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In vitro Studies Inducing Genetic Variation in Grape Vine (*Vitis vinifera* L.) using Gamma Irradiation and Sodium Azide

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ABSTRACT

This investigation was carried out during two successive experiments (2013 and 2014) at the tissue culture laboratory, Horticulture Research Institute, Giza, Egypt. The aim of this study was to induce variations in Red Roomy grape vine cultivar through in vitro mutagenesis by treating the axillary buds with two mutagenic, i.e. gamma rays at doses 0, 2, 4 and 6 Krad and varying concentrations of sodium azide 0, 0.01, 0.02 and 0.03 mg/L. The study applied ISSR analysis for discovery of genetic polymorphism among the variants and detect molecular markers associated with mutations and genetic variations. The results of analysis of variance indicated that the studied characters (explant height, shoot thickness, No. of leaves, root length, root thickness, No. of roots, survived explants and acclimized plants) were influenced by both gamma rays and sodium azide doses and gave significant differences caused by different treatments. However, mutagenic treatments with 2 or 4 Krad gamma rays and sodium azide at 0.02 mg/L enhanced all studied characters except for both survived plants % and acclimated plants %which the control was showed the highest values. The results indicated that the maximum increase in all studied characters induced by the dose of 4 Krad gamma rays followed by 2 krad and 0.02 mg/L sodium azide as compared to the other doses, while control had the highest values for survived plants % and acclimated plants %. At the molecular level, ISSR analysis has been used to study the genetic variation at genetic expression. The ISSR primers 14A, HB-08, HB-10, HB-13, HB-14 and HB-15 exhibited different amplification patterns in the variants. In ISSR analysis banding patterns, there were absent bands, whereas mutation induction was 37 and 31% by using doses of 6 Krad gamma rays and sodium azide, respectively. The results showed that the variability among studied explants through tissue culture and gamma rays as well as sodium azide mutagenesis confirmed by using ISSR markers for Red Roomy grape vine. On the other hand, using ISSR markers for construction of genetic variations and exhibiting of the molecular genetic diversity for these explants supported the use of marker-assisted selection (MAS) in grape vine cultivars breeding programs.

Key words: Grapevine, ISSR molecular markers, gamma irradiation, sodium azide, mutation induction.

Introduction

Vitis is an economically one of the most important fruit crops for table foods and a processing agent for wines and dry fruits. Genetic improvement of vitis has been accomplished by clonal selection of spontaneous bud mutation and breeding (Olmo 1942 and Einset and Pratt, 1975). However, the occurrence of bud mutations is random, which limits directed crop improvement. Breeding has limited application for genetic improvement because of extreme heterozygosity of the vitis genome, which is fostered by inbreeding depression (Winkler *et al*. 1974). Mutations are induced by physical e.g. gamma radiation and chemical e.g. ethyl methane sulfonate mutagen treatment of both seed and vegetatively propagated crops. The mutagen treatment breaks the nuclear DNA and during the process of DNA repair mechanism, new mutations are randomly induced and heritable. The changes can occur also incytoplasmic mutations such as flower colour, flower shape, disease resistance, early flowering types. Genetic variation is the starting point of any breeding programme. Genetic variation may already be present in nature, may be obtained after several years of selection, or may be produced through hybridization (for seed propagated crops). These techniques have presented positive results in the improvement of genetic variability, enlarging genotypic classes and facilitating the practices of selection and to study gamma-ray induced variation in tree architecture (Predieri *et al*., 1998) and in traits connected to fruit production (Predieri and Zimmerman, 2001) and in vitro leaf-shoot regeneration and Somaclone selection for sodium chloride tolerance in quince and pear reported by (Marino and Molendini, 2005) and inducing genetic variation in pear (*Pyrus communis*) using in vitro chemical mutagenesis (Nahla and Rayan, 2007) and *in vitro* studies on genetic variations of some plum cultivars using gamma irradiation from Cobalt 60 (Rayan, A.O. *et al*., 2010). The first objective of the present investigation was to study the effect of gamma rays and sodium azide to induce mutations in Red Roomy grape vine cultivar. The second objective was to detect molecular markers associated with mutations and genetic variations using ISSR analysis.

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Materials and Methods

This investigation was carried out at the tissue culture laboratory, Horticulture Research Institute, Giza, Egypt during 2013 and 2014 seasons in order to investigate the influence of various doses of gamma irradiation and different concentrations of sodium azide.

Gamma irradiation and sodium azide treatments:

Axillary buds were treated with two mutagenic agents, i.e. gamma irradiation as a physical mutagen and sodium azide as a chemical mutagen. Mutagenic treatments with gamma rays were conducted by irradiating grape axillary buds with 2, 4 and 6 Krad with a dose rate 1.36rad/second from cobalt 60 in gamma cell 220. On the other hand mutagenic treatments with sodium azide (NaN_3) were conducted by soaking of axillary buds with three concentrations, i.e. (0.00, 0.01, 0.02 and 0.03 mg/L) for six hours at 4˚C.

Table 1: Medium composition for *in vitro* starting material, multiplication and rooting.

Medium composition	Starting material	Multiplication	Rooting
MS salts	2314(1/2MS)	2314(1/2MS)	2314(1/2MS)
Myo-Inositol	2.8g/l	2.8g/l	2.8g/l
Thiamine HCl	2.5 mg/l	2.5 mg/l	2.5mg/l
N6-benzylaminopurine (BAP)	1 mg/l	2 mg/l	---
Indole 3-butyric acid (IBA))	---	---	0.5 mg/l
Sucrose	30 g/l	30 g/l	20 g/l
Agar	7g/l	7g/l	7g/l

Culture Medium:

The medium consists of half strength (Murashige and Skoog 1962) supplemented with myo-inositol 2.8g/l + thiamine HCl 2.5 mg/l+ BAP 0.1 mg/l+ 30 g/l sucrose + 7.0 g/l agar and pH was adjusted to 5.6. Media was autoclaved at 121˚C for 20 minutes. Cultures were incubated in growth chamber at 27±1˚C under 16 hr light and 8 hr dark. Explants were taken from *in vitro* plantlets cultured on a modified MS medium, axillary buds were excised and cultivated on MS medium supplemented with myo-inositol 2.8g/l + thiamine HCl 2.5 mg/l+ BAP 2 mg/l+ 30 g/l sucrose + 7.0 g/l agar and pH was adjusted to 5.6. The multiple shoot cultures obtained were separated into individual shoots and transferred onto rooting medium for 4-6 weeks. Thefollowing Vegetative growth data were recorded.

1-Plant height (cm)
2-Shoot thickness (cm)
3-Number of leaves
4-Root length (cm)
5-Root thickness (cm)
6-Number of root
7-Percentage of survived plant
8-Percentage of acclamized plant

Transfer to Greenhouse:

After proliferation success the shoots were transferred on rooting MS medium supplemented with 2 mg/l IBA and maintained under in vitro conditions (ventilation of vessels). The plants were transplanted into small plastic pots containing peatmoos and perlite 2:1 (v/v) in greenhouse for three months. The experiment was conducted again to certain the obtained results.

DNA Isolation Procedure:

Fresh tissue parts were collected separately from different treatments and control.The bulked DNA extraction was performed using DNeasy Mini Kit (QIAGEN). Isolation protocol of DNA according to (Williams *et al*.1990)

Inter- simple sequence repet PCR (ISSR-PCR) procedure:

PCR reactions were conducted using 6 ISSR primers. Their names and sequences are shown in Table (2).

Table 2: Sequence and primers codes of the random primers used to study variation.

No	Name	Sequence	No	Name	Sequence
1	14A	5 CTC TCT CTC TCT CTC TTG 3	4	HB-13	5` GAG GAG GAG GC 3`
2	HB-08	5 CTC TCT CTC TCT CTC TTG 3	5	HB-14	5` GAG GAG GAG GC 3`
3	HB-10	5 GAG AGA GAG AGA GG 3	6	HB-15	5` GTG GTG GTG GC 3`

Statistical analysis:

Each treatment included five jars containing four explants. Each experiment was repeated three times. Data were subjected to analysis of variance by MSTAT-C (1990) computer statistical analysis program. Differences among treatments were tested by LSD at the 5% level of significance (P=0.05). The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers. The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) to calculate genetic similarity and to construct dendrogram tree among the studied treatments. Calculation was achieved using Dice similarity coefficient (Dice, 1945) as implemented in the computer program SPSS-10.

Results and Discussion

Different doses of gamma rays (2, 4 and 6 krad) and concentrations of sodium azide were applied to study their effects on vegetative growth of induced plants (plant height, shoot thickness, number of leaves, root length, root thickness, number of roots, and percentages of survived acclimated plants) for the two seasons of study. As shown in table (3) and fig (1and 2) significant increase was observed in all studied vegetative growth characters (except for of survived and acclimated plants, percentage control found the highest values by using of gamma rays (2, 4 and 6 krad) and concentrations of sodium azide (0.01, 0.02 and 0.03 mg/L). Data in table (3) and fig (1 and 2) also indicated that treatments with 2 and 4 krad gamma rays and sodium azide with 0.02 mg/L enhanced all studied vegetative growth / plant except survived and acclimated plants percentage. Dose of 4 krad gamma rays showed the maximum values for characters of vegetative growth followed by 2 krad and 0.02 mg/L sodium azide as compared to the other treatments in both seasons. While the lowest increase was observed with concentration 0.03 mg/L sodium azide in all vegetative growth characters per plant as compared to the other treatments and control.

Growth parameters of Red Roomy Vitis Vinefra L. i.e. plant height, shoot thickness, number of leaves, root length, root thickness, number of roots were presented in table (3) and fig (1). As for the plant height data in table (3) and fig (1) illustrated that the plant height of the plantlets were significant by raised with 4 krad gamma rays gaving 11.81 and 12.43 cm for the first and second seasons, respectively as compared to control and other treatments. The treatment 0.03 mg/L sodium azide showed the lowest values of plant height (7.33 and 7.98 cm for the first and second seasons, respectively as compared to control and other treatments. Concerning shoot thickness, the effect of 4 krad gamma rays induced significant increases (0.25 and 0.28 cm, respectively for first and second seasons as compared to control or other treatments. While, the lowest shoot thickness was obtained from treatment of 0.03 mg/L sodium azide (0.14 and 0.16 cm) for first and second seasons, respectively.

Regarding the number of leaves, results in table (3) and fig (1) indicated that the treatment 4 krad gamma rays induced significant enhancement on number of leaves (10 and 12) for the first and second seasons, respectively. While the treatment 0.03 mg/L sodium azide resulted in the lowest number of leaves (4.67 and 6.33) for the first and second seasons, respectively as compared to control and the other treatments.

Regarding the root length, root thickness and number of roots, results in table (3) and fig (2) illustrated that root length, root thickness and number of roots were significantly raised with treatment 4 k/rad gamma rays, whereas induced significant increase in root length 8.49 and 9.09 cm for the first and second seasons, respectively as well as resulted in the greatest root thickness (0.22 and 0.25 cm) for the first and second seasons, respectively as compared to control and other treatments. From table (3) and fig (1); the number of roots took the same trend of the root length and root thickness. Data in table (3) and fig (1) indicated that treatment of 0.01 mg/L sodium azide gave the lowest values of root length (6.37 and 7.08 cm) for the first and second seasons, respectively. As for the root thickness and number of root, data in table (3) and fig. (1) indicated that treatment of 0.03 mg/L sodium azide induced the lowest values of both root thickness (0.14 and 0.16 cm) and number of roots (13.64 and 15.00 cm) for the first and second seasons, respectively compared to control and the other treatments.

Concerning survived plants percentage, the effect of all treatments (gamma rays and sodium azide) was lower than control, whereas control recorded the highest values (50.0 and 54.4%) for the first and second seasons, respectively. While treatment 0.03 mg/L sodium azide gave the lowest values (12.33 and 14.1%) for the first and second seasons, respectively. The same trend was taken with acclimated plants percentage for the

first and second seasons. While the lowest values obtained by using treatment 0.03 mg/L sodium azide (9.67 and 10.60%) for the first and second seasons, respectively.

In this respect, similar findings were obtained by Nahla and Rayan (2007), Rayan *et al*. (2010), Mba, (2012) and Siamak *et al*. (2012).

Table 3: Vegetative growth / plant (plant height, shoot thickness, number of leaves, root length, root thickness, number of roots, percentages of survived acclimated plants) as affected by using gamma rays and sodium azide during seasons 2013 and 2014

Treatment	Conc. (mg/L)	Plant height (cm)		Shoot thickness (cm)		No. of leaves		Root length (cm)		Root thickness (cm)		No. of root		(%) Survived plants		(%)Acclimated Plants	
		2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
Control	0.00	9.88	10.03	0.17	0.19	8.00	9.33	7.12	7.80	0.16	0.18	15.00	16.33	50.00	54.40	42.00	42.77
Gamma rays	2 krad	8.78	9.43	0.18	0.22	8.00	9.67	6.73	7.36	0.18	0.21	21.33	22.67	22.33	24.13	18.33	19.90
	4 krad	11.81	12.43	0.25	0.28	10.00	12.00	8.49	9.09	0.22	0.25	26.00	27.00	26.33	28.53	21.00	23.00
	6 krad	7.96	8.60	0.18	0.23	6.67	9.00	6.50	6.99	0.18	0.20	20.67	22.00	18.33	19.30	16.00	17.93
Sodium azide concenteration	0.01mg/l	7.72	8.40	0.15	0.16	6.00	7.67	6.37	7.08	0.16	0.19	14.64	16.00	15.00	17.33	12.33	13.24
	0.02mg/l	8.57	9.30	0.17	0.20	6.00	7.33	6.64	7.23	0.21	0.23	16.67	18.33	19.67	20.47	15.33	16.67
	0.03mg/l	7.33	7.98	0.14	0.16	4.67	6.33	6.51	7.15	0.14	0.16	13.64	15.00	12.33	14.10	9.67	10.60
L.S.D (0.05)		0.57	0.19	0.03	0.01	1.11	0.84	0.75	0.21	0.04	0.01	1.90	0.94	2.89	0.54	2.00	0.51

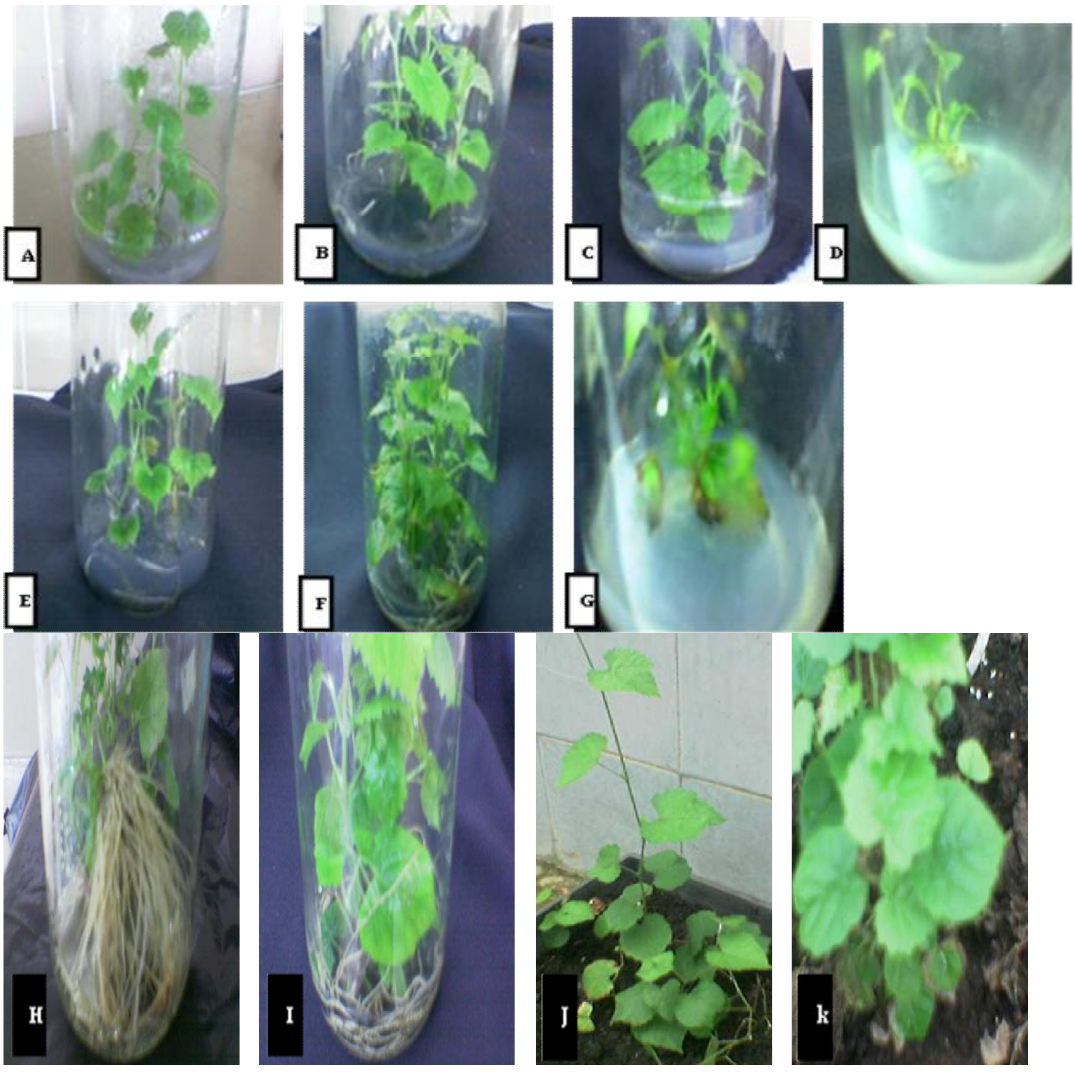

Fig. 1: Effect of sodium azide (A) control, (B) 0.01 mg/L, (C) 0.02 mg/L, (D) 0.03 mg/L SA and gamma irradiation (E) 2 Krad, (F) 4 Krad and (G) 6 K/rad. Also effect of gamma Irradiation and Sodium Azide on rooting (H) 4 Krad and (I) 0.02 mg/L SA and acclamized plant

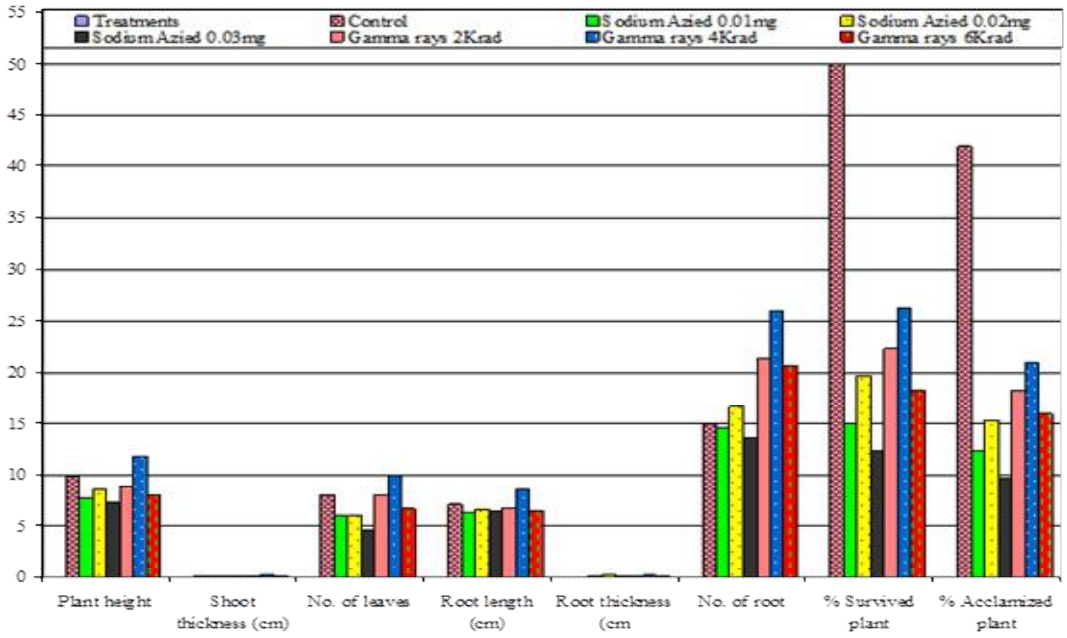

Fig. 2: Histogram show the effect of gamma irradiation and sodium azide at various doses on different characters growth of grapevine (*Vitis Vinifera* L)

Issr-dna analysis:

DNA analysis was done by using six ISSR primers (14A, HB-08, HB-10, HB-13, HB-14 and HB-15) for the similarities between the grape vine (*Vitis vinifera L.*) using gamma irradiation and sodium azide as shown in figure (3) and tables (4,5,6,7,8 and 9) which revealed that ISSR of grape vine DNA using the six selected primers generated a total of 30 polymorphic loci with approximately 100% polymorphism (Tables 4 and 5). The total number of 30 ISSR bands were obtained; of these 30 bands, 20 bands were polymorphic (66.67%) and 10 monomorphic (33.33%). The highest number of amplicans was generated. Polymorphism levels differed from one primer to the other. The number of bands per primer varied between 2 (14A), 8(HB-08), 6(HB-10 and HB-14), 5(HB-13) and 3 for HB-15. While the size of the bands ranged between 160 and 940 bp with an average 5 bands per primer. Both primer 14A, HB-08 and HB-14 obtained (50%), primer HB-10 obtained (66.66%), primer HB-13 gained (40%). On the other hand, primer HB-15 had no polymorphism (0.0%). The numbers of total amplified fragments, polymorphic bands, monomorphic and unique bands, polymorphic and monomorphic percentages for the grape vine using gamma irradiation and sodium azide, using the six primers are shown in Table (10). There were some specific fragments discriminated each seven grape vine treatment from the others as follows:

The dendrogram tree and the similarity indices among the grape vine using gamma irradiation and sodium azide utilizing ISSR markers presented in table (11) and figure (3), using dice computer package. The strongest relation was scored between No. 4 and No. 7 (similarity of 100%).

Primer HB-8 showed three specific fragments, as positive marker and the two other were negative markers. On the other hand primer HB-14 showed three specific fragments one of them was negative and the two other were negative. Primers 14A, HB-10, HB-13 and HB-15 did not show any specific fragments.

Genetic similarity and dendrogram based on ISSR analysis:

The ISSR data were used to estimate the genetic similarity among the seven grape vine by using UPGMA computer analysis as presented in Table (12). The highest similarity index (100%) was recorded between treatment No. 4 and No. 7, while the lowest or no similarity was detected between treatment No. 3 and No. 4. A dendrogram for the genetic relationship among the seven treatments of grape vine was drawn in fig. (4). The results of ISSR are in harmony with Younis *et al*. (2011), Aborekab (2013) and Aborekab *et al*. (2014).

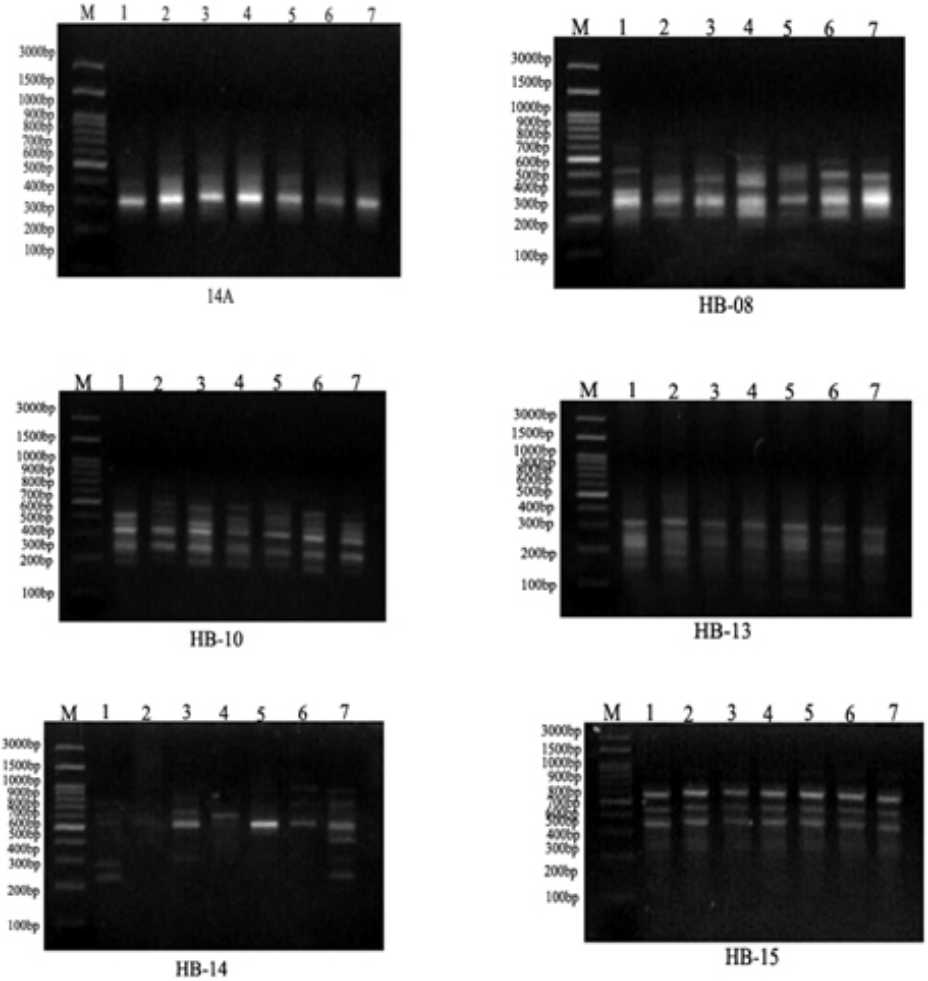

Fig 3: ISSR profiles of the seven treatments of grape vine (*Vitis vinifera* L.) amplified with sex primers (14A, HB-08, HB-10, HB-13, HB-14 and HB-15) for each analyses

Table 4: Survey of ISSR using 14Aprimer in seven treatments of grape vine (*Vitis vinifera L.*) 1 or 0 means percent or absence of band 14A

Band No.	M.W bp	Cultivars						
		1	2	3	4	5	6	7
1	375	0	1	1	1	1	0	0
2	295	1	1	1	1	1	1	1
Total		1	2	2	2	2	1	1

Table 5: Survey of ISSR using HB-08 primer in seven treatments of grape vine (*Vitis vinifera* L.) 1 or 0 means percent or absence of band HB-08.

Band No.	M.W bp	Cultivars						
		1	2	3	4	5	6	7
1	475	0	0	0	1	0	0	0
2	420	1	1	1	1	1	1	0
3	390	0	0	0	0	1	1	1
4	350	0	1	1	1	1	0	0
5	280	1	1	1	1	1	1	1
6	260	1	0	0	0	1	0	0
7	230	0	1	1	1	1	1	0
8	205	1	0	0	0	0	0	0
Total		4	4	4	5	6	4	2

Table 6: Survey of ISSR using HB-10 primer in seven treatments of grape vine (Vitis vinifera L.) 1 or 0 means percent or absence of band HB-10

Band No.	M.W bp	Cultivars						
		1	2	3	4	5	6	7
1	485	0	1	1	1	1	1	0
2	425	1	0	0	0	0	0	1
3	395	0	0	1	1	1	1	1
4	370	1	1	1	1	1	1	1
5	270	1	1	1	1	1	1	1
6	190	1	0	1	1	1	1	0
Total		4	3	5	5	5	5	4

Table 7: Survey of ISSR using HB-13 primer in seven treatments of grape vine (Vitis vinifera L.) 1 or 0 means percent or absence of band HB-13.

Band No.	M.W bp	Cultivars						
		1	2	3	4	5	6	7
1	340	1	1	1	1	1	1	1
2	270	1	1	1	1	1	1	1
3	230	1	1	1	1	1	1	1
4	195	1	1	0	0	0	0	1
5	160	1	1	1	1	0	0	0
Total		5	5	4	4	3	3	4

Table 8: Survey of ISSR using HB-14 primer in seven treatments of grape vine (Vitis vinifera L.) 1 or 0 means percent or absence of band HB-14.

Band No.	M.W bp	Cultivars						
		1	2	3	4	5	6	7
1	940	0	0	0	0	0	1	1
2	640	0	1	1	1	1	1	1
3	540	1	0	1	0	1	1	1
4	420	0	0	0	0	0	0	1
5	300	1	1	1	1	0	1	1
6	240	1	0	0	0	0	0	1
Total		3	2	3	2	2	4	6

Table 9: Survey of ISSR using HB-15 primer in seven treatments of grape vine (Vitis vinifera L.) 1 or 0 means percent or absence of band HB-15.

Band No.	M.W bp	Cultivars						
		1	2	3	4	5	6	7
1	590	1	1	1	1	1	1	1
2	450	1	1	1	1	1	1	1
3	350	1	1	1	1	1	1	1
Total		3	3	3	3	3	3	3

Table 10: Primers sequences names, polymorphic, monomorphic bands and polymorphism percent detected by ISSR analysis in grape vine (Vitis vinifera L.) using gamma irradiation and sodium azide.

Primer name	Amplified bands (loci)	Monomorphic bands	Polymorphic bands	Polymorphic %
14 A	2	1	1	50.00
HB-08	8	1	4	50.00
HB-10	6	2	4	66.66
HB-13	5	3	2	40.00
HB-14	6	-	3	50.00
HB-15	3	3	-	0.00
Total	30	10	14	

Table 11: ISSR analysis (total bands, polymorphic bands, monomorphic bands, unique bands, polymorphic and monomorphic percent

Analysis ISSR	Total bands	Polymorphic band	Monomorphic band	Unique band	Polymorphic %	Monomorphic %
	30	14	10	6	66.67	33.33

Table 12: Genetic similarity matrices among grape vine (Vitis vinifera L.) using gamma irradiation and sodium azide, accessions as computed according to Dice coefficient from ISSR.

	1	2	3	4	5	6
1						
2	0.73					
3	0.69	0.16				
4	0.84	0.16	0.00			
5	0.84	0.47	0.15	0.29		
6	0.79	0.57	0.23	0.38	0.23	
7	0.63	0.89	0.84	1.00	0.84	0.47

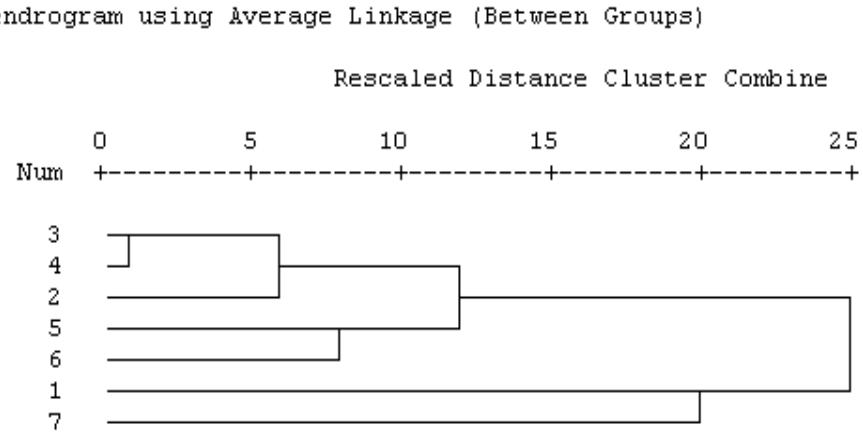

Fig. 4: Dendrogram for the grape vine (*Vitis vinefera L.*) using gamma irradiation and sodium azide accessions constructed from the ISSR data using unweighted pair-group arithmetic (UPGMA) and similarity matrices computed according to Dice coefficient

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