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IN VITRO STUDIES OF DIFFERENT CHEMICAL MUTAGENS ON SOME APPLE ROOTSTOCK (MALUS DOMESTICA BORKH)

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ABSTRACT

This study aimed to induce genetic variations through callus initiation and regeneration using leaves of two apple rootstocks (MM106 and Balady) cultured on MS medium supplemented with two chemical mutagens (EMS and NaN_3). Variations were genetically discriminated using biochemical (protein and isozymes) and molecular (RAPD-PCR) analyses. The percentage of callus survival was decreased due to the applied of both mutagens at 0.03 mg/l [54.0 and 55.5 %] in comparison with control [80.0 %], as well as, reduced the regenerated plants percentage. Vegetative parameters of the regenerated plantlets were affected by mutagens e.g., percentage of multiple shoots, number of leaves/plant and number of roots/plant. Effect of EMS at 0.01mg/l was increased some parameters if compared with control [5.50, 4.66, 6.00, 4.66, 4.50 and 3.16 %], respectively, for multiplication percentage, number of leaves and number of roots per plant. However, EMS and NaN_3 at 0.03 mg/l decreased all the vegetative parameters. Protein and isozymes (peroxidase and poly phenyl oxidase) analyses were used to studying genetic variation of gene expression. Protein banding patterns of Balady and MM106 revealed a total number of five bands, one common band (monomorphic band) was detected (20.0% monomorphism). In addition, the remaining four bands were polymorphic (80.0% polymorphism). Few bands appeared or absent in control and some treatments are considered as specific molecular markers for these treatments. On the other hand, isozymes banding patterns represent differences in density of bands with different mutagenic concentrations compared with control. However, RAPD analysis had successfully generated reproducible polymorphic products. The generated profiles revealed levels of polymorphism in Balady rootstock. These appeared thirty eight total amplified fragments distributed between twenty were polymorphic (52.6%) and eighteen were monomorphic fragments. However, the total number of amplified fragments in MM106 rootstock and its treatments were thirty six which containing eighteen polymorphic fragments (50%).

Keywords: Chemical mutagens, Ethyl-methanesulphonate (EMS), Isozymes, RAPD-PCR, Rootstock, Sodium azide (NaN_3).

INTRODUCTION

Apple is one of the most important fruit crops cultivated in Egypt. This subfamily includes approximately 1000 species in 30 genera characterized by the distinctive fruit, the pome, and a base chromosome number; x = 17 (Evans and Campbell, 2002). Improvement apple traits, such as; fruit quality, yield, and disease resistance, needed more efforts. Inducing mutations in plants with different methods such as irradiation and chemical substances has been used in biotechnology and breeding programs. Using chemical mutagens via *in vitro* cultures could accelerate the breeding programs, in which heterozygosity and long juvenile period make genetic improvement by

conventional method means long lasting and difficult particularly in these species (Caboni *et al.,* 2002). *In vitro* leaf-shoot regeneration and inducing genetic variations using chemical mutagens were reported before in pear by Awad and Rayan (2007), as well as, in some plum cultivars using gamma irradiation of Cobalt 60 by Abou Rekab *et al*., (2010) and Rayan, *et al.*, (2010) and in Red Roomy Grape using gamma irradiation and sodium azide (Rayan, *et al.*, 2014). Mutagenesis has already been used in fruit crops to introduce many useful traits affecting plant size, blooming time, fruit ripening, fruit color, self-compatibility, and resistance to pathogens (Janick and Moore, 1996), and in traits related to fruit production (Predieri and Zimmerman, 2001). SDS-protein banding patterns are efficiently used to identify plant genotypes via obtaining different molecular genetic markers in various fruit species such as apple (Menendez *et al*., 1982). Isozyme markers provide a convenient method for detecting genetic changes. Moreover, they have been used in apple as biochemical markers for cultivars identification (Weeden and Lamb 1985; Barnes 1993) and for the identification of clonal apple rootstocks (Battle and Alston 1994). RAPD-PCR analysis can be used to identify many useful polymorphisms quickly and efficiently. In addition, it has tremendous potential for use in cultivar identification i.e., apple (Koller *et al.*, 1993). This study aimed to induce *in vitro* genetic variations in apple rootstock using different chemical mutagens to be used thereafter for selection of specific traits.

MATERIALS AND METHODS

This work was conducted in the laboratory of Fruit and Ornamentals Breeding Department and Biotechnology Research lab, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt. All tissue culture experiments were carried out during 2013 and 2014 seasons.

Explants Preparation

Leaves of two apple rootstocks (*Malus domestica* Borkh); MM106 and Balady were used as source of explants for callus initiation, regeneration, multiplication and rooting throughout this study. Explants were washed by tap water, then soaked in three different concentrations (0.01, 0.02 and 0.03mg/l) of ethyl-methanesulphonate (EMS) and sodium azide (NaN_3) for 24 h. Explants were then sterilized by immersion in 70% ethanol for 5 min, followed by 20% sodium hypochlorite supplemented with 150 mg/L of ascorbic acid for 20 min, and rinsed four times in sterile distilled water supplemented with 150 mg/L of ascorbic acid.

Mode of excision

Margins of expanded leaves (10-12 mm long) were removed and the remaining part was cut transversely to the midrib into two portions. Then the leaf portions were dissected into small pieces (0.3 cm) and placed with the adaxial surface in contact with different MS media (Murashige and Skoog, 1962).

Establishment and regeneration medium

Leaf segments were placed with the adaxial surface in contact with MS medium supplemented with 3% sucrose, 0.7% agar, 2.0 mg/l BA, 0.2 mg/l

IBA and the pH was adjusted to 5.6. Explants were grown at 25°C using 16 h light photoperiod with a light intensity of 2000 lux provided by cool white fluorescent tubes. The subculture was performed every four weeks using the same medium. Then the maintained calluses were transferred to the same previous MS medium supplemented with 0.5 mg/l IBA and 3.0 mg/l BA. Number of shoots per callus piece were recorded after two months from transferring the callus to the regeneration medium.

Shoot multiplication and elongation:

After plant regeneration, cultures were grown on MS medium supplemented with 3% sucrose, 0.7% agar, 3.0 mg/l BA and the pH was adjusted to 5.6. In this experiment, shoots (0.8-1 cm long) were placed vertically in medium. Shoots were multiplied and subcultured in jars every four weeks intervals. Shoot elongation (cm) was measured after 30 days.

Root formation

The uniform shoots with 2-3 cm long were collected from subculture medium of each rootstock and transferred to MS medium supplemented with 2% sucrose, 0.7% agar, 0.5 mg/l IBA and the pH was adjusted to 5.6. At the first four days, shoots were cultivated on the above mentioned medium in the dark, at 22ºC. Later, rooting was encouraged under a photoperiod of 16 h of cool white fluorescent light (40 µE $m^{-2}s^{-1}$) at 22ºC. Number of roots and root length were determined after 50 days of treatment.

Biochemical Analysis

Protein electrophoresis

Sodium dodecylsulphate polyacrylamide gel (SDS-PAGE) was used to studying protein profiles of the two apple rootstocks and their treatments. Water soluble protein fractionation was performed on 12% (W/V) vertical slab gels using BIORAD Techware 1.5 mm according to the method of Laemmli (1970) as modified by Studier (1973). Molecular weight of protein bands was relatively estimated using a wide range of molecular weight related to protein marker (Fermentas comp.).

Isozymes electrophoresis

Extraction of isozymes was adopted as described by Jonathan and Weeden (1990). Native–polyacrylamide gel electrophoresis (Native-PAGE) was performed on 12% (W/V) slab gels (Davis 1964). Then, gels were stained according to Tanksely and Rick (1980) for peroxidase (Px) isozyme and poly phenyl oxidase (PPO). The stained gels were incubated at 37 °C in dark conditions for complete staining after adding the appropriate substrates and staining solutions.

Molecular analysis

Randomly amplified polymorphic DNA-Polymerase chain reaction (RAPD-PCR) analysis:

DNA Extraction

Young and freshly excised leaves were collected separately from the two apple rootstocks and their regenerated treated plantlets. DNA extraction was performed using DNeasy Plant Mini Kit (QIAGEN) as described by Williams *et al.,* (1990).

DNA amplification

Amplification reactions were performed in 25µl total volume according to Williams *et al.,* (1990). Amplification procedures were carried out in Techni TC-512 PCR thermocycler, programmed as follows; initial predenaturation step of one cycle at 95 ºC for 5 minutes, followed by 35 cycles of 30 seconds at 96 ºC, 30 seconds at 37 ºC and 30 seconds at 72 ºC, carried out by a final cycle at 72 ºC for 5 minutes. PCR products were run at 100 V for one hour on 1.5 % agarose gels.

PCR reaction was conducted using twenty-one arbitrary 10-mer primers. Only five primers succeeded to generate reproducible polymorphic DNA fragments. Their codes and sequences are shown in Table 1.

Table 1. List of RAPD primers used and their nucleotide sequences.

No.	Primer code	Sequence
1	OP-C04	5´ TCGGCCATAG 3`
2	OP-C12	5´ CCTTGACGCA 3`
3	OP-C15	5 AGGGAACGAG 3 `
4	OP-E15	5 ´GGTGATCAGG 3`
5	OP-M01	5`GGG CGG TAC T3`

Protein, isozyme, and RAPD gels analysis

Protein, isozyme, and RAPD gels were processed using quantity one software (Bio-Rad) which identifies DNA fragments using an optimized set of parameters (as reported in quantity one user guide for version 4.2 Windows Bio-Rad Laboratories) which was manually adjusted by visual inspection.

Statistical analysis

Experiments were designed in completely randomized design. Each treatment was performed in six jars containing five explants and each experiment was replicated three times. Data were subjected to analysis of variance by MSTAT-C (1990) computer statistical analysis program. Duncan's multiple range test at 5% level of significance (p=0.05) was used for means comparisons according to Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Table 2 and Figure 1 illustrated the influence of different chemical mutagens on survival percentage of callus regeneration and multiple shoots of two apple rootstocks. It was obvious from the results that chemical mutagens reduced callus survival percentage, which varied from 66.67 to 54.00 % compared with control (80%). Mutagen concentrations appeared that the percentage of callus survival showed a dose-response. Concerning the effect of rootstocks, there were insignificant differences between them. The interactions between the two studied variables revealed that control gave the highest significant value (80 %) in both rootstocks followed by EMS at 0.01 mg/l (69.67 %). While the lowest significant mean was obtained with EMS at 0.03 mg/l (50 %).

The same previous trend was achieved with the percentage of regeneration, which appeared the highest significant mean at control treatment. Chemical mutagens showed a dose-response with the ability of

regeneration. The lowest significant mean obtained with EMS and NaN_3 at 0.03 mg/l (33.17 and 33.00 %), respectively. Concerning the effect of rootstocks, MM106 gave highest significant mean (42.76 %). The interactions between the variables revealed that the lowest significant mean (26.67 %) was obtained with EMS on Balady at 0.03 mg/l.

Numbers of multiplicated shoots recorded the highest significant mean with EMS treatment at 0.01 mg/l (5.50), while both concentrations of NaN_3 at 0.02 and 0.03 mg/l gave the lowest means (3.66 and 3.50) without significant differences between them. In addition, there were insignificant differences between the two rootstocks. Interactions between the two variables scored the highest significant mean with MM106 cultured on EMS at 0.01 mg/l (5.66). The lowest mean was recorded with Balady cultured on NaN_3 at 0.02 and 0.03 mg/l (3.33 and 3.33), respectively.

Table 2. Effect of EMS and NaN_3 on *in vitro* callus survival, regeneration and multiplication percentages of MM106 and Balady apple rootstocks.

Traits in tissue culture	Treatment mg/l	Balady	MM 106	Mean
Percentage of callus survival	Control	80.00 a	80.00 a	80.00 A
	EMS (0.01)	69.67 b	63.67 b-d	66.67 B
	EMS (0.02)	62.67 b-d	60.00 c-e	61.33 C
	EMS (0.03)	58.00 c-e	50.00 f	54.00 D
	NaN_3 (0.01)	65.33 bc	64.33 bc	64.83 BC
	NaN_3 (0.02)	60.00 c-e	60.00 c-e	60.33 C
	NaN_3 (0.03)	54.67 ef	56.33 d-f	55.50 D
	Mean	64.33 A″	62.14 A″	
Percentage of regeneration	Control	52.00 a	49.33 ab	50.00 A
	EMS (0.01)	42.67 b-e	45.67 a-d	44.17 B
	EMS (0.02)	31.00 fg	42.33 b-e	36.67 C
	EMS (0.03)	26.67 g	39.67 de	33.17 C
	NaN_3 (0.01)	48.67 a-c	44.67 a-d	46.67 AB
	NaN_3 (0.02)	42.33 b-e	41.33 c-e	41.83 B
	NaN_3 (0.03)	29.67 fg	36.33 ef	33.00 C
	Mean	39.00 B ″	42.76 A ″	
No. of multiplicated shoots	Control	4.33 b-e	5.00 a-c	4.66 B
	EMS (0.01)	5.33 ab	5.66 a	5.50 A
	EMS (0.02)	4.66 a-d	4.33 b-e	4.50 B
	EMS (0.03)	4.00 c-e	4.00 c-e	4.00 BC
	NaN_3 (0.01)	4.33 b-e	4.00 c-e	4.16 BC
	NaN_3 (0.02)	3.33 e	4.00 c-e	3.66 C
	NaN_3 (0.03)	3.33 e	3.66 de	3.50 C
	Mean	4.19 A ″	4.38 A ″	

Means followed by the same letters are not significantly different from each other at 5% level.

Data given in Table 3, Figure 1 and Figure 2 showed the influence of chemical mutagens on shoot elongation, number of leaves, number of roots

per plant and root length. The results showed that there were inversely relationship between mutagens concentrations and shoot elongation. The highest significant mean was recorded with control (8.90 cm), followed by a gradual decrease from (8.23 to 6.68 cm). Concerning the rootstocks, MM106 scored the highest significant mean (7.81 cm). On the other hand, interactions between the two variables gave the highest significant mean with control of Balady (9.00 cm). Shoot elongation varied from 8.40 to 6.30 cm depending on the level of chemical mutagens for two rootstocks. The lowest mean value obtained with Balady rootstock cultured on NaN_3 at 0.02 and 0.03 mg/l (6.46 and 6.30 cm), respectively, without significant difference.

Table 3. Effect of EMS and NaN_3 on *in vitro* vegetative growth of MM106 and Balady apple rootstocks.

Traits in tissue culture	Treatment mg/l	Balady	MM 106	Mean
Shoot elongation (cm)	Control	9.00 a	8.80 ab	8.90 A
	EMS (0.01)	8.06 cd	8.40 bc	8.23 B
	EMS (0.02)	7.30 e-g	8.06 cd	7.68 C
	EMS (0.03)	7.16 e-g	7.73 de	7.45 CD
	NaN_3 (0.01)	6.73 gh	7.46 ef	7.10 DE
	NaN_3 (0.02)	6.46 h	7.20 e-g	6.83 EF
	NaN_3 (0.03)	6.30 h	7.06 fg	6.68 F
	Mean	7.29 B ″	7.81 A ″	
No. of leaves / plant	Control	4.66 b-d	4.66 b-d	4.66 BC
	EMS (0.01)	6.66 a	5.33 bc	6.00 A
	EMS (0.02)	4.00 cd	4.00 cd	4.00 CD
	EMS (0.03)	4.66 b-d	3.66 d	4.16 B-D
	NaN_3 (0.01)	5.66 ab	4.33 cd	5.00 B
	NaN_3 (0.02)	3.66 d	3.33 d	3.50 D
	NaN_3 (0.03)	4.33 cd	3.66 d	4.00 CD
	Mean	4.81 A ″	4.14 B ″	
No. of roots / plant	Control	3.00 d	3.33 cd	3.16 BC
	EMS (0.01)	4.00 bc	5.00 a	4.50 A
	EMS (0.02)	3.00 d	4.00 bc	3.50 B
	EMS (0.03)	2.66 d	3.00 d	2.83 C
	NaN_3 (0.01)	4.00 bc	4.33 ab	4.16 A
	NaN_3 (0.02)	2.66 d	3.33 cd	3.00 BC
	NaN_3 (0.03)	3.00 d	3.00 d	3.00 BC
	Mean	3.19 B ″	3.71 A ″	
Root length (cm)	Control	4.66 a	4.50 a	4.58 A
	EMS (0.01)	3.60 cd	4.16 b	3.88 B
	EMS (0.02)	3.33 de	4.03 b	3.68 BC
	EMS (0.03)	3.06 ef	4.03 b	3.55 CD
	NaN_3 (0.01)	3.13 ef	3.90 bc	3.51 CD
	NaN_3 (0.02)	3.16 ef	3.60 cd	3.38 D
	NaN_3 (0.03)	2.93 f	3.20 ef	3.06 E
	Mean	3.41 B ″	3.92 A ″	

Means followed by the same letters are not significantly different from each other at 5% level.

The number of leaves affected by chemical mutagen concentrations showed that the plantlets treated with EMS at 0.01 mg/l gave the highest significant mean (6.0), as well as, the means of leaves number ranged from 6.0 to 3.5. Regarding the rootstocks, Balady scored high significant mean (4.81) compared with MM106 (4.14). Interactions between the two variables showed that the highest significant mean recorded with Balady cultured on EMS at 0.01 mg/l. While, MM106 cultured on EMS at 0.03 mg/l, NaN_3 at 0.02 mg/l and NaN_3 at 0.03 mg/l gave the lowest significant means (3.66, 3.33 and 3.66), respectively. Beside that, NaN_3 at 0.02 mg/l gave the same trend with Balady (3.66).

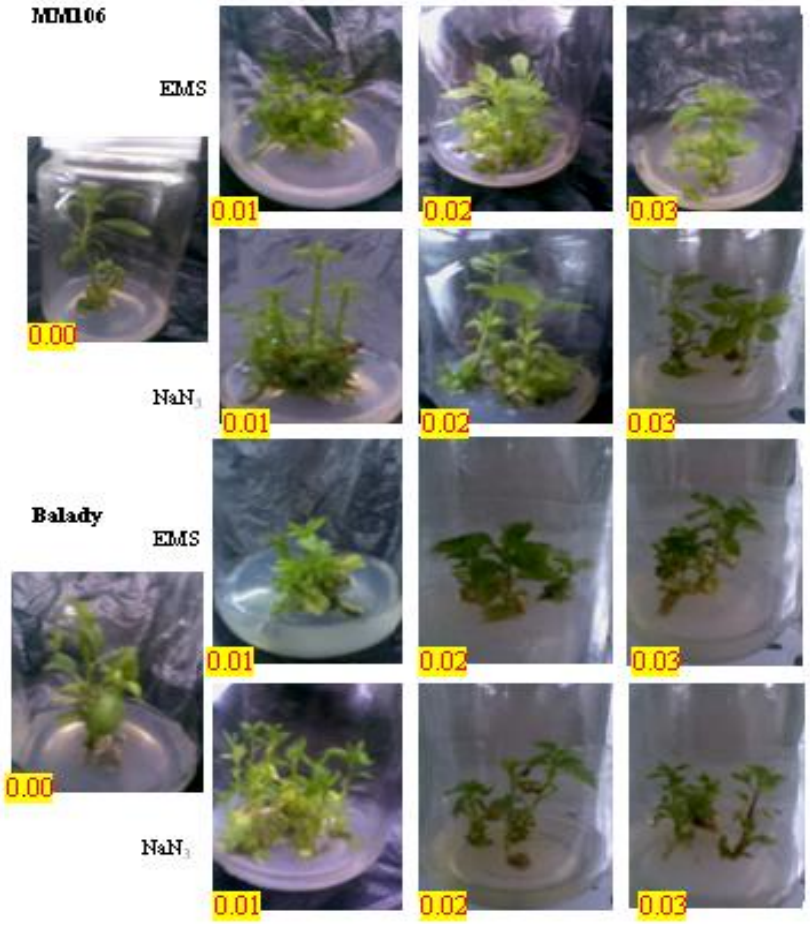

Figure 1. Effect of different concentrations of EMS and NaN_3 on MM106 and Balady apple rootstocks.

On the other hand, both treatments of EMS and NaN_3 mutagens at 0.01 mg/l gave the highest significant mean of roots number per plant (4.5 and 4.16), respectively. While EMS at 0.03 mg/l scored the lowest mean (2.83). Regarding the influence of rootstocks, MM106 gave the highest significant mean (3.71). Concerning the interactions, MM106 with EMS at 0.01 mg/l gave the highest significant mean (5.0) compared with the lowest mean of Balady rootstock cultured on EMS at 0.03 mg/l (2.66).

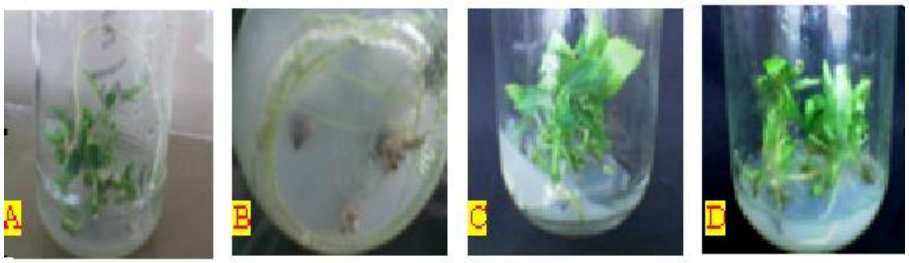

Figure 2. Different potential of root traits at four months from culture in MM106 and Balady apple rootstocks cultured on MS medium containing 0.5mg/l IBA.

Moreover, the highest significant mean of root length was achieved with control (4.58 cm). While there were an opposite relationship between the root length and mutagen concentrations. The lowest significant mean was recorded with EMS at 0.03 mg/l (3.06 cm). Concerning the influence of mutagens on rootstocks, MM106 gave the highest significant mean (3.92 cm) compared with Balady. Regarding interactions between the two variables, control in both rootstocks scored the highest significant means (4.66 and 4.50 cm) for Balady and MM106, respectively. The lowest mean was recorded with NaN_3 at 0.03 mg/l with Balady rootstock (2.93 cm).

These results were supported by the works conducted before in pear by Awad and Rayan (2007), who found that EMS at 0.01 mg/l reduced the percentage of callus formation, regeneration, shoot elongation and root length if compared with control, while, EMS and NaN_3 at (0.01 and 0.02 mg/l) increased the percentage of multiplication, number of leaves and roots per plant if compared with control. Moreover, the works conducted on red roomy grape by Rayan *et al.*, (2014) revealed that NaN_3 at 0.01, 0.02 and 0.03 mg/l reduced number of leaves and roots per plant, as well as, root length.

SDS-Protein banding patterns

SDS-PAGE of Balady and MM106 apple rootstocks and their treatments with EMS (E) and NaN_3 (S) revealed a total number of five protein bands with molecular weight (MW) ranging from about 21 to 29 KDa. One common band (monomorphic band) was detected, while, the remaining four bands were polymorphic with 80.0% polymorphism (Table 4 and Figure 3).

In Balady rootstock, data represent a band with 22 KDa that was a common band in control and all treatments. On the other hand, there was absence of band in control and each of treatments; E1, E2, S1, S2 and S3 which present in E3 with 29 KDa. In addition, at 27 KDa, bands were present

at control, E1, E2, E3, S1 and S2 but absent in S3. While, the band with 23 KDa was present in each of control, E1 and E3 but absent in other treatments.

Data of MM106 rootstock explained differences in present and absent bands in treatments compared with control, such as, the band with 21 KDa was present only in S3 and absent in control and other treatments which considered as specific marker for this treatment. While, there were absent bands in control, E3, S1, S2 and S3 and present in E1 and E2 with 23 and 29 KDa, respectively. Conversely, there were two bands present in each of control and in all treatments, which considered as common bands at 22 and 27 KDa, respectively. These results are in accordance with Rayan *et al.*, (2010), who demonstrated variations in protein banding patterns in some plum cultivars using gamma irradiation of Cobalt 60 and in Red Roomy Grape using gamma irradiation and sodium azide (Rayan *et al.*, 2014).

Peroxidase Banding Patterns

Table (5) and Figure (4) represent peroxidase electrophoresis banding patterns from fresh leaves of Balady and MM106 apple rootstocks and their treatments with EMS (E) and NaN_3 (S).

In Balady rootstock, there were differences in bands density between control and some treatments that have a reduction in banding density. In Px1 with relative mobility 0.3 there were reducing in band density to moderate density in each of E3, S2 and S3 if compared with control and other treatments which had high density of bands. In addition, each of Px2, Px3, Px8 and Px10 appeared reduction in band density with relative mobility 0.4, 0.43, 0.67 and 0.8, respectively. There were some treatments manifesting moderate density of bands if compared with control and other treatments, which achieved high density bands. While, some treatments (E2 and S3) illustrated low density of bands in Px4 and Px5 with relative mobilities 0.46 and 0.5, respectively, if compared with control and other treatments which had high density of bands. On the other hand, there were similarity in density of bands in control and all treatments in each of Px6, Px7 and Px8 with relative mobilities 0.6, 0.63 and 0.67, respectively. Meanwhile, there was absent of band in S2 treatment only and present in control and other treatments at Px12 with relative mobility 0.9. Among MM106 rootstock, data illustrated similarity in band density at control and all treatments; Px2, Px3, Px6, Px7, Px8 and Px9 with relative mobilities 0.4, 0.43, 0.6, 0.63, 0.67 and 0.7, respectively. While, there were absent bands in S3, E2, E3 and S2 at Px4, Px5, and Px12 with relative mobilities 0.47, 0.5 and 0.9, respectively. There were differences in banding patterns density in some treatments if compared with control and other treatments; S1, E2, and E1 at Px1, Px4, Px5, Px10 and Px11 with relative mobilities 0.3, 0.46, 0.5, 0.8 and 0.85, respectively.

Poly Phenyl Oxidase Banding Pattern

Table (6) and Figure (5) represent poly phenyl oxidase electrophoresis banding patterns among fresh leaves of Balady and MM106 apple rootstocks and their treatments with E (EMS) and S (NaN_3).

In Balady rootstock, there were differences in banding patterns density between control and some treatments that have a reduction in banding density. In each of treatments; E2, E3, S1, S2 and S3 at PPO1, PPO2, PPO3, PO4, PPO5, PPO6, PPO7 and PPO8 there were a reduction in banding density with relative mobilities 0.1, 0.2, 0.3, 0.4, 0.45, 0.5, 0.7 and 0.8, respectively. On the other hand, there was absent band in S3 at PPO3 with 0.3 relative mobility which were present in control and other treatments.

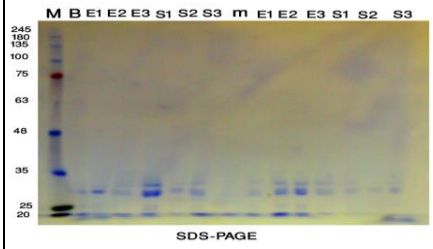

Fig. 3. SDS- leaf protein banding patterns for two apple rootstocks treated with two chemical mutagens.

Table 4. Densitometric analysis for leaf protein of two apple rootstocks treated with two chemical mutagens.

Band No.	M.W KDa	B						M						
		C	E1	E2	E3	S1	S2	S3	C	E1	E2	E3	S1	S2	S3
1	29	0	0	0	1	0	0	0	0	1	1	0	0	0	0
2	27	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3	23	1	1	0	1	0	0	0	0	1	1	0	0	0	0
4	22	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5	21	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Total		3	3	2	3	2	2	1	2	4	4	2	2	2	3

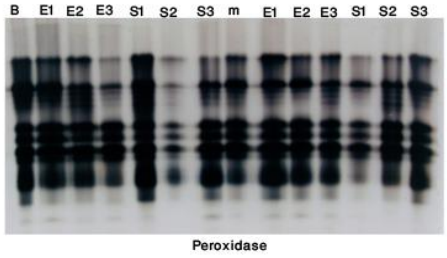

Fig. 4. Leaf peroxidase isozyme banding patterns for two apple rootstocks treated with two chemical mutagens.

Table 5. Densitometric analysis for leaf peroxidase isozyme of two apple rootstocks treated with two chemical mutagens.

Peroxidase Groups	R.M	B						M						
		C	E1	E2	E3	S1	S2	S3	C	E1	E2	E3	S1	S2	S3
gx1	0.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx2	0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx3	0.43	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx4	0.46	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx5	0.5	1	1	1	1	1	1	1	1	1	-	1	-	1	1
gx6	0.6	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx7	0.63	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx8	0.67	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx9	0.7	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx10	0.8	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx11	0.85	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx12	0.9	1	1	1	1	1	-	1	1	1	1	1	1	-	1

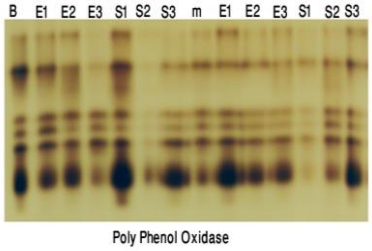

Fig. 5. Leaf poly phenyl oxidase isozyme banding patterns for two apple rootstocks treated with two chemical mutagens.

Table 6. Densitometric analysis for leaf poly phenyl oxidase isozyme of two apple rootstocks treated with two chemical mutagens.

Poly Phenyl oxidase Groups	R.M	B						M						
		C	E1	E2	E3	S1	S2	S3	C	E1	E2	E3	S1	S2	S3
gx1	0.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx2	0.2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx3	0.3	1	1	1	1	1	-	-	-	-	-	1	-	1	1
gx4	0.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx5	0.45	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx6	0.5	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx7	0.7	1	1	1	1	1	1	1	1	1	1	1	1	1	1
gx8	0.8	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Results of MM106 rootstock obviously showed similarity in band density in control and all treatments at PPO1 only with relative mobility 0.1. While, there were present bands in some treatments compared with control and other treatments of S2 and S3 at PPO3 with relative mobility 0.3. On the other hand, there were differences in banding pattern density in some treatments which appeared density reduction in; E1, E3, S1, and S2 at PPO1, PPO3, PPO7 and PPO8 with relative mobilities 0.1, 0.4, 0.7 and 0.8 respectively, if compared with control.

The results obtained herein are in harmony with Rayan *et al.*, (2010) and Abo Rekab *et al*., (2010), who established that peroxidase and poly phenyl oxidase banding patterns represent differences in density of bands with increase or decrease and absent of bands in treatments in comparison with control in plum and date palm cultivars.

RAPD Analysis

At the polymorphism level, a high level of polymorphism was generated utilizing the five RAPD primers as shown in Tables (7 and 8) and Figure (6). A total number of bands obtained in Balady rootstock and its treatments were 38 amplified fragments (TAF). From them, twenty fragments were polymorphic (PF) (52.6%) and eighteen were monomorphic. The highest number of bands was generated from S3 (31 fragments), while control generated the lowest (20 fragments). The highest number of bands, generated from primer OP-M01 (10 fragments), while the lowest was generated from primer OP-C15 (5 fragments). Also, control represented negative specific markers which were absent only using the following primers; OP-C04 at 995 bp, Primer OP-C15 at 265 bp and primer OP-M01 at 245, 470 and 940 bp, respectively. While, EMS treatment (E1) illustrated negative specific marker (absent band) using primer OP-C12 at 970 bp, however, NaN_3 treatment (S3) was present only which is considered as positive specific marker for the following primers; OP-C04 at 125 bp, primer OP-C15 at 700 bp and primer OP-M01 at 200 bp.

A total number of bands appeared in MM106 rootstock were 36 amplified fragments (TAF). From them eighteen fragments were polymorphic (PF) (50%) and eighteen were monomorphic. The highest number of fragments was generated from each of control and E1 (30 fragments), while S3 generated the lowest fragments (21). The highest number of bands was generated from primer OP-C04 (9 fragments), while the lowest was generated from primer OP-C12 (4 fragments).

Table 7. Specific RAPD markers for Balady apple rootstocks treated with two chemical mutagens (EMS and NaN_3).

Primers code	Range of M.W (bp)	TAF	MF	PF	SM	Polymorphism (%)
RAPD primers						
OP-C04	125-995	9	5	4	C^-(995bp),$S3^+$(125bp)	44.4
OP-C12	190-970	7	3	4	$E1^-$ (970bp)	57.1
OP-C15	130-700	5	3	2	$S3^+$(700bp), C(265bp)	40
OP-E15	200-1080	7	5	2	------	28.2
OP-M01	200-940	10	2	8	3C (245,470and940bp) $S3^+$ (200bp)	80
Total		38	18	20	9	52.6

Table 8. Specific RAPD markers for MM106 apple rootstocks treated with two chemical mutagens (EMS and NaN_3).

Primers code	Range of M.W (bp)	TAF	MF	PF	SM	Polymorphism (%)
RAPD primers						
OP-C04	125-995	9	5	4	C^+ (510bp)	44.4
OP-C12	190-970	5	3	2	-----	40
OP-C15	130-700	6	3	3	-----	50
OP-E15	200-1080	6	3	3	-----	50
OP-M01	200-940	10	4	6	$S3^-$ (940bp), $E2^-$ 280bp)	60
Total		36	18	18	3	50

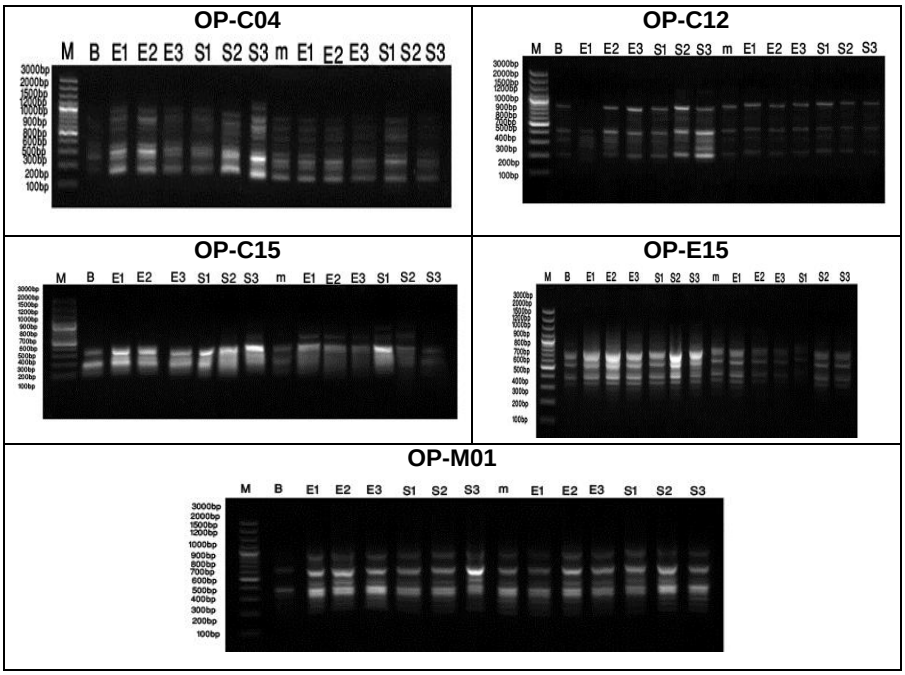

Fig 6. RAPD fragments of the two apple rootstocks and their treatments with two chemical mutagens.

Also, control represented positive specific markers which appeared only using primer OP-C04 at 510 bp. While, EMS treatment (E2) illustrated negative specific marker (absent band) using primer OP-M01 at 280 bp. However, NaN_3 treatment (S3) appeared the same trend using primer OP-M01 at 280 bp. These results of RAPD are in harmony with Rayan *et al*., (2010) and Rayan *et al*., (2014), who found that gamma radiation and sodium azide induced molecular genetic variations in plum and grape vine cultivars.

Finally, a specific advantage of mutation induction is to develop a range of mutant lines via biochemical and molecular genetic analyses. All variant regenerated plantlets were acclimatized to be transplanting into the permanent field for future evaluation of their genetic stability to be used in breeding programs and selection procedures.

In conclusion, All RAPD primers used in the present study allowed for enough distinction between each of Balady and MM106 rootstocks and their treatments with EMS and NaN_3 as used before in previous work (Awad and Rayan, 2007). Moreover, the molecular marker may be the easier criteria to distinguish genetic variations induced by chemical mutagens.

Overall, it can be concluded that the high concentration of chemical mutagen caused a negative influence on all studied parameters. Increasing chemical mutagens concentrations was reflected on the behavior of different explants under *in vitro* conditions.

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دراسات معملية لمطفرات كيميائية مختلفة على بعض أصول التفاح

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تم استخدام ثلاثة تركيزات مختلفة لاثنين من المطفرات الكيماوية (الايثيل ميثان سلفونيت، الصوديوم أزايد). استخدمت هذه المطفرات على أوراق أصلين من التفاح هما البلدي، MM106 ، في محاولة لإنتاج بعض الاختلافات الوراثية مع متابعة هذه الاختلافات باستخدام تقنية RAPD-PCR. استخدمت هذه المطفرات بتركيزات هى : صفر، 0.01، 0.02، 0.03 ملجم / لتر ، وذلك باستخدام تقنية زراعة الأنسجة. أظهرت النتائج أن نسبة البقاء للكالس تتناقص تدريجيا بزيادة تركيز المادة المطفرة وخاصة عند تركيز 0.03 ملجم/لتر من كلا المادتين محل الدراسة حيث وصلت نسبة الكالس الى 54 ، 5,55 % لكلا من EMS ، NaN_3 على التوالي مقارنة بتجربة المقارنة التي وصلت إلى 80 %. بالإضافة الى ذلك تأثرت كل القياسات الخضرية المأخوذة (عدد الأفرع الناتجة، عدد الأوراق لكل نبات، عدد الجذور لكل نبات). فكلما زاد تركيز المادة المطفرة أدى إلى نقص تلك القياسات ماعدا استخدام EMS بتركيز 0.01 والذي أدى الى زيادة ملحوظة في عدد الأفرع الناتجة وعدد الأوراق وعدد الجذور للنبات حيث كانت 5,5 ، 6 ، 4,5 على التوالي ، في حين سجلت تجربة المقارنة 4,66 ، 4,66 ، 4,16 على التوالي لنفس القياسات.

وباستخدام تحليل البروتينات الكلية الذائبة والمشابهات الإنزيمية (البيروكسيديز، البولي فينيل أوكسيديز) لدراسة الاختلافات الوراثية على مستوى التعبير الجيني، وجد عدد 5 حزم بروتينية كان منها حزمة واحدة متشابهة في الأصلين ومعاملاتهما (نسبة 20 % تشابه)، بينما الحزم المتبقية وهي عدد 4 حزم كانت مختلفة في الأصلين ومعاملاتهما (نسبة الإختلاف 80 %). وأما على مستوى المشابهات الإنزيمية فقد ظهرت اختلافات في درجة كثافة الحزم الناتجة مع التركيزات المختلة من المطفرات المستخدمة مقارنة بتجربة المقارنة. كما أظهرت تقنية RAPD-PCR وجود درجة من الاختلاف في أصل التفاح البلدي ومعاملاته فكان إجمالي عدد الحزم الناتجة 38 حزمة منها عدد 20 حزمة متباينة (نسبة تباين 52.6 %) أما الحزم المتبقية وهي 18 حزمة كانت متشابهة. وكان إجمالي عدد الحزم الناتجة مع أصل تفاح MM106 ومعاملاته 36 حزمة منها عدد 18 حزمة متباينة (نسبة تباين 50 %).