists reported that breeding for tolerance against stress factors has been possible by using a combination of mutagenesis, *in vitro* techniques and molecular markers (Yaycili and Alikamanoglu, 2012). The mutation breeding via gamma radiation is an effective and highly successful approach for fruit breeding purposes and generation of commercial cultivars (Predieri and Di Virgilio, 2007). In addition, *in vitro* successful selection for drought tolerance using polyethylene glycol (PEG) as selection agent has been applied to several crops (El-Agamy *et al.*, 2009). Moreover, molecular markers are important tools in precisely detecting the

effect of gamma radiation since they identify genetic polymorphism at the DNA level. These markers have been used to study genetic dissimilarity in many crop species (Pestana *et al.*, 2011). Inter Simple Sequence Repeat (ISSR) markers provide a reliable and rapid detection means for the screening of mutants (Wu *et al.*, 2011) and overcome many technical limitations of RFLP and RAPD analyses (Devarumath *et al.*, 2002).

On the other hand, grape (*Vitis vinifera* L.) is an old deciduous temperate fruit crop, widespread and highly valuable because of its nutritional characteristics as a natural source of sugar, vitamins and fibers (Gomes *et al.*, 2004). Egypt ranked the 12th on the world production while Syria ranked the 27th according to (FAO STAT, 2011). "Black Matrouh" is one of the most popular table grapes in Egypt. It has good tolerance to soil salinity and irrigation water due to its adapting to the nature of the growth area in Mersa Matrouh. As grape area extended greatly in reclaimed regions, the need to water conservation and utilization of plants adapted to drought conditions has become urgent (Rajashekar *et al.*, 1995). Thus, the present study aimed to regenerate plantlets more tolerant to drought stress from grape cv. "Black Matrouh" through *in vitro* mutagenesis by using gamma irradiation as an inducing factor, and to detect the genetic variation between irradiated and non-irradiated (control) plants by ISSR analysis.

Materials and Methods:

This study was carried out in Tissue Culture Laboratory and Laboratory of Molecular Genetics, National Gene Bank, Agricultural Research Center, Giza, Egypt, and at Atomic Energy Commission, Nuclear Research Center, Anshas, Egypt, during the period 2012-2014.

Plant Material:

Shoot tip explants from local grape (*Vitis vinifera* L.) cv. "Black Matrouh" were cultured on full strength MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mgl^{-1} benzyl amino purine (BAP) as an establishment medium. The individual plantlets were then multiplied on three-quarter strength MS medium supplemented with 0.75 mgl^{-1} BAP and 0.5 mgl^{-1} indole-3-buteric acid to get aseptic plant materials for *in vitro* mutagenesis treatments. Well developed shoots (1.0 cm) were transferred to half strength MS medium with 0.5 mgl^{-1} naphthalene acetic acid for rooting stage. The cultures were kept in growth room at 25±2°C and under photoperiods of 16 h per day supplied by fluorescent lamp (four lamps per shelf) to provide light intensity of 3000 lux at explants level (Al Mousa *et al.*, Under Press).

***In vitro* mutagenesis:**

Shoot tip explants (5 mm) from well established *in vitro* shoots of grape cv. "Black Matrouh" were isolated and irradiated with different doses of gamma rays (0, 10, 20, 30, 40 and 50 Gray) from gamma irradiation cell with a ^{60}Co source at dose rate of 13.38 Gy. min^{-1} in Cyclotron Unit.

Determination of radio-sensitivity response to different gamma rays doses:

Irradiated and non-irradiated shoot tips were cultured on multiplication medium, mentioned above, for 4 weeks to raise M_1V_1. Then, the individual plantlets were dissected and cultured on the same medium up to M_1V_3, because a number of vegetative propagation sub-cultures (M_1V_1 to M_1V_n) after irradiation are necessary to allow the mutated sectors to grow and develop in non-chimerical shoots. Lethal dose (LD_{50}) was calculated as the dose of gamma rays that reduces the survival percentage of irradiated meristems to 50% of non-irradiated controls in M_1V_1 after treatment (Heinze and Schmidt, 1995). Survival percentage (%), number of shoots/explant and shoot length (cm) were determined for every gamma rays dose at the end of every sub-culture (4 weeks for each).

***In vitro* selection for drought tolerance:**

a. Effect of different gamma rays doses on adventitious shoot induction under drought stress:

Irradiated and non-irradiated explants were dissected from the individual plantlets in M_1V_3 and recultured on multiplication medium containing polyethylene glycol [PEG (6000)] at different

concentrations (0, 5 and 10 gl^{-1}) for 4 times at monthly intervals. Survival percentage (%), number of shoots/explant and shoot length (cm) were determined as means after four sub-cultures on multiplication medium (4 weeks for each).

b. Effect of different gamma rays doses on rooting of proliferated shoots under drought stress:

Shoots (1.0 cm) proliferated from each treatment in the previous experiment were cultured individually on rooting medium, mentioned above, containing PEG (6000) at 0, 5 and 10 gl^{-1} concentrations. Rooting percentage (%), number of roots/shoot explant and main root length (cm) were determined after 5 weeks on rooting medium.

DNA extraction:

DNA extraction was carried out using five to six healthy young shoots collected from each gamma rays dose. Genomic DNA was extracted and purified using the DNeasy plant Mini Kit following the manual instructions (DNeasy Plant Handbook, 2012). Quality and quantity of DNA were estimated using spectrophotometer and by gel electrophoresis. Each sample was diluted to 50 $ng.\mu l^{-1}$ in TE (Tris-EDTA) buffer and stored at -20°C.

ISSR analysis:

Twenty ISSR primers were used to estimate the genetic variability among these treatments. Inter Simple Sequence Repeats analysis was carried out in a total volume of 25 µl reaction volume containing 2X ready mix (Emerald Amp Max PCR master mix), 25 pM oligonucleotide primer and 50 ng genomic DNA. The following temperature profile was used: 5 min initial denaturation of the template DNA at 95ºC, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at annealing temperature (Ta) of the primer (as shown in Table 10), and 1 min extension at 72°C, and ending with 10 min of additional extension at 72°C. The amplification products were visualized in an ultraviolet transilluminator, after horizontal electrophoresis in 2.5% agarose gel (SIGMA company), and photographed by gel documentation system (Alpha Innotech).

Data analysis:

Comparison of variability caused by gamma irradiation was carried out based on the presence or absence of fragment produced by ISSR amplification. The sizes of fragments were estimated by 100-bp ladder marker which was run along with the amplified products. ISSR bands were scored as present (1) or absent (0). The molecular results were analyzed using the Phoretix 1D Pro software from nonlinear Dynamics. Similarity matrix between treatments was determined using Jaccard's (1908) similarity coefficient. The coefficients were utilized to construct a dendrogram using the unweighted pair group of arithmetic means algorithm (UPGMA).

Statistical analysis

The experiments were arranged as factorial experiment in simple randomized design with three replicates, ten explants for each replicate. Effect of treatments was tested by ANOVA with least significant difference (LSD) calculated at 0.05 level of significance, using Waller and Duncan (1969). Collected data were statistically analyzed using IBM SPSS 22.0 (Software Package for Statistics and Simulation, 2013).

Results:

Determination of radio-sensitivity response to different gamma rays doses:

Table (1) clears that survival percentage of irradiated explants shows linear decreasing trend with increasing irradiation dose. Meanwhile, the low dose (10 Gy) of gamma rays exhibites high survival (88.89%) without significant difference compared to control plants (100%). Regeneration response of irradiated explants was very low beyond 20 Gy and decreased with increasing sub-culturing number. Furthermore, all explants irradiated with 50 Gy were not able to regenerate new shoots at the 3rd sub-culture (M_1V_3) as a friable callus was produced which turned brown and failed to survive later (Fig. 1). The dose 50 Gy was observed as the LD_{50} dose for "Black Matrouh" shoot tips.

Table 1: Effect of irradiation with different gamma-rays doses on survival percentage of "Black Matrouh" shoot tip explants cultured on multiplication medium for 3 successive sub-cultures

Gamma-rays (Gy)	Survival percentage of explants			Mean (A)
	M_1V_1	M_1V_2	M_1V_3	
Non-irradiated	100.00[a]	100.00[a]	100.00[a]	**100.00[A]**
10	93.33[ab]	90.00[abc]	83.33[bcd]	**88.89[A]**
20	90.00[abc]	60.00[ef]	60.00[ef]	**70.00[B]**
30	76.67[cd]	43.33[gh]	33.33[ghi]	**51.11[C]**
40	73.33[de]	30.00[hi]	26.67[ij]	**43.33[C]**
50	46.67[fg]	13.33[jk]	0.00[k]	**20.00[D]**
Mean (B)	**80.00[A]**	**56.11[B]**	**50.56[B]**	
LSD $_{0.05}$	A	= 11.21		
	B	=7.81		
	AxB	=13.52		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

The number of axillary shoots regenerated from non-irradiated explants and explants irradiated with 10 and 20 Gy was higher and significantly different from those regenerated from higher doses (30, 40 and 50 Gy) that adversely affected the multiplication rate as shown in Table (2). Meanwhile, shoot induction increased significantly with increasing the sub-culture number. The highest significant number of shoots/explant (3.50) was recorded in explants irradiated with 10 Gy at the 3rd sub-culture.

Table 2. Effect of irradiation with different gamma rays doses on number of shoots/explant of "Black Matrouh" cultured on multiplication medium for 3 successive sub-cultures

Gamma rays (Gy)	Number of shoots/explant			Mean (A)
	M_1V_1	M_1V_2	M_1V_3	
Non-irradiated	2.07[g]	2.73[cde]	2.93[bc]	**2.58[a]**
10	1.50[h]	2.43[ef]	3.50[a]	**2.48[a]**
20	1.27[hij]	2.57[de]	3.13[b]	**2.32[a]**
30	1.10[j]	2.13[fg]	2.87[bcd]	**2.03[b]**
40	1.13[ij]	1.47[hi]	2.60[cde]	**1.73[c]**
50	1.07[j]	1.17[hij]	0.00[k]	**0.75[d]**
Mean (B)	**1.36[C]**	**2.08[B]**	**2.51[A]**	
LSD $_{0.05}$	A	= 0.28		
	B	=0.20		
	AxB	=0.35		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

With regard to the effect of different gamma rays doses on shoot length, results in Table (3) indicates that 10 Gy was the most effective dose for stimulating shoot elongation, while increasing radiation dose decreased significantly the shoot length. Although shoot length increased with increasing the sub-culture number in all treatments, explants irradiated with 50 Gy completely failed to survive at the 3rd sub-culture. However, the longest shoots (1.48 cm) were recorded at the 3rd sub-culture in explants irradiated with 10 Gy.

Table 3. Effect of treatment with different gamma rays doses on shoot length (cm) of "Black Matrouh" shoot tip explants cultured on multiplication medium for 3 successive sub-cultures

Gamma rays (Gy)	Shoot length (cm)			Mean
	M_1V_1	M_1V_2	M_1V_3	
Non-irradiated	1.13^{defg}	1.33^{abc}	1.38^{abc}	$\mathbf{1.28^{ab}}$
10	1.24^{cde}	1.42^{ab}	1.48^{a}	$\mathbf{1.38^{a}}$
20	1.08^{efgh}	1.23^{cde}	1.28^{bcd}	$\mathbf{1.20^{b}}$
30	1.14^{def}	1.24^{cde}	1.28^{bcd}	$\mathbf{1.22^{b}}$
40	0.97^{ghi}	1.12^{defg}	1.02^{fghi}	$\mathbf{1.04^{c}}$
50	0.92^{hi}	0.91^{i}	0.00^{j}	$\mathbf{0.61^{d}}$
Mean	$\mathbf{1.08^{B}}$	$\mathbf{1.21^{A}}$	$\mathbf{1.07^{B}}$	
LSD $_{0.05}$	A	= 0.13		
	B	=0.09		
	AxB	=0.16		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

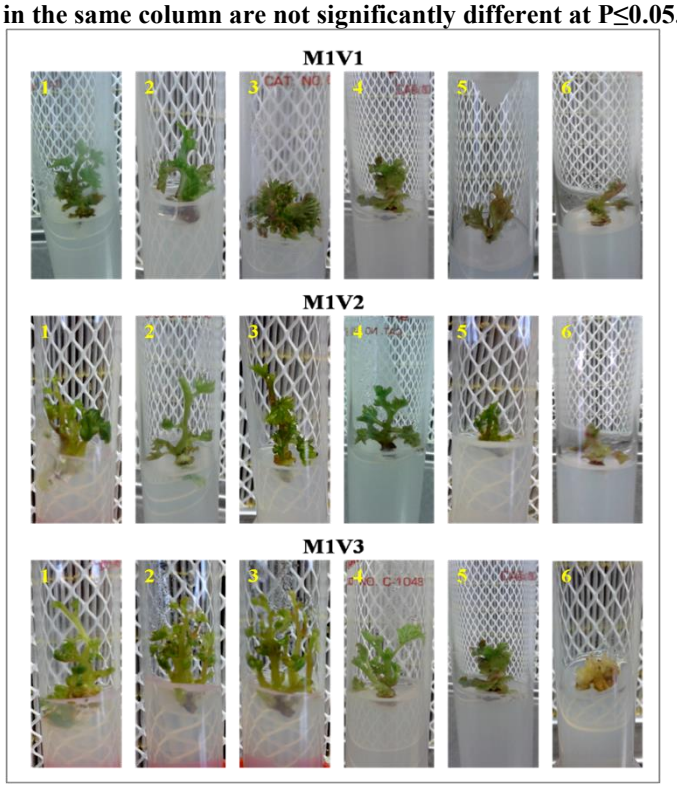

Fig. 1. Effect of different gamma rays doses on number of shoots/explant and shoot length (cm) of "Black Matrouh" for 3 successive vegetative regenerations in multiplication medium

1= Non-irradiated (control) **3= 20 Gy** **5= 40 Gy**
2= 10 Gy **4= 30 Gy** **6= 50 Gy**

In vitro selection for drought tolerance

a. Effect of different gamma rays doses on adventitious shoot induction under drought stress

The gamma rays dose of 50 Gy was lethal as all explants died, as showed in the previous experiment, so it was discarded from this experiment. Results in Table (4) demonstrates that the low doses (10-20 Gy) of gamma rays stimulated survival percentage of "Black Matrouh" explants under drought stress, but raising irradiation dose led to progressive decrease in this parameter. Furthermore, survival percentage decreased with increasing PEG concentration in the multiplication medium. Explants irradiated with 20 and 10 Gy exhibited significantly higher survival percentages compared to non-irradiated explants on medium with 5 gl^{-1} PEG (80.00, 73.33 and 53.33%, respectively).

Table 4. Effect of different gamma rays doses on survival percentage of "Black Matrouh" shoot tip explants under drought stress after 4 sub-cultures on multiplication medium

Gamma rays (Gy)	Survival percentage of explants			Mean (A)
	PEG- 0 gl^{-1}	PEG- 5 gl^{-1}	PEG- 10 gl^{-1}	
Non-irradiated	100.00^{a}	53.33^{de}	53.33^{de}	**68.89^{ab}**
10	100.00^{a}	73.33^{c}	66.67^{cd}	**80.00^{a}**
20	100.00^{a}	80.00^{bc}	53.33^{de}	**77.78^{a}**
30	100.00^{a}	53.33^{de}	40.00^{ef}	**64.44^{b}**
40	93.33^{ab}	46.67^{ef}	33.33^{f}	**57.78^{b}**
Mean (B)	**98.67^{A}**	**61.33^{B}**	**49.33^{C}**	
LSD $_{0.05}$	A	=11.79		
	B	= 9.13		
	AxB	=14.44		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

Similar trend was observed for shoot number/explant, which decreased gradually with increasing PEG concentration from 0, 5 to 10 gl^{-1} as illustrated in Table (5) and Fig (2). On the other hand, 20 Gy gamma rays dose had the most stimulative effect on adventitious shoot induction under drought stress, followed by 10 Gy gamma rays dose and non-irradiated explants without significant differences. Meanwhile, high doses (30 and 40 Gy) reduced the number of proliferated shoots/explant. On PEG-free medium, explants irradiated with 20 Gy had formed the highest number of shoots (4.07) with significant difference compared to non-irradiated explants (3.50). The lowest number of shoots (1.83) was obtained from explants irradiated with 40 Gy that cultured on medium with 10 gl^{-1} PEG.

Table 5. Effect of different gamma rays doses on number of shoots/explant of "Black Matrouh" under drought stress after 4 sub-cultures on multiplication medium

Gamma rays (Gy)	Number of shoots/explant			Mean (A)
	PEG- 0 gl^{-1}	PEG- 5 gl^{-1}	PEG- 10 gl^{-1}	
Non-irradiated	3.50^{bc}	2.53^{efg}	1.73^{i}	**2.59^{abc}**
10	4.00^{ab}	2.60^{defg}	1.67^{i}	**2.76^{ab}**
20	4.07^{a}	2.73^{def}	2.07^{ghi}	**2.96^{a}**
30	3.13^{cd}	2.30^{fgh}	1.80^{hi}	**2.41^{bc}**
40	2.93^{de}	1.93^{hi}	1.87^{hi}	**2.24^{c}**
Mean (B)	**3.53^{A}**	**2.43^{B}**	**1.83^{C}**	
LSD $_{0.05}$	A	= 0.45		
	B	= 0.34		
	AxB	= 0.55		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

Results in Table (6) clears that low doses (10-20 Gy) of gamma rays enhanced shoot elongation under drought stress, whereas increasing gamma dose from 30 to 40 Gy reduced significantly the shoot length. Meantime, increasing PEG concentration had negative effects on shoot elongation. No significant differences were observed between non-irradiated explants and explants irradiated with 10 or 20 Gy under each concentration of PEG. The highest shoots (1.74 cm) were noticed in explants irradiated with 10 Gy that cultured on PEG-free medium. Whereas, the shortest shoots (0.78 cm) were noticed in explants irradiated with 40 Gy that cultured on medium with 5 gl^{-1} PEG.

Table 6. Effect of different gamma rays doses on shoot length (cm) of "Black Matrouh" under drought stress after 4 sub-cultures on multiplication medium

Gamma rays (Gy)	Shoot length (cm)			Mean (A)
	PEG- 0 gl^{-1}	PEG- 5 gl^{-1}	PEG- 10 gl^{-1}	
Non-irradiated	1.70^{ab}	1.43^{cde}	1.30^{de}	**1.48^{a}**
10	1.74^{a}	1.58^{abc}	1.43^{cde}	**1.59^{a}**
20	1.58^{abc}	1.50^{bcd}	1.25^{e}	**1.44^{a}**
30	1.46^{cd}	1.37^{de}	0.83^{f}	**1.22^{b}**
40	1.32^{de}	0.78^{f}	0.89^{f}	**1.00^{c}**
Mean (B)	**1.56^{A}**	**1.33^{B}**	**1.14^{C}**	
LSD $_{0.05}$	A	= 0.16		
	B	= 0.13		
	AxB	= 0.20		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

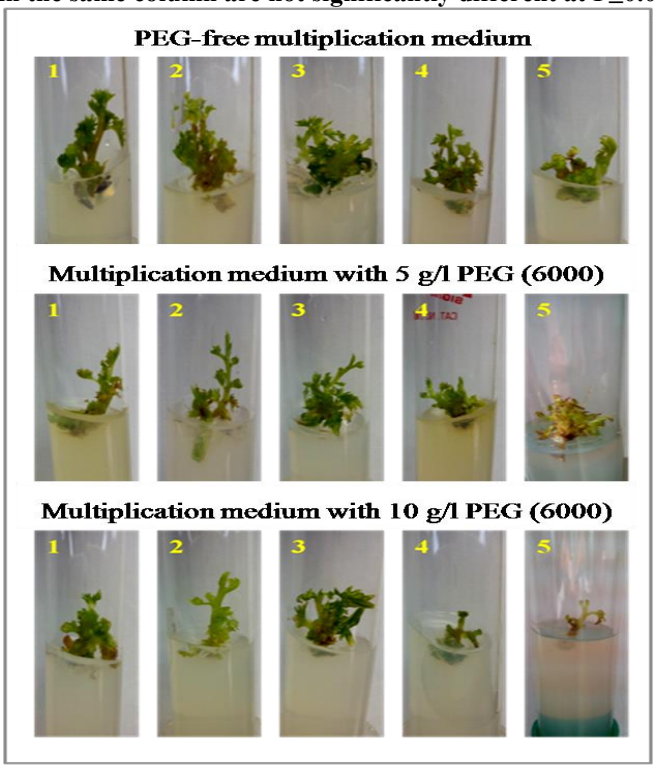

Fig. 2. Effect of different gamma rays doses on number of shoots/explant and shoot length (cm) of "Black Matrouh" under drought stress after 4 sub-cultures on multiplication medium (4 weeks for each).

1= Non-irradiated (control) 2= 10 Gy
3= 20 Gy 4= 30 Gy 5= 40 Gy

b. Effect of different gamma rays doses on rooting of proliferated shoots under drought stress

Results in Table (7) clears that explants irradiated with 20 Gy recorded the highest rooting percentage without significant differences compared to non-irradiated explants and explants irradiated with 10 Gy. Rooting percentage significantly decreased with increasing gamma-rays doses to 30 and 40 Gy and with increasing PEG concentration in rooting medium. Explants irradiated with 20 Gy significantly exhibited higher rooting percentage compared to non-irradiated explants on medium with 10 gl^{-1} PEG (66.67 and 33.33%, respectively). Otherwise, no significant differences were observed between explants irradiated with 10 or 20 Gy and non-irradiated explants on PEG-free medium or medium with 5 gl^{-1} PEG.

Table 7. Effect of different gamma rays doses on rooting percentage of "Black Matrouh" shoot explants under drought stress after 5 weeks on rooting medium

Gamma rays (Gy)	Rooting percentage			Mean (A)
	PEG- 0 gl^{-1}	PEG- 5 gl^{-1}	PEG- 10 gl^{-1}	
Non-irradiated	93.33^{a}	66.67^{cde}	33.33^{gh}	**64.44^{a}**
10	80.00^{abc}	63.33^{cdef}	43.33^{gh}	**62.22^{a}**
20	86.67^{ab}	73.33^{bcd}	66.67^{cde}	**75.56^{a}**
30	56.67^{defg}	46.67^{fgh}	33.33^{h}	**45.56^{b}**
40	50.00^{efh}	43.33^{gh}	33.33^{h}	**42.22^{b}**
Mean (B)	**73.33^{A}**	**58.67^{B}**	**42.00^{C}**	
LSD $_{0.05}$	A	= 13.69		
	B	= 10.60		
	AxB	= 16.77		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

Gradual increase in PEG concentration caused a significant decrease in the number of roots formed per shoot explant in the same irradiation dose (Table 8, Fig 3). On the other hand, the higher dose of gamma rays (40 Gy) significantly reduced root induction compared to non-irradiated explants, while no significant differences were observed among other treatments. The highest numbers of roots (4.00, 4.43 and 4.10) were noticed on PEG-free medium from non-irradiated explants and explants irradiated with 10 and 20 Gy, respectively. Explants irradiated with 40 Gy that cultured on medium with 10 gl^{-1} PEG formed the lowest number of roots (1.73).

Table 8. Effect of different gamma rays doses on number of roots/shoot explant of "Black Matrouh" under drought stress after 5 weeks on rooting medium

Gamma rays (Gy)	Number of roots/shoot explant			Mean (A)
	PEG- 0 gl^{-1}	PEG- 5 gl^{-1}	PEG- 10 gl^{-1}	
Non-irradiated	4.00^{ab}	3.13^{cd}	2.00^{f}	**3.04^{ab}**
10	4.43^{a}	3.23^{cd}	2.22^{ef}	**3.29^{a}**
20	4.10^{a}	3.33^{cd}	2.27^{ef}	**3.23^{ab}**
30	3.50^{bc}	2.90^{d}	1.97^{f}	**2.79^{bc}**
40	2.77^{de}	2.20^{ef}	1.73^{f}	**2.23^{c}**
Mean (B)	**3.76^{A}**	**2.96^{B}**	**2.03^{C}**	
LSD $_{0.05}$	A	= 0.48		
	B	= 0.37		
	AxB	= 0.59		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

Root length exhibited a similar trend. Results in Table (9) clears that low doses (10 and 20 Gy) of gamma rays stimulated the root length under drought stress without significant differences compared to non-irradiated explants. Meanwhile, high doses (30 and 40 Gy) reduced significantly this parameter. In addition, increasing PEG concentration had negative effects on root length of explants in the same irradiation dose. No significant differences were observed between explants irradiated with 10 or 20 Gy and non-irradiated explants under each concentration of PEG. The longest roots (2.85 cm) were obtained from explants irradiated with 20 Gy and cultured on PEG-free medium, while the shortest roots (1.01 cm) were observed from explants irradiated with 40 Gy and cultured on medium with 10 gl^{-1} PEG.

Table 9. Effect of different gamma rays doses on root length (cm) of "Black Matrouh" shoot explants under drought stress after 5 weeks on rooting medium

Gamma rays (Gy)	Root length (cm)			Mean (A)
	PEG- 0 gl^{-1}	PEG- 5 gl^{-1}	PEG- 10 gl^{-1}	
Non-irradiated	2.75^{a}	2.00^{b}	1.50^{de}	**2.08^{a}**
10	2.71^{a}	1.98^{bc}	1.70^{cd}	**2.13^{a}**
20	2.85^{a}	2.13^{b}	1.58^{de}	**2.19^{a}**
30	2.15^{b}	1.42^{def}	1.14^{fg}	**1.57^{b}**
40	2.17^{b}	1.33^{ef}	1.01^{g}	**1.50^{b}**
Mean (B)	**2.53^{A}**	**1.77^{B}**	**1.39^{C}**	
LSD $_{0.05}$	A	= 0.24		
	B	= 0.18		
	AxB	= 0.29		

A: Gamma rays dose, B: Vegetative generation subculture (M_1V_n).
Means with the same letter in the same column are not significantly different at P≤0.05.

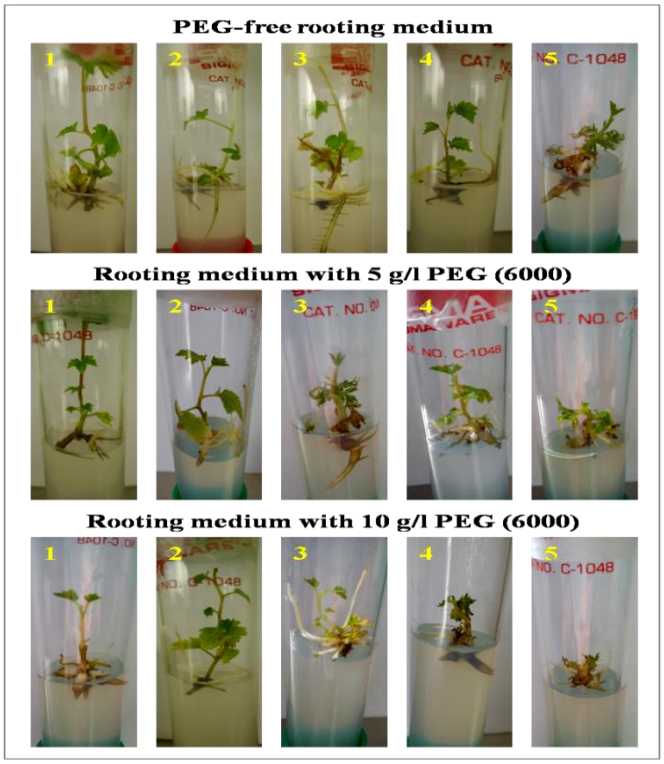

Fig. 3. Effect of different gamma rays doses on number of roots/shoot and root length (cm) of "Black Matrouh" shoot explants under drought stress after 5 weeks in rooting medium.
1= Non-irradiated (control) 2= 10 Gy
3= 20 Gy 4= 30 Gy 5= 40 Gy

ISSR-PCR analysis for genetic variability:

At the end of ISSR analysis, all the 20 primers used could amplify and produced distinct and clear bands in all experimental treatments. A total of 244 DNA bands were amplified in the non-irradiated (control) and irradiated plants with an average frequency of 12.2 bands per prime. The total number of scorable bands produced per individual primer ranged between 6 in (HB-10) and 22 in (ISSR-4). The size of amplified bands ranged between 159 bp in (889) and 2392 bp in (17898-B) as shown in Table (10). Out of the twenty ISSR primers used, three primers (842, 809 and HB-10) were monomorphic, while the remaining seventeen primers were polymorphic. There were 167 monomorphic bands with an average of 8.35 bands/primer, while only 77 bands were polymorphic with an average of 3.85 bands/primer. There was a wide variation in the range of polymorphic bands produced by the primers. Primers (889 and HAD) produced the highest number of polymorphic bands (10), while primer (17898-A) produced the lowest number of polymorphic

bands (1). The polymorphism percentage ranged between 71.43% in primer (17898-B) and 16.67% in primers (811 and BEC), with an average of 31.54% indicating that DNA mutation took place in various degrees between treatments. Out of the 77 polymorphic bands, there were only 18 unique bands (one band in control plants, two bands in plants irradiated with 10 Gy, eight bands in plants irradiated with 20 Gy, two bands in plants irradiated with 30 Gy and five bands in plants irradiated with 40 Gy).

Primer 17898-B revealed a total of 7 amplified bands; of which five bands were polymorphic. One unique band at size 2392 bp appeared only in non-irradiated plants (control) whereas disappeared from all irradiated plants while a new unique band at size 1397 bp was manifested in plants irradiated with 20 Gy (Fig. 4).

Primer 852 revealed a total of 10 amplified bands of which five bands were polymorphic. The most promising results were the manifestation of two unique bands at sizes 606 and 721 bp in plants irradiated with 20 Gy (Fig. 4).

Primer ISSR-35 revealed a total of 11 amplified bands of which two bands were polymorphic. Those two polymorphic bands at sizes 491 and 716 bp were unique in plants irradiated with 20 Gy (Fig. 4).

Primer 834 revealed a total of 10 amplified bands of which three bands were polymorphic. The most promising result was the manifestation of new unique band at size 874 bp in plants irradiated with 20 Gy. Primer 889 revealed a total of 20 amplified bands of which ten bands were polymorphic. The most promising result was the manifestation of new unique band at size 253 bp in plants irradiated with 20 Gy (Fig. 4). Primer HB-9 revealed a total of 12 amplified bands of which four bands were polymorphic. The most promising result was the manifestation of new unique bands at size 836 bp in plants irradiated with 20 Gy.

Table 10. The number of amplicons, polymorphic bands and level of polymorphism among "Black Matrouh" grape (*Vitis vinifera* L.) treatments as revealed by the twenty ISSR primers

ISSR primers	Primer Sequence 5`____3`	Ta (ºC)	Size range (bp)	No. of amplicons		Polymorphism%	Unique bands		Gamma dose (Gy)				
				Total	polymorphic		No.	MW (bp)	control	10	20	30	40
17898-B	$(CA)_6$ GT	40	733-2392	7	5	71.43%	2	2392	+				
								1397			+		
17899-B	$(CA)_6$ GG	42	471-1336	9	4	44.44%	1	595					+
17898-A	$(CA)_6$ AC	40	536-1218	7	1	14.29%	0						
17899-A	$(CA)_6$ AG	40	297-1192	14	5	35.71%	0						
844-A	$(CT)_8$ AC $(AG)_8$ T	53	365-1339	8	3	37.50%	1	491					+
807		42	335-1176	14	3	21.43%	1	639					+
811	$(GA)_8$ C	42	315-1371	12	2	16.67%	0						
852	$(TC)_8$ AA	42	606-1451	10	5	50.00%	2	721			+		
								606			+		
AW-3	$(GT)_7$ AG	42	369-1429	10	4	40.00%	1	946					+
ISSR-35	TCGA $(CA)_7$	53	280-1209	11	2	18.18%	2	716			+		
								491			+		
834	$(AG)_8$ CT	53	295-1414	10	3	30.00%	1	874			+		
841	$(GA)_8$ TC	53	226-1196	14	8	57.14%	1	666		+			
842	$(GA)_8$ TG	53	224-1905	18	0	0.00%	0						
809	$(AG)_8$ G	53	221-1032	13	0	0.00%	0						
ISSR-4	CGA $(CA)_7$	53	243-1712	22	6	27.27%	1	329				+	
889	AGG $(AC)_7$	53	159-1442	20	10	50.00%	1	253			+		
HB-9	(GT)6 GG	40	357-1108	12	4	33.33%	2	836			+		
								589				+	
HB-10	(GA)6 CC	40	327-882	6	0	0.00%	0						
BEC	(CA)7 TC	42			2	16.67%	1	807					+
HAD	CT (CCT)3 CAC	42	448-1846	15	10	66.67%	1	1846		+			
Total				244	77		18		1	2	8	2	5
Average			159-2392	12.2	3.85	31.54%							

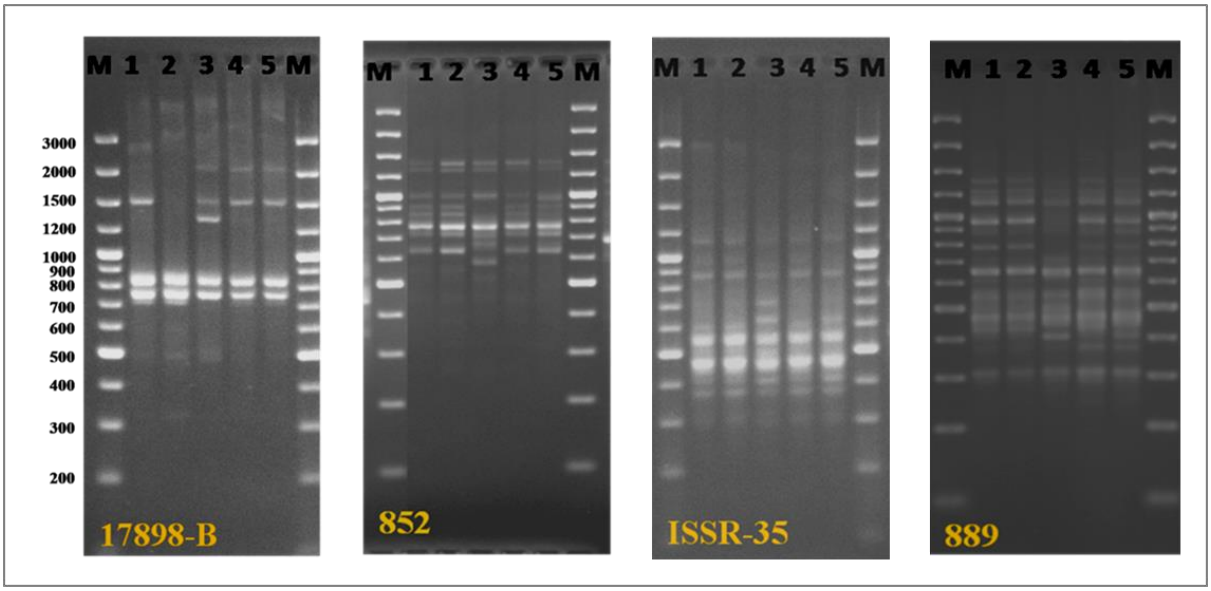

Fig. 4. ISSR marker profiles for the non-irradiated plants (control) and plants irradiated with different doses of gamma rays as detected by the four primers (17898-B, 852, ISSR-35, 889) in 2.5% agarose gel.

M = 100 bp ladder marker **2= 10 Gy** **4= 30 Gy**
1 = Non-irradiated (control) **3= 20 Gy** **5= 40 Gy**

The Jaccard's similarity coefficient values calculated from ISSR data ranged between 0.88 to 1.00, as can be seen in Table (11). It was clear that the genetic similarity between plants irradiated with 20 Gy gamma rays and non-irradiated plants (control) was as far as 88% indicating that 20 Gy gamma rays was the best dose for grape mutagenesis. The genetic distance of variation plants with gamma rays treatment were between 5% (with 10 Gy treatment) and 12% (with 20 Gy treatment) which showed that the impact of mutagen agent on grape mutagenesis was little.

Table 11. Similarity coefficient matrix of *in vitro* "Black Matrouh" grape (*Vitis vinifera* L.) explants after irradiation with different gamma rays doses as a mutagenic treatment

Treatment	Non-irradiated (control)	10 Gy	20 Gy	30 Gy	40 Gy
Non-irradiated (control)	1.00				
10 Gy	0.95	1.00			
20 Gy	0.88	0.88	1.00		
30 Gy	0.94	0.92	0.90	1.00	
40 Gy	0.89	0.89	0.91	0.93	1.00

On the basis of the similarity matrix, ISSR dendrogram obtained from UPGMA cluster analysis was divided into two main clusters separating plants irradiated with 20 Gy gamma rays on its own at 89% similarity with other treatments. The other main cluster included the rest of treatments. This cluster was further subdivided into two sub-clusters at 91% similarity; the first subcluster composed of two treatments at 95% similarity which indicated that plants irradiated with 10 Gy were closely related to non-irradiated plants (control). The second subcluster was about 93% similarity between plants irradiated with 30 and 40 Gy (Fig. 5).

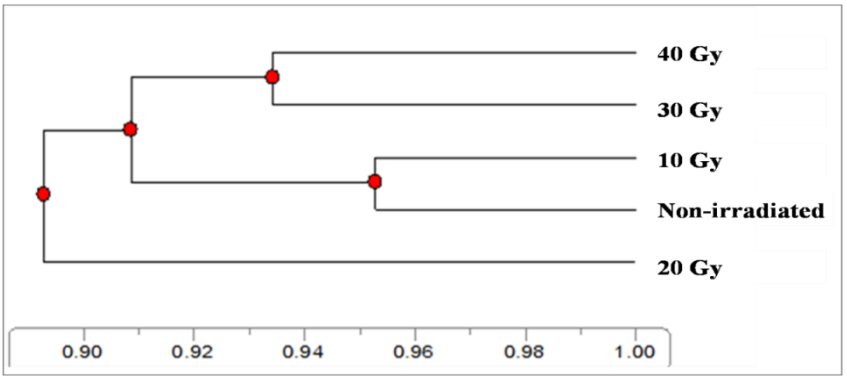

Fig. 5. Dendrogram showing the genetic relationships among the gamma irradiated and non-irradiated plants of "Black Matrouh" (*Vitis vinifera* L.) based on the analysis of ISSR data

Discussion:

Drought is one of the major environmental stresses that affect plant growth and productivity in many parts of the world (Shao *et al.*, 2006). Combined use of mutagenesis, tissue culture and molecular markers techniques can greatly facilitate the selection and isolation of tolerant lines. Mutation induction through gamma radiation is a way of increasing novel genetic variability. The first step of *in vitro* mutagenesis with gamma rays is the assessment of the most suitable dose/doses which commonly based on growth parameters as a result of the treatment (Patade and Suprasanna, 2008). The radio-sensitivity of shoot tip explants was found to be directly proportional to the irradiation dose. Survival percentage, number of shoots/explant and shoot length were negatively correlated to increased gamma rays dose. Similarly, Patil and Patil (2005) had proved that survival percentage was higher at shorter duration of gamma mutagenic treatment.

The higher dose of gamma rays (50 Gy) was not suitable due to the serious necrosis it caused to the explants, furthermore all explants irradiated with 50 Gy were not able to regenerate new shoots at the 3rd sub-culture generation (M_1V_3) as a friable callus was produced which turned brown and failed to survive later. Thus, the LD_{50} for "Black Matrouh" grape had done at 50 Gy. The harmful effect of exposure to high doses may be attributed to the indirect damage of the radiation on metabolic system by way of various radicals in irradiated cells. It is worth considering that the more OH^- produced the more radiosensitive achieved. The tissues differ in their sensitivity to radiation because of the difference in the number of OH^- generated by irradiation (Wada *et al.*, 1998).

However, irradiated explants with 10 Gy caused insignificant reduction in survival percentage and shoot number while enhanced significantly shoot length compared to non-irradiated explants. Such stimulatory effects of low dose irradiation on multiplication of *in vitro* shoots were also observed in earlier studies in grape (Al-Dhaher, 2010; Abdel Gawad *et al.*, 2011) and banana (Harb *et al.*, 2005). In this line, Predieri and Di Virgilio (2007) mentioned that doses lower than LD_{50} favor plant recovery after treatment. In contrary, the use of higher dose increases the probability to induce mutation. Gamma radiation can be useful for the alteration of physiological characters (Kiong *et al.*, 2008). The biological effect of gamma rays is based on the interaction with atoms or molecules in the cell, particularly water, to produce free radicals (Kovacs and Keresztes, 2002). These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending on the radiation dose (Ashraf *et al.*, 2003). The relatively low-doses ionizing irradiation on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yields (Chakravarty and Sen, 2001; El-Beltagi *et al.*, 2011).

In addition, the retarding effect of gamma rays was prominent within a sub-culture generation and among sub-culture generations as the survival percentage decreased gradually with increasing sub-culture number. However, elevated shoot number and shoot length were observed with increasing the sub-culture number indicating a stimulatory effect on proliferation of the tissue. These results are in agreement with those of Rosati *et al.* (1989) who cleared that irradiation had a depressive effect in M_1V_1 and M_1V_2 while in M_1V_3 it stimulated multiplication of grape cv. "Trebbiano R.". In

addition, Madboly (2000) reported that survival percentage of irradiated banana explants decreased with increasing sub-culture, whereas the ability of explants to produce shoots and shoot elongation were enhanced with increasing the sub-culture number.

To induce drought stress, irradiated and non-irradiated explants were dissected from the individual plantlets in M_1V_3 and sub-cultured on medium containing PEG (6000). Results concerning the effect of drought stress demonstrated an obvious decline in growth parameters (survival percentage, number of shoots/explant and shoot length) and rooting parameters (rooting percentage, number of roots/explant and root length) in experimented explants of "Black Matrouh" with increasing PEG concentration. Similarly, Harb *et al.* (2005) pointed out that the gradual increase in PEG was negatively correlated with survival percentage, leaves and roots number of *in vitro* micropropagated banana (*Musa acuminate*). El-Agamy *et al.* (2009) reported a high decrease in survival percentage and plant height associated with the increased PEG concentrations. Abdel Gawad *et al.* (2011) noticed that the increase of PEG in the medium from 0 to 8 gl^{-1} showed a significant decrease in number of leaves and roots per plantlet of grape rootstocks "Freedom" and SO4". While, the PEG concentration of 10 gl^{-1} dehydrated the plantlets as they could not survive more than a week.

The decrease in survival percentage under stress conditions may be due to the energy spent to maintain turgor pressure at the expense of growth (Nieman *et al.*, 1988) or the decrease in the availability of water to plants (Gunes *et al.*, 1996). On the other hand, Ullah *et al.* (1993) demonstrated that the reduction in shoot growth might be due to the decrease in transpiration and photosynthesis under stress conditions. The retardant in plant growth may be also explained by the great portion of energy will be used for water stress tolerance than for growth and biomass production of the organism (Harb *et al.*, 2005). The reduction in roots number might be due to the imbalances in phytohormone levels under stress conditions which may affect the biosynthesis or the destruction of plant hormones *i.e.* the increase in ethylene concentration (Slocum *et al.*, 1984) and ABA accumulation (Shakirova and Bezrukova, 1998) or the reduction in endogenous IAA levels (Dunlap and Binzel, 1996). In addition, the ability of plant itself to absorb water may be affected under stress conditions or directly affected the plant biochemical process due to toxicity (Harb *et al.*, 2005).

On the other hand, growth and rooting parameters were greatly affected by the interaction between different PEG concentrations and irradiation doses. For each gamma rays dose, a gradual decrease in all rested parameters was obviously noticed with increasing PEG concentration. Furthermore, irradiating explants with 20 Gy caused significant enhancements, compared to non-irradiated explants, in survival percentage on medium with 5 gl^{-1} PEG, shoot number on PEG-free medium and rooting percentage on medium with 10 gl^{-1} PEG. Meanwhile, high doses (30 and 40 Gy) adversely affected all parameters under each concentration of PEG. In this line, Harb *et al.* (2005) pointed out that exposure to gamma radiation at 10 and 20 Gy alone or prior to PEG had stimulative effects on the growth and the number of roots/plantlet. El-Shafey *et al.* (2009) mentioned that the pre-exposure to gamma rays had alleviated the harmful effect of drought on rice calli.

To determine alterations in DNA following gamma irradiation, DNA was isolated from *in vitro* shoots of both non-irradiated and irradiated plantlets of "Black Matrouh" grape. Out of the 20 primers used, five primers (852, ISSR-35, 834, 889 and HB-9) generated 8 unique bands in plants irradiated with 20 Gy dose of gamma rays that could be considered as potential markers for initial estimation and selection of drought tolerance in grape. In this concern, Jain (2005) recommended the development of molecular database as an instrument for predicting expected mutations. Molecular markers are important tools in precisely detecting the effects of gamma radiation since they identify genetic polymorphism at the DNA level and have been used to study genetic dissimilarity in many crop species (Pestana *et al.*, 2011). Especially in the mutation breeding, ISSR markers provide a reliable and rapid detection means for the screening of mutants (Wu *et al.*, 2011).

The present study on ISSR analysis of genetic variation among the *in vitro* irradiated and non-irradiated grape cultures cleared the presence of changes on DNA molecular level which prove the effectiveness of gamma-rays as a mutagen for grape improvement and suggest that 20 Gy gamma rays was the best dose for grape mutagenesis.

Conclusion:

The use of mutation induction (gamma-rays) combined with *in vitro* and molecular marker techniques is an interesting approach to generate information for obtaining promising grape cultivars with good agronomic characteristics. This study concluded that mutation technique has shown to be very useful in grape improvement. Plantlets irradiated with 20 Gy gamma rays dose were more tolerant to drought stress than non-irradiated plantlets and the exposure of *in vitro* explants to 20 Gy dose of gamma rays is a potential source of genetic variability and can potentially be employed to develop drought tolerant plants *in vitro*.

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استحداث الطفرات مخبرياً لزيادة تحمل الجفاف والتوصيف الجزيئي في العنب (*Vitis vinifera* L.) صنف "مطروح أسود"

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الملخص:

نُفذت هذه الدراسة في البنك القومي للجينات وهيئة الطاقة الذرية/مصر، خلال الأعوام 2012– 2014، بهدف إنتاج نباتات من العنب أكثر تحملاً للجفاف. تم تعريض القمم النامية من صنف العنب "مطروح أسود" لجرعات مختلفة (0–50 جراي) من أشعة جاما وتم الحصول على ثلاثة أجيال خضرية M_1V_1 إلى M_1V_3. أُضيف البولي اتيلين جليكول بتراكيز 0، 5 و 10 غ/ل لأوساط الزراعة بهدف استحداث إجهاد الجفاف. أوضحت نتائج هذه الدراسة أن حساسية القمم النامية للأشعة ازدادت بزيادة الجرعة من أشعة جاما، وتبين أن الجرعة المميتة عند 50 جراي. على نقيض ذلك، لوحظ زيادة في عدد الأفرع الناتجة واستطالتها بزيادة عدد مرات النقل. تحت ظروف الاجهاد المائي، سببت الزيادة التدريجية في تركيز البولي ايتلين جليكول إلى انخفاض واضح في معايير النمو والتجذير ضمن كل جرعة من أشعة جاما. يمكن للنباتات أن تتحمل التأثير الضار للجفاف بتعريضها لأشعة جاما بجرعة 20 جراي. من بين 20 بادئ من التكرارات التتابعية البسيطة الداخلية المستخدمة، استطاعت 6 بادئات (17898–B, 852, ISSR–35, 834, 889 and HB–9) أن تنتج 8 أليلات فريدة في النباتات المعرضة لأشعة جاما بجرعة 20 جراي. يمكن اعتبار هذه الاليلات كواسمات محتملة للتقييم المبدئي والانتخاب لتحمل الجفاف في العنب.

الكلمات المفتاحية: العنب، أشعة جاما، الجرعة المميتة LD_{50}، الجفاف، البولي اتيلين جليكول PEG، التكرارات التتابعية البسيطة الداخلية ISSR.