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GENETICAL STUDIES ON BANANA (*Musa ssp*) TISSUE CULTURES I. MORPHOLOGICAL AND MOLECULAR CHARACTERISZATION OF THE MUTANTS INDUCED BY CHEMICAL MUTAGENESIS (EMS AND NaN_3) THROUGH TISSUE CULTURE TECHNIQUE

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BSTRACT:

This work aimed to characterize the mutants induced by some chemical mutagens in banana (*Musa acuminate cv. Grand Nain*). Healthy *in vitro* plantlets were exposed to two chemical mutagenesis (EMS at 0, 100, 150 and 200 mM or NaN_3 at 0, 1, 2 and 3 mg/l) for 21 days and after three subcultures of the treatment without chemical mutagenesis were characterized. The results showed that when the banana plantlets treated with the different dose of EMS or NaN_3, the percentage of plantlets survival was decreased (66.66 and 33.33 %) for the 200 mM of EMS and 3 mg/l of NaN_3, respectively, in comparison with control (100 %). Moreover, analysis of variance indicated that the studied characters (number of multiplication shoots, shoot height, shoot thickness, number of leaves/plant, leaf area, number of roots/plant, root length and root thickness) were affected by the mutagenesis. NaN_3 at (1 mg/l) increased number of leaves/plant, number of roots /plant and root length) (4.81, 14.33 and 8.15cm), respectively compared with control (2.99, 13.33 and 2.80cm), respectively, also EMS at 100 mM increased (leaf area, root length and root thickness) (17.04cm2, 6.56cm, 0.15cm) respectively compared with control (12.97, 2.80cm and 0.12cm), respectively with same parameters. While the highest average of shoot thickness (1.07

cm) was recorded with EMS at 150 mM. The higher concentration of EMS or NaN_3 leaded to reduction all vegetative parameters. At the molecular level, using 7 primers ISSR technology allowed measure the genetic distance, relationship and similarity between the selected mutants of banana lines comparison with the untreated plantlets. Sixty-eight ISSR amplified bands were obtained (ranged from 237 to 1591 bp). The total number of polymorphic bands was 17 bands (25%polymorphism) while the total number of monomorphic bands was (75%monomorphism). In ISSR analysis banding patterns, there were absent bands, whereas mutation induction was 25% and 16.17% by using EMS and NaN_3, respectively. Using ISSR markers for detecting of genetic variations and exhibiting of the molecular genetic diversity for these explants supported the use of marker-assisted selection (MAS) in banana cultivars breeding programs.

Key words: *mutation induction,* ISSR molecular *markers, EMS Ethyl methanesulphonate , NaN_3 (sodium azide),.*

INTRODUCTION

Banana is one of the most important tropical fruit crops in the world. It is grown in every humid tropical region and constitutes the fourth largest fruit crop of the world. Banana is popular due to their typical sweet aromatic taste, large fruits with thick skin and its high yield. Banana production occupies an important share in the total fruit of Egypt. The total area of banana in the world attained about 5,103,033 ha (about 12,601,105 fed.) with an annual production of about 107,401,205 tons in Egypt, the total cultivated area of banana was about 67881 fed and produced about 1,129,777 tons FAO (2013).

The breeding of most banana cultivars is very difficult due to genetic sterility and triploidy level, hence, producing hardly any seeds. In addition, it is further complicated by low seed germination of hybrid banana plants (Javed *et al*., 2004). Induced mutation has been utilized as a tool to generate variation and breeding in a number of vegetative propagated crops such as some plum cultivars (Abou Rekab *et al*., 2010) and (Rayan, *et al*., 2010), and banana (Okole *et al* 2000, Hautea *et al*., 2004, and Predieri., 2001).

In banana, several DNA marker techniques have been used to investigate genetic relationships between *Musa* accessions, and to determine differences in somaclonal variants and radiation-induced mutants **(**Hautea *et al*., 2004). The molecular markers included randomly amplified polymorphic DNA (RAPD) (Pillay *et al.,* 2001), amplified fragment length polymorphism (AFLP) **(**Ude *et al*., 2002), and microsatellites markers **(**Creste *et al*., 2004). This study aimed to induce *in vitro* genetic variations in banana using different chemical

mutagenesis 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 2014 and 2015 seasons.

Source of the plant materials:

Healthy suckers about 50-70 cm in length were carefully cut from mother plants (*Musa acuminate cv. Grand Nain*) were collected from open-air banana fields and the suckers transferred to the laboratory.

Preparation and sterilization of explants:

The leaves and roots of healthy banana suckers (cv. Grand-Nain) were removed, and the basal part of the suckers were cut at 4cm above apical meristem tip after that it washed with tap water for 30 min, explants were surface sterilized by soaking in Clorox (commercial bleach) 70% for 30 minutes then, explants were rinsed several times in sterilized distilled water containing 0.1 g/l citric and 0.1 g/l ascorbic acids. The surface sterilized explants were then aseptically transferred to sterilized petri dishes, individual explants were then excised using fragments of a razor blade attached to a scalpel handles. Explant (1cm length and 1 cm diameter) were soaked in ethanol 70% for 30 sce. Finally rinsed in sterile distilled water 3 times before transferred to the cultured medium. Individual explants were cultured in Pyrex glass jars containing MS **(**Murashige and Skoog 1962) basal medium with vitamins supplemented with 30g/l sucrose , 7 g/l agar 3mg/l BA and adjusted to pH 5.7. The cultures were incubated at 24±2 C^o under photo period cycle of 16/8 h. as light/dark. Light intensity was used at 3000 lux. with white fluorescent tubes.

Induced mutation by using chemical mutagens:

After three subcultures on the basal MS medium (initiation medium) *in vitro* banana regenerated shoots were transferred to MS medium supplemented with 30 g/l sucrose, 7 g/l ager 5 mg/l BA and different concentrations of NaN_3 at (0, 1, 2, and 3 mgl^{-1}), or ethyl methane sulphonate at (EMS) (0, 100, 150 and 200 mM) and incubated for 21 days. The treated shoots were transferred to MS medium without chemical mutagenesis for three subcultures. The following data was recorded: survival percentage, no. of multiplicted shoots, shoot length(cm), shoot thickness(cm), no. of leaves, leaf area, no. of roots, root length (cm) and root thickness(cm).

DNA Isolation Procedure:

Fresh tissue parts were collected separately from different selected clones from the 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 Repeat DNA-PCR (ISSR-PCR) procedure:

PCR reactions were conducted using 7 arbitrary 5-mer primers. Their names and sequences are shown in Table (1).

Table (1): List of the primer names and their nucleotide sequences used in the study of molecular analysis of obtained *in vitro* banana cv. *Grand Nian* plantlets for ISSR procedure.

NO.	Primer codes	Sequence (5` to 3`)
1	14A	5´ CTC TCT CTC TCT CTC TTG 3`
2	44B	5´ CTC TCT CTC TCT CTC TGC 3
3	HB-08	5` GAG AGA GAG AGA GG 3`
4	HB-09	5´ GTG TGT GTG TGT GC 3`
5	HB-10	5´ GAG AGA GAG AGA CC 3`
6	HB-12	5´ CAC CAC CAC GC 3`
7	HB-15	5´ GTG GTG GTG GC 3`

RESULTS AND DISCUSSION

The effect of different concentrations of EMS was recorded in Table (2) and Fig. (1). The results showed that increasing of EMS concentrations leaded to decrease in plantlets survival compared to control (100 % survival). The plantlets produced on medium contain low concentration of EMS (100mM) showed high survival percentage comparing with control 83.33 and 100% respectively. While, by increasing the concentration of EMS to 200 mM resulted in the lowest survival percentage 66.66%. Growth parameters of banana plantlets i.e no. of multiplicted shoot, shoot length(cm), shoot thickness, no. of leaves, leaf area, no. of roots, root length and root thickness were presented in Table (2) and Fig (1). Clear significant differences were found between plantlets treated with different concentrations of EMS in average of shoot length. The untreated plantlets showed the highest average of shoot length (5.22 cm). While, increasing the concentration of EMS to 200 mM exhibited the lowest average of shoot length (1.85cm). Also the increasing of EMS concentrations leaded to decrease in number of multiplicated shoots compared to control and other treatment, while no significant difference was recorded between the effects of different doses of EMS on shoot thickness. These results are in agreement with previous findings in banana, chrysanthemum, petunia and soybean (Omar *et al*., 1989; Bhagwat and Duncan, 1997; Hofmann *et al*., 2004; Latado *et al*., 2004; Berenschot *et al*., 2008). Hofmann *et al*. (2004) whom reported a reduction in survival of soybean cell cultures with increasing dose of EMS. Khawale *et al*. (2007) also reported a decline in the regeneration capacity of shoots in

grapevine as the dose of mutagenic agent increased

Regarding the number of leaves and leaf area the treatment of 150mM EMS induced significant enhancement on number of leaves (4.11) compared to control and other treatment while the highest average of leaf area (17.04 cm^2) was recorded with 100mM EMS and, the lowest (5.49 cm^2) was recorded with the high level of EMS 200mM. On the other hand, the treatment 100mM EMS showed the highest values for root length and root thickness (6.56cm and 0.15 cm, respectively). In contrast, the treatment of 200mM EMS resulted in the lowest root length and root thickness, while no significant difference were found between different doses of EMS in root numbers.

The percentage of survived plants decreased by increasing the concentration of Sodium azide were 1, 2 and 3mg /L was 86.66%, 66.66% and 33.33% respectively (Table 2). Treatment with different concentration of sodium azide significantly decreased the number of multiplicated banana shoots (Table 2). Also, shoot length was decreased (2.1cm) in concentration of 3 mg L^{-1} compared to the control (5.22cm), while there were no significant differences between effects of different doses of sodium azide on shoot thickness. The highest average of shoot thickness 0.73 cm was recorded with the control and sodium azide at 2mgL^{-1}. However, the lowest average (0.46 cm) was recorded with the high level of sodium azide at (3mgL^{-1}) In contrast, number of leaves was increased to (4.81and 3.63) with two concentrations (1 and 2 mg L^{-1}) of sodium azide, respectively compared with the control (2.99). While the treatment 3 mgL^{-1} sodium azide resulted in the lowest leaf area (2.36 cm^2) as compared to control and the other treatments. These results were in agreement with those of with EL-Sayed ***et al.*** (2011) reported a reduction in survival of banana cv. Grand-Nain shoot tips with increasing dose of sodium azide.

Regarding the number of roots, root length and root thickness results in Table (2) and Fig (1) illustrated that number of roots and root length were significantly raised with treatment, where induced significant increase in root length (8.15 cm) compared to the control (2.8 cm) and other treatments. Data in Table (2) indicated that treatment of 3 mg/L sodium azide gave the lowest values of root length (1.53 cm). while there were no significant difference between effect of different doses of sodium azide in root thickness, As for the root thickness and number of root, data in Table (2) indicated that treatment of 3 mg/L sodium azide induced the lowest values of both root thickness (0.09 cm) and number of roots (7.33) compared to control and the other treatments.

Table (2): Effect of different concentrations of EMS and sodium azide on survival percentage and quantitative characters of banana *in vitro* shoot.

Treatment	Survival %	Plantlets quantitative characters							
		No. of multiplicated shoots	Shoot length (cm)	Shoot thickness (cm)	leaves number	Leaf area (^{cm2})	Roots number	Root length (cm)	Root thickness (cm)
Control	100	5.75^{a}	5.22^{a}	0.73^{a}	2.99^{bc}	12.97^{ab}	13.33^{a}	2.80^{b}	0.12^{b}
EMS (100 mM)	83.33	4.25^{ab}	4.06^{a}b	0.75^{a}	2.40^{b}	17.04^{a}	13.00^{a}	6.56^{a}	0.15^{a}
EMS (150 mM)	75.00	3.00^{b}	3.05^{cb}	1.07^{a}	4.11^{a}	10.61^{b}	11.00^{a}	5.50^{a}	0.10^{b}
EMS (200 mM)	66.66	3.25^{b}	1.85^{c}	0.52^{a}	3.54^{ac}	5.49^{b}	12.00^{a}	2.83^{b}	0.10^{b}
Means		3.50	2.99	0.78	3.35	11.05	12.00	4.96	0.12
NaN_3 (1 mgl-1)	86.66	3.00^{b}	3.39^{b}	0.65^{a}	4.81^{a}	8.76^{a}	14.33^{a}	8.15^{a}	0.10^{b}
NaN_3 (2 mgl-1)	66.66	5.00^{a}	3.30^{b}	0.73^{a}	3.63^{b}	9.13^{a}	13.33^{a}	4.75^{ab}	0.11^{b}
NaN_3 (3 mgl-1)	33.33	3.00^{b}	2.10^{b}	0.46^{a}	2.74^{c}	2.36^{b}	7.33^{b}	1.53^{b}	0.09^{b}
Means		3.67	2.93	0.61	3.73	6.75	11.66	4.81	0.10

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

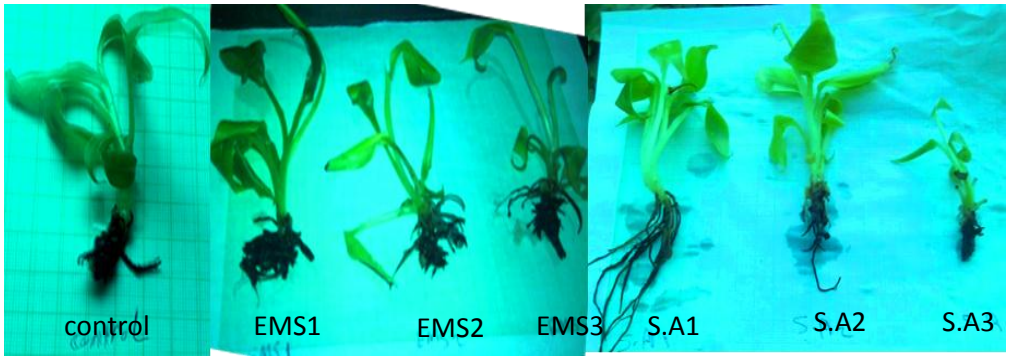

Fig. (1): The effect of different concentration of EMS and sodium azide on micropropagation banana in vitro shoots (EMS1 100 mM EMS; EMS2 150 mM EMS; EMS3 200mM EMS; S. A1 1 mg/l sodium azide; S.A2 2 mg/l sodium azide; S.A3 3 mg/L sodium azide)

The ISSR analysis was performed on the forty-nine DNA samples representing six banana mutant lines and control using seven primers (14A, 44B, HB-08, HB-09, HB-10, HB-12 and HB-15). Table (3) and Fig. (2) showed the results and data revealed that total number of ISSR bands was 68 bands, ranged from 237 to 1591 bp. The total

number of polymorphic bands was 17 bands this represents a level of polymorphism about of (25%), while the total number of monomorphic bands was 51bands this represents a level of monomorphism about of (75%).

Polymorphism levels were differed from one primer to the other. The number of polymorphic marker varied among the different primers. Primer HB-08, HB-12 and HB-15 generated one polymorphic band represents a level of polymorphism about of 7.692%, 20% and 9.091%respectively with fragment size of 598 bp for primer HB-08, with fragment size 668 bp for primer HB-12 and with fragment size 1258 bp for primer HB-15. While the number of polymorphic markers varied among the primer 14A, 44B and HB-10 generated 4, 2 and 3 polymorphic bands respectively, represents a level of polymorphism about of 40%, 18.182% and 42.857% respectively. On the other hand, primer HB-09 exhibited the highest polymorphism percentage so it gave five fragments with molecular weight 1516, 1228, 870, 553 and 324 bp were polymorphic with 45.455 % polymorphism.

The similarity indices (Si) between the control and six selected mutant banana lines derived from the treatment by two chemical mutagenesis, based on the DNA fragment generated by using seven random primers 14A, 44B, HB-08, HB-09, HB-10, HB-12 and HB-15 presented in Table (3). UPGMA cluster analysis Fig. (3) showed that control and EMS2 line nearly clustered together and showed similarity Si= 0.926, as well as SA1 and SA2 lines nearly clustered together and showed similarity Si= 0.985. In ISSR analysis banding patterns, there were absent bands, whereas mutation induction was 25% and 16.17 by using doses of EMS and sodium azide, respectively

Table (3): ISSR analysis from the DNAs of banana (*Musa acuminata cv. Grand Nain)* Seven primers.

Primer Code	Total Amplified Fragments	Length range bp	Poly-morphic Bands	Poly-morphism (%)	Mono-morphic bands	Mono-Morphism (%)
14A	10	287-1147	4	40	6	60.00
44B	11	283-1196	2	18.182	9	81.818
HB-08	13	237-1591	1	7.692	12	92.308
HB-09	11	324-1516	5	45.455	6	54.545
HB-10	7	447-1500	3	42.857	4	57.143
HB-12	5	354-668	1	20	4	60.00
HB-15	11	305-1383	1	9.091	10	90.909
Total	68	237-2591	17	25	51	75.00

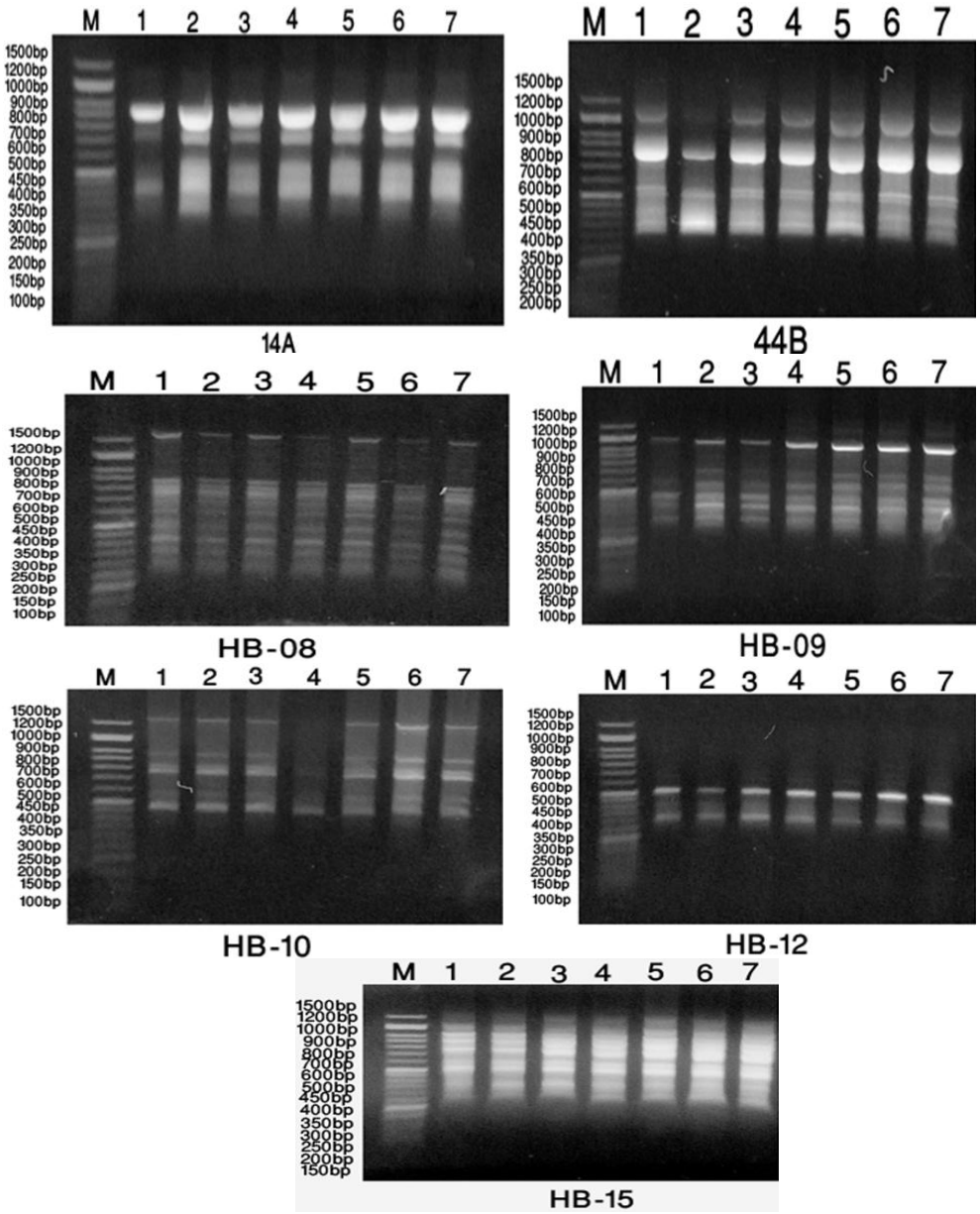

Fig. (2): ISSR-PCR band patterns generated by seven primers (14A, 44B, HB-08, HB-09, HB-10, HB-12 and HB-15) Lane M DNA ladder (150 – 1500 bp) plus, lanes (2, 3 and 4), for (1 00, 150 and 200mM) of EMS and lanes (4, 5 and 6) for (1, 2, and 3 mgl-1 NaN3) compared with control (lane 1).

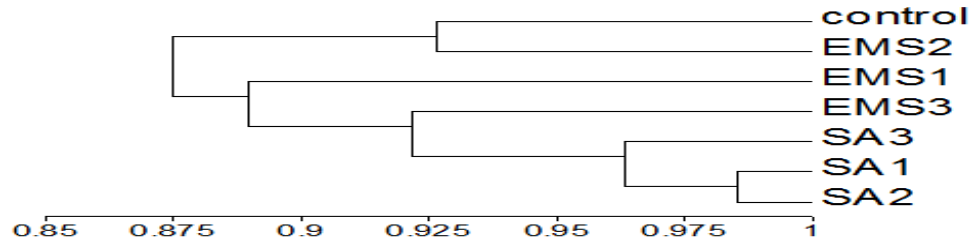

Fig. (3): Dendrogram for 6 lines of banana (Musa acuminata cv. Grand Nain) induced via EMS and sodium azide mutagens in vitro and parental genotype as control. Data were constructed from ISSR marker using UPGMA and Si computed according to Dice coefficient.

Table (4): Similarity index as percentage (pairwise comparison) among the 6 banana lines induced via *in vitro* mutation treatments, based on ISSR analysis.

Similarity index	Control	EMS1	EMS2	EMS3	SA1	SA2	SA3
Control	1.000						
EMS1	0.824	1.000					
EMS2	0.926	0.897	1.000				
EMS3	0.809	0.868	0.882	1.000			
SA1	0.882	0.882	0.926	0.926	1.000		
SA2	0.868	0.897	0.912	0.941	0.985	1.000	
SA3	0.853	0.912	0.897	0.897	0.971	0.956	1.000

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دراسات وراثية علي مزارع انسجة الموز

1 – الصفات المورفولوجية والجزيئيه للطفرات المستحدثة بالمطفرات الكيماوية (الايثيل ميثان سلفونيت و الصوديوم أزايد) من خلال تقنية زراعة الانسجه

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تهدف هذه الدراسة الي محاولة استحداث الطفرات في نباتات الموز (Musa acuminate) صنف الجراندنين من خلال تقنية زراعة الأنسجة بتعريض نباتات الموز في مرحلة التضاعف لاثنين من المواد المطفرة وهما الايثيل ميثان سلفونيت بتركيز 100 ,150, 200 مل مولر) و الصوديوم أزايد بتركيز (1، 2، 3 ملجم /لتر) مع متابعة هذه الاختلافات باستخدام تقنية ISSR-PCR . و أظهرت النتائج أن نسبة البقاء للنباتات تتناقص تدريجيا بزيادة تركيز المادة المطفرة وخاصة عند تركيز 200 ميلمولر من EMS والتركيز 3 ملجم/لتر من NaN3 حيث وصلت نسبة بقاء النباتات الى (66,66% ، 33,33 %) على التوالي مقارنة بتجربة المقارنة التي وصلت إلى 100 %. بالإضافة الى ذلك تأثرت كل القياسات الخضرية المأخوذة (عدد الأفرع الناتجة، طول وسمك الفرع ،عدد الأوراق لكل نبات، مساحة الورقة، عدد الجذور لكل نبات، وطول وسمك الجذر). حيث أدى استخدام الصوديوم أزايد بتركيز 1ملجم / لتر إلى زيادة في نسبة القياسات المورفولوجية (عدد الاوراق، عدد الجذور، وطول الجذر) حيث كانت (4.18، 14.33، 8.15سم) علي التوالي في حين سجلت تجربة المقارنة (2.99، 13.33، 2.8سم) علي التوالي لنفس للقياسات. كما أدى استخدام الايثيل ميثان سلفونيت بتركيز 100ملى مولر إلى زيادة في نسبة القياسات المورفولوجية (مساحة الورقة، طول الجذر، وسمك الجذر) حيث كانت (17.04 سم2 ، 6.56، 0.15 سم) علي التوالي في حين سجلت تجربة المقارنة (12.97 سم2، 2.8 سم، 0.12 سم) علي التوالي لنفس للقياسات بينما استخدام التركيزات العالية من كلا المادتين أدي الي انخفاض ملحوظ في جميع هذه القياسات. وعلي المستوي الجزيئي تم تحديد التباينات الحادثة علي مستوي ال DNA للنباتات المعاملة بالماد المطفرة ونباتات تجربة المقارنة. وأجري هذا التحليل باستخدام تقنية ال ISSR باستخدام 7 من البادئات وظهرت النتائج ان إجمالي عدد الحزم الناتجة 68 حزمة منها عدد 17 حزمة متباينة (نسبة تباين 25 %) أما الحزم المتبقية وهي 51 حزمة كانت متشابه. كما دلت النتائج علي ان نسبة التباين النتاجة من المعاملة بالاثيل ميثان سلفونيت25%بينما ان نسبة التباين النتاجة من المعاملة بالصوديوم أزايد 16.17% فقط.