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Effect of diethyl sulphate and sodium azide on tolerance of ex vitro banana to salt stress

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Abstract : Banana is a cash crop sensitive to the salinity and problem increases by water shortage especially in arid and semi-arid regions and this can severely limit plant growth and productivity. The aim of current study was to develop mutants of banana Grande Nain cv. tolerant to salinity stress. In vitro shoots of banana Grande Nain cv. were treated with different concentrations of diethyl sulphate (DES) and sodium azide (NaN_3) during the multiplication stage (in the third subculture) and then with NaCl in the rooting stage. After then, the transplanted banana was more stressed by adding the sea water to the irrigation water every time for 6 months. Three factors were investigated in this study, mutagen type, concentrations plus duration and salinity levels. The recorded vegetative growth parameters after six months in acclimatization stage showed that DES had less negative impact on the vegetative growth parameters than NaN_3. In addition, the low concentration of DES and NaN_3 gave healthy ex vitro plants of 22.88 and21.60 cm as plant height/plant, 5.78 and 7.00 as number of leaves/plant, 24.62 and 21.80cm^2 as leaf area, 5.22 and 6.89 as number of roots/plant, and 7.31 and 2.98 cm as psudostem circumference respectively at all duration periods. However, using the high concentration of DES (400µm) and NaN_3 (8µm) produced the most viable plants at 10 min. Concerning the effect of salinity levels in the greenhouse, all measured growth parameters decreased by increasing salinity levels. There was a great variance regarding the double interactions between the three studied factors, but the triple interaction among the three factors indicated that DES at 100 and 200 µM for 10, 20 or 30 min as well as at 400 µM for 10 or 20 min with all studied salinity levels produced the best results when compared with the other treatments. Also, a fingerprinting based on ISSR was used to detect any genetic variation occurred in the stressed plants. Using different mutagens under different salinity levels, on the other hand, led to induce a great effects in terms of ions accumulation (K^+ and Na^+) and proline contents. According to PCR-ISSR and SDS-PAGE test results; there were genetic variations in the stressed plants as a result to use DES mutagen. This study could be a base to use diluted sea water in irrigating banana plants produced from in vitro culture and treated with DES mutagens.

Key words: Banana cv. Grande Nain, diethyl sulphate, DES, Sodium azide, NaN_3, mutagens, salinity, vegetative growth, ISSR.

Introduction

Banana (*Musa spp*.) is an important staple food crop for many people in the tropical zones of the world. Banana provide nourishment and a well balanced diet to millions of people around the globe and contribute to livelihood through crop production, processing and marketingJain[1].

Excess salt in the soil or in irrigation water is one of the biggest problems in agriculture since almost all cultivated plants are sensitive to it Jose *et al*[2]. In this concern, Israeli *et al.*[3] studied the effect of sodium salt on growth and productivity of banana and found that increasing of salt concentration in the irrigation water led to a marked decrease in the growth and productivity of field grown plants.

Genetic modification of crop plants to improve their salt tolerance is a possible way for increasing their productivity especially for regions of the world where arable lands must be extended to marginal area and sometimes irrigated with saline water Dorion*et al*.[4]

Due to the difficulty of improving banana with conventional methods, alternative approaches such as mutation induction have been pursued. The main advantage of mutation induction in vegetatively propagated crops is the ability to change one or a few characters without changing the remaining characters in the genotype Broertjes and Van- Harten[5].

Two mutation induction systems were used for banana improvement. The first one was based on in vivo sucker irradiation before meristem tip isolation and culture, yielding a low number of mutants De Guzman *et al*.[6]The second one was based on in vitro techniques for mutation induction, recovering mutant plants, avoiding or reducing chimerism and micropropagation of desirable mutants Novak *et al*.[7].

Several methods of selection for enhanced salt tolerant genotypes have been developed Tal[8]. Approaches based on biochemical, molecular and physiological studies of tissue culture can provide more reliable means for the development of salt-tolerant plants Winicov and Bastola[9]. Plant material selected from salt stressed cultures can be used to establish plantations in saline soil Shin *et al*.[10]

The aim of this work was to induce mutagenesis in the in vitro produced banana Grande Nain cv. plantlets by using two chemical mutagens Diethyl sulphate $(C_2H_5)_2SO_4$ (DES) and Sodium Azide (NaN_3) to determine the optimal concentrations and duration of exposure to produce in vitro mutants for salt stress.

Materials and Methods

Tissue culture and transplantation parts were carried in the tissue culture laboratory and in the greenhouse of the experimental farm in the Horticulture Research Institute (HRI), Agricultural Research Center (ARC) while the fingerprinting and biochemical measurements were in the Plant Biotechnology Dept., National Research Center (NRC), Giza, Egypt during the periods from 2012 till 2015.

Plant materials and treatments

The uniform in vitro shoots of banana Grande Nain cv. were selected from the multiplication stage (from the third subculture).These shoots were treated with two mutagens during the fourth subculture of the multiplication stage. The two mutagens Diethyl Sulphate (DES) and Sodium Azide (NaN_3) were tested at ten concentrations. Each was for (control, low concentration (100 µM for DES and 2 µM for NaN_3) for 10, 20 and 30 min, medium (200 µM for DES and 4 µM for NaN_3) for 10, 20 and 30 min and high concentration (400 µM for DES and 8 µM for NaN_3) for 10, 20 and 30 min). Then these in vitro shoots treated with three salinity levels of NaCl (500, 1000 and 1500 ppm) in the subculture of the rooting stage. After transplanting plants were stressed more by adding sea water every time with the irrigation water as per need.

Culture medium composition

Murashige and skoog[11](MS) basal medium was used as a basal nutrient medium. The basal medium was supplemented with 4 mg l^{-1} BA 6-Amino Benzyl Purine (BA)+ 30 g I^{-1} sucrose + 2gI^{-1} gel as imitation medium for culturing the sterilized shoot tip explants, pH of the medium was adjusted to 5.6 ±0.2. The medium

was distributed into the culture jars (325 ml) where each jar contained 45 ml of the medium. The jars were immediately capped with polypropylene closers then were autoclaved at 121^{o}C at 15 lbs/ Inch for 20 min.

Culture incubation conditions

Shoot tip explants were incubated in the growth room at day and night temperature of 27 ±2^{o}C. Light was provided by florescent lamps giving intensity of 1500 Lux for 16 hours per day.

Mutagens application

In the fourth subculture two mutants Diethylsulphate (DES) chemical formula $(C_2H_5)_2SO_4$ and Sodium Azide (NaN_3) were added after they were autoclaved at 121°C at 15 Ibs/Inch for 20 min. Uniform multiplied shoot tips explants were longitudinally dissected and dipped into aqueous solutions of DES at 100 µM, 200 µM and 400 µM for 10, 20 and 30 min. As well as NaN_3 was used at 2µM, 4µM and 8 µM for 10, 20 and 30 min. in addition to the control treatment which was left without dipping in any mutagens. All treatments were then transferred on multiplication medium for 4 weeks.

Individual shoots were separated and transferred into the rooting MS basal medium supplemented with 0.1 mg l^{-1} Naphthalene Acetic Acid (NAA), 1.0 gl^{-1} Activated Charcoal (AC). The prepared fresh media was usually left a single week before culture for the rooting medium (The 6th subculture) for contamination check. The plantlets were stressed for salinity by adding NaCl that were equals to 500, 1000 and 1500 ppm.

Acclimatization procedure

Rooted plantlets were shifted to the plastic green house at HRI in order to carry out the acclimatization stage, the following procedure was followed:

1. Rooted plantlets were washed with running tap water to remove all traces of agar.
2. The plantlets were immersed in a fungicide solution Topsin M 70 (0.1% for three min).
3. The individual plantlets were planted in trays contained a mixture of clay loamy soil, peatmoss and sand at ratio of 1: 1: 1 (v: v: v).
4. The plantlets were watered and covered with a transparent white polyethylene sheet.
5. Plants were being irrigated every three days with a tap water
6. After three months of transplanting, the survived plant were watered with sea water at three concentrations 500 (13.8 ml sea water/l of tap water), 1000 (27.6 ml sea water/l of tap water) and 1500 ppm (41.4 ml/l of water) at three days intervals. Irrigation was done at the field capacity.

Data recorded

At the end of this study the morphological parameters were recorded as follows:

1. Plant height (cm)
2. Plant stem circumference (cm)
3. Number of leaves/ plant
4. Number of roots /plant
5. Leaf area (cm^2). The third leaf from top was measured, according to the formula reported by Obiefuna and Ndubizu[12]Leaf area = L×W× 0.83 where:

L= the length of leaf
W= the maximum width
0.83= coefficient

Therefore, the genetic parameters such as DNA finger printing based on ISSR analysis was used, protein analysis (SDS – PAGE) Damasco *et al.*[13]were recorded.

Experimental layout and Statistical Analysis

The used experimental design was completely randomized design in a factorial experiment (three factors) as described by Snedecor and Cochran[14] at 5% probability level. Data obtained were statistically analyzed using MSTAT- Computer Program MSTAT Development Team[15]

Measurement of Proline Content

Proline content was estimated colorimetrically according to the method of Renisch*et al*.[16]. Hundred mg of tissue was ground in liquid nitrogen and homogenized in 5 ml of 3% w/v sulfosalicylic acid and centrifuged at 5000 x g for 15 min. The supernatant was used for proline estimation using ninhydrin reagent which was prepared by dissolving 0.5 g of ninhydrin in 30 ml acetic acid and 20 ml of distilled water with continuous stirring. For 1 ml aliquot, 1 ml of ninhydrin reagent was added and the mixture was heated on a boiling water bath for 20 min. Then the samples were cooled at room temperature. On attaining the room temperature, the contents of test tube were shaken vigorously with 4 ml of toluene. Upper pink coloured organic phase was separated from the lower aqueous phase and absorbance of organic phase was read at 520 nm using toluene as a blank. Standard curve was prepared using graded concentrations of L-proline and data were expressed as µmoles g^{-1} fresh weight.

Ion Analysis

Sodium and potassium contents were determined from the oven-dried material. Hundred mg of the dry material was extracted in 50 ml of deionized water with continuous shaking and estimated on microprocessor based Ion Analyzer (Elico, India) using ion specific electrode (Na, K and Cl).

Total soluble Protein Extraction and Estimation

Soluble protein in banana leaves extract was determined according to the coomassie G-250 Dye binding method Bradford[17]. A 100 µl of extract was taken and to it 1.5 ml of Bradford's reagent was added. After 5 min incubation at room temperature, absorbance was noted at 595 nm against a reagent blank. Calculations were made using standard curve prepared from the graded concentrations of bovine serum albumin (BSA). The same protein extract was used to load onto the gel on equal protein quantity basis.

Electrophoresis

The SDS-PAGE was carried out using the method of Laemmli[18] using 12% (w/v) separating and 4% (w/v) stacking gels. Five µl of samples were electrophoresed on 12% acrylamide (Sigma) gel for 3 h at 30 mA using a small electrophoresis chamber (BioRad, USA). In each gel a wide range molecular weight marker (Fisher) was included. The gels were stained in 0.25% Coomassie Brilliant Blue R250 (Sigma) in methanol:acetic acid:distilled water (5 : 1 : 5) for 90 min with gentle shaking. Then the gels were destained in methanol:acetic acid:distilled water (2 : 3 : 35) overnight .

DNA Isolation

Total DNA was extracted using the protocol of CTAB method of Murray andThampson[19] and used immediately for PCR or frozen at -20 °C for later use. four ISSR primers that had a high level of polymorphism, were used for polymerase chain reaction (PCR) amplification.

ISSR analysis

Each 25 µL of the reaction contained 100 ng of genomic DNA, 10 × PCR reaction buffer, 25 mM MgCl2 , 25 m MdNTP mix, 100 pmol of each primer, and 1.5 units of Taq DNA polymerase (Fermentas). PCR was performed in a Gene Amp PCR system 9700, PE Applied Biosystems. The initial step of 95 °C for 3 min was followed by 35 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, and a final cycle at 72 °C for 10 min. ISSR amplification products were separated by gel electrophoresis in 1.5% agarose gels and stained with ethidium bromide.

Results and Discussion

The current study aimed at using subsequent varietal stresses on the banana plants during different in vitro and ex vitro growth stages. These stresses started with treating the in vitro multiplying shoots with different concentrations with two mutagenesis DES (100,200 and 400 µm) and NaN_3 (2,4 and 8 µm) for different periods of time (10, 20 and 30 min). Then all treatments in rooting stage were exposed to 3 different concentrations of NaCl at 500, 1000 and 1500 ppm. Keeping in consideration that the in vitro plants died when exposed to 3000 ppm (preliminary results) this why the high concentration was discarded before to start the experiment. Eventually, the plants were stressed more by adding the sea water into the irrigation water (two times per week in the net house) for all plants for a period of 6 months.

1. Morphological characteristics:

A. Effect of mutagens

Data presented in Table (1) showed that DES was more effective than NaN_3 in increasing all of the studied traits during acclimatization stage. DES significantly recorded the highest values of plant height as recorded 22.13 cm, pseudostem circumference/plant (3.71 cm), number of leaves/plant (5.63), leaf area/plant (16.99 cm^2) and number of roots /plant (5.19). NaN_3 recorded only 19.45 cm for plant height, 2.37 cm for pseudostem circumference/plant, 5.00 for number of leaves/plant, 15.77 cm^2 for leaf area/plant and finally 5.19 for number of roots /plant.

Table 1. Effect of mutagens, concentrations plus durations and salinity levels on the vegetative growth characteristics of banana Grand Nain during acclimatization stage.

Treatments	Plant height (cm)	Pseudostem circumference/plant (cm)	Number of leaves/plant	Leaf area/plant (cm^2)	Number of roots /plant (cm)
Mutagens (A)					
DES	22.13	3.71	5.63	16.99	5.99
NaN_3	19.45	2.37	5.00	15.77	5.19
L.S.D. at 0.05	0.24	0.69	0.16	1.27	0.22
Concentrations plus duration (B)					
Control	15.16	2.33	4.33	14.01	3.89
Low[z] for 10min	23.00	2.90	5.78	16.88	5.78
Low for 20min	23.91	3.37	5.89	19.21	6.61
Low for 30min	22.24	5.15	6.39	23.21	6.06
Medium[y] for 10min	21.15	2.93	5.17	12.88	5.22
Medium for 20min	21.73	2.73	5.44	15.76	5.44
Medium for 30min	21.29	2.61	5.11	14.86	5.11
High[x] for 10min	20.86	3.12	5.94	16.12	6.78
High for 20min	19.98	2.76	4.61	16.19	6.39
High for 30min	18.58	2.50	4.50	14.69	4.61
L.S.D. at 0.05	0.53	1.54	0.37	2.84	0.50
Salinity levels[w] (C)					
500 ppm	23.52	3.04	5.63	18.46	5.88
1000 ppm	19.95	3.00	5.43	19.15	5.77
1500 ppm	18.90	3.09	4.88	11.53	5.12
L.S.D. at 0.05	0.29	0.85	0.20	1.55	0.27

Z:100 µm for DES or 2 µm for NaN_3, Y: 200 µm for DES or 4 µm for NaN_3, X: 400 µm for DES or 8 µm for NaN_3, W:NaCl.

The above mentioned results are in agreement with many author findings on banana, Jain [20]showed that inducing mutations chemically by using DES stimulated growth of plantlets of micropropagated Grand Nain banana plants. Predieri[21] reported that inducing mutation chemically in tissue cultured Williams banana plants by DES had an announced promotion on growth relative to untreated plants. Jain[1]investigated that treating

explants of Grand Nain banana with diethyl sulphate increased number of plantlets leaves comparing with the control. In this concern, Spina *et al.*[22] stated that treating shoot tips of in vitro grown cultures of Grand Nain banana with DES greatly was accompanied with enhancing height of plantlet as well as producing vigorous plantlets treated. On other plants Krupa-Małkiewicz[23] reported that DES was more effective than NaN_3, EMS and MMS in increasing all studied characters of *Kalanchoe hybrid.*

B. Effect of mutagen concentration plus durations:

Table (1) showed the effect of mutagen plus durations on some vegetative growth traits of in vitro produced banana plantlets during acclimatization stage. Data indicated that low concentration (100 µM for DES and 2 µM for NaN_3) for 30 min significantly produced the highest values in terms of pseudostem circumference/plant, number of leaves/plant and leaf area/plant (5.15 cm, 6.39 cm and 32.21 cm^2, respectively). Moreover, plant height was significantly affected by different concentrations and durations. Low concentration (100 µM for DES and 2 µM for NaN_3) for 20 min significantly produced the tallest plants as recorded 23.91 cm. Although high concentration (400 µM for DES and 8 µM for NaN_3) for 10 min increased the number of roots to the highest values (6.78), this increase was insignificant. On the other hand, control (without any mutagens) and high concentration of the used mutagens (400 µM for DES and 8 µM for NaN_3) for 30 min produced the lowest values for all the studied traits.

These results were in agreement with those obtained by Spina *et al.*[22]Jain[20] and Jain[1] on banana plants.

C. Effect of salinity levels:

Illustrated data in Table (1) indicated that salinity level at 500 ppm was more effective than other levels in promoting some vegetative growth characteristics, such promotion was significant in case of plant height (23.52 cm) and number of leaves/plant (5.63) and insignificant in case of number of roots/plant (5.88). In this concern, salinity level at 1000 ppm produced the highest insignificant value and recorded (19.15). In contrast to the previous results, Pseudostem circumference/plant insignificantly increased to the highest value when treated with salinity level at 1500 ppm (3.09 cm). Excepting Pseudostem circumference/plant, salinity level at 1500 ppm produced the lowest values of the other studied characters.

Negative effect of salinity on banana plants was studied by many authors. Palaniappam and Yerriswamy[24] conducted a field experiment on Monthan banana (*Musa sp.* ABB Group) using saline irrigation water (0.3, 2.5 and 5.0 dS/m). Plant height decreased as salinity increased from 0.3 to 5.0 dS/m. Mahmoud[25] stated that the presence of sodium chloride in the culture media at 0.25 to 0.75% caused a reduction on all growth characters of micropropagated banana cv. Grand Nain, Gros Michel and Williams over the check treatment the reduction was associated with increasing sodium chloride in the culture.Gomes *et al.*[26] found that increasing NaCl concentrations resulted in a reduction in leaf area and dry weight for most banana genotypes (Calcutta 4, Pacovan, Nanicao, Caipira and FHIA-18).Herb *etal.*[27]determine that sea salt rate was negatively correlated with growth parameters (survival percentage, shoots and pseudostem height, leaf and root numbers and shoot fresh and dry weights) of banana cv. Williams grown in vitro as well as under greenhouse conditions. Ismail *et al.*[28]. Studied the effects of different salt concentrations on the produced transgenic banana plants, results showed lower decrease in the percentage of survived plants, pseudostem diameter and leaf area with an increase of salt concentrations in case of transgenic plants compared with the controls. Junior *et al.*[29]analysed growth variables including leaf area, fresh and dry biomass, biomass allocation and growth rate of ten diploid banana genotypes (*Musa spp*.) grown under salinity conditions. In most genotypes, salinity caused reductions in almost all variables.

D. Effect of interaction between mutagens and concentrations plus soaking durations:

Data in Table (2) showed the effect of interaction between mutagenes (DES and NaN_3) and concentrations plus durations on some vegetative growth parameters. In general, treating in vitro produced plantlets with DES regardless concentration or duration was more effective than NaN_3 in increasing the most studied character values. DES at 100µM for 10 min produced the tallest plants (24.87 cm) and for 30 min produced the highest values of pseudostem circumference/plant (7.31 cm) and leaf area (24.62 cm^2). While, DES at 400 µM produced the highest number of leaf/plant (7.44) and number of roots/plant (8.67). DES at 400 µM for 20 min shared the previous treatment and produced the highest number of roots/plant and gave the same

value (8.67).NaN_3 at high concentration (8 µM) for different durations and control produced the lowest values in most cases.

Table 2. Effect of interaction between mutagens and concentrations plus durations on the vegetative growth characteristics of banana Grand Nain during acclimatization stage.

Mutagens / Conc. &durations	Plant height (cm)		Pseudostem circumference /plant (cm)		Number of leaves/plant		Leaf area/plant (cm^2)		Number of roots /plant (cm)	
	DES	NaN_3	DES	NaN_3	DES	NaN_3	DES	NaN_3	DES	NaN_3
Control	15.16	15.16	2.33	2.33	4.33	4.33	14.01	14.01	3.89	3.89
Low[z] for 10min	24.87	21.13	3.13	2.67	5.78	5.78	14.49	19.27	6.00	5.56
Low for 20min	24.60	23.22	3.95	2.78	5.44	6.33	14.20	24.23	5.89	7.33
Low for 30min	22.88	21.60	7.31	2.98	5.78	7.00	24.62	21.80	5.22	6.89
Medium[y] for 10min	21.89	20.40	3.26	2.61	5.11	5.22	13.72	12.04	5.00	5.44
Medium for 20min	22.21	21.25	3.27	2.20	6.22	4.67	17.94	13.58	5.78	5.11
Medium for 30min	23.98	18.60	3.16	2.06	6.11	4.11	19.40	10.31	5.78	4.44
High[x] for 10min	21.98	19.74	4.13	2.12	7.44	4.44	18.68	13.55	8.67	4.89
High for 20min	22.59	17.36	3.51	2.01	5.22	4.00	16.12	16.25	8.67	4.11
High for 30min	21.16	16.00	3.06	1.95	4.89	4.11	16.73	12.64	5.00	4.22
L.S.D. at 0.05	0.75		2.18		0.52		4.01		0.70	

Z:100 µm for DES or 2 µm for NaN_3, Y: 200 µm for DES or 4 µm for NaN_3, X: 400 µm for DES or 8 µm for NaN_3, W:NaCl.

Similar results were obtained by Spina*et al.*[22] who reported that treating shoot tips of in vitro grown cultures of Grand Nain banana with DES (diethyl sulphate) at 100 µM for 20 minutes greatly was accompanied with enhancing height of plantlet as well as producing vigorous plantlets treated. Jain[20] showed that inducing mutations chemically by using DES at 200 µM for 20 minutes stimulated growth of plantlets of micropropagated GrandNain banana plants.Jain[1] found that in vitro propagation of Grand Nain banana, treating explants with diethyl sulphate at 100 µM for 20 minutes was accompanied with producing plantlets with higher number of leaves comparing with the control.

E. Effect of interaction between mutagens and salinity levels:

Regarding the interaction between mutagens and salinity levels, data in Table (3) showed that, combined treatment between DES and salinity level at 500 ppm significantly increased plant height and number of leaves/plant to the highest values (25.38 cm and 6.07, respectively), such increment was significant. In this regard, DES combined with salinity level at 1000 ppm produced the highest values of leaf area/plant (20.41 cm^2) and number of roots/plant (6.33), this effect was insignificant when compared with DES and salinity level at 500 ppm and significant when compared with the remainder treatments. NaN_3 combined with salinity level at 1500 ppm produced the lowest values for all studied traits.

Table 3. Effect of interaction between mutagens and salinity levels on the vegetative growth characteristics of banana Grand Nain during acclimatization stage.

Salinity levels[w] / Mutagens	500 ppm	1000 ppm	1500 ppm
	Plant height (cm)		
DES	25.38	21.18	19.84
NaN_3	21.66	18.72	17.97
L.S.D. at 0.05	0.409		
	Pseudostem circumference/plant (cm)		
DES	3.51	3.49	4.12
NaN_3	2.56	2.51	2.05
L.S.D. at 0.05	1.195		
	Number of leaves/plant		
DES	6.07	5.73	5.10
NaN_3	5.2	5.13	4.67
L.S.D. at 0.05	0.282		
	Leaf area/plant (cm^2)		
DES	18.37	20.41	12.20
NaN_3	18.55	17.9	10.86
L.S.D. at 0.05	2.197		
	Number of roots /plant (cm)		
DES	6.07	6.33	5.57
NaN_3	5.70	5.20	4.67
L.S.D. at 0.05	0.384		

F. Effect of interaction between concentrations plus durations and salinity levels:

Effect of combined treatment between mutagen concentrations plus durations and salinity levels on some vegetative growth of ex vitro produced banana plants during acclimatization stage was shown in Table (4). Low concentration (100 µM for DES and 2 µM for NaN_3) for 20 min combined with salinity levels at 500 ppm produced the highest values of plant height and number of roots/plant and recorded 26.51 cm and 7.50, respectively. In this concern, low concentration (100 µM for DES and 2 µM for NaN_3) for 30 min combined with salinity levels at 500 ppm increased number of leaves/plant and leaf area the highest values (6.83 cm^2 and 26.31, respectively). Pseudostem circumference/plant reached to the maximum value (8.02) when low concentration (100 µM for DES and 2 µM for NaN_3) for 30 min + salinity level at 1500 ppm was applied. The lowest values of all experiment traits were obtained when any of mutagens were used (control) in addition to high concentration (400 µM for DES and 8 µM for NaN_3) + salinity levels at 1500 ppm, especially on plant height (16.60 cm), number of leaves/plant (4.00) and number of roots/plant (4.33).

Table 4. Effect of interaction between concentrations plus durations and salinity levels on the vegetative growth characteristics of banana Grand Nain during acclimatization stage.

	Plant height (cm)			Pseudostem circumference/plant (cm)			Number of leaves/plant			Leaf area/plant (cm^2)			Number of roots /plant (cm)		
Salinity levels[w] Conc. &durations	**500 ppm**	**1000 ppm**	**1500 ppm**	**500 ppm**	**1000 ppm**	**1500 ppm**	**500 ppm**	**1000 ppm**	**1500 ppm**	**500 ppm**	**1000 ppm**	**1500 ppm**	**500 ppm**	**1000 ppm**	**1500 ppm**
Control	18.99	13.50	13.00	2.00	3.00	2.00	4.00	5.00	4.00	14.10	20.63	7.30	3.67	4.33	3.67
Low[z] for 10min	24.15	22.99	21.87	3.25	2.86	2.59	5.83	6.00	5.50	17.90	20.32	12.42	6.50	5.83	5.00
Low for 20min	26.51	23.94	21.29	3.14	3.64	3.33	6.17	6.17	5.33	21.07	22.19	14.39	7.50	6.50	5.83
Low for 30min	25.39	21.54	19.80	3.71	3.70	8.02	6.83	6.50	5.83	26.31	26.01	17.31	6.33	6.33	5.50
Medium[y]for 10min	24.13	19.83	19.49	3.42	2.81	2.56	5.50	5.17	4.83	13.70	15.73	9.21	5.83	5.17	4.67
Medium for 20min	23.04	21.77	20.39	2.96	2.82	2.42	6.00	5.33	5.00	22.05	15.73	9.49	6.17	5.50	4.67
Medium for 30min	22.98	20.97	19.94	2.84	2.74	2.26	5.17	5.33	4.83	17.53	16.82	10.23	5.83	5.00	4.50
High[x] for 10min	24.76	18.99	18.85	3.41	3.12	2.85	6.50	6.00	5.33	17.60	18.30	12.45	6.83	7.33	6.17
High for 20min	23.81	18.30	17.83	3.03	2.78	2.46	5.17	4.50	4.17	17.49	19.76	11.32	5.17	7.17	6.83
High for 30min	21.47	17.68	16.60	2.59	2.54	2.39	5.17	4.33	4.00	16.85	16.04	11.18	5.00	4.50	4.33
L.S.D. at 0.05	0.92			2.67			0.63			4.91			0.86		

Z:100 µm for DES or 2 µm for NaN_3, Y: 200 µm for DES or 4 µm for NaN_3, X: 400 µm for DES or 8 µm for NaN_3, W:NaCl.

G. Effect of interaction between mutagens, concentrations plus durations and salinity levels:

It is obvious from data presented in Table (5) that regardless of high concentration and long duration (400 µM for 30 min), DES at other concentrations and durations when combined with different salinity levels produced the highest values of the most studied vegetative growth characteristics compared to NaN_3. The only exception was for leaf area which was increased to the maximum value (30.99 cm^2) by using NaN_3 at 2 µM for 20 or 30 min when combined with salinity level at 500 ppm then DES at 100 µM for 30 min when combined with salinity level at 1000 ppm with insignificant differences between these two treatments. On the other hand, it could be noticed that DES increased the most studied vegetative growth characteristics of in vitro produced banana plantlets exposed to high salinity level (1500 ppm) above control at the same salinity level for example DES at 100 µM for 10 min in case of plant height (23.33 cm), DES at 100 µM for 30 min for pseudostem circumference/plant and leaf area (13.47 cm and 20.18 cm^2), DES at 400 µM for 10 min in case of number of leaves/plant (6.67) and DES at 400µM for 20 min in case of number of roots/plant (9.67). The same previous characteristics of untreated banana plantlets (control) exposed to salinity level at 1500 recorded only 13 cm for plant height, 2.00 cm for pseudostem circumference/plant, 7.30 cm^2 for leaf area, 4.00 for number of leaves/plant and 3.67, for number of roots/plant.

Table 5. Effect of interaction between mutagens, concentrations plus durations and salinity levels on the vegetative growth characteristics of banana Grand Nain during acclimatization stage.

Mutagens (A)	Concentrations plus durations (B)	Plant height (cm)			Pseudostem circumference /plant (cm)			Number of leaves/plant			Leaf area/plant (cm^2)			Number of roots /plant (cm)		
		Salinity levels [w] (C)														
		500 ppm	1000 ppm	1500 ppm	500 ppm	1000 ppm	1500 ppm	500 ppm	1000 ppm	1500 ppm	500 ppm	1000 ppm	1500 ppm	500 ppm	1000 ppm	1500 ppm
DES	Control	18.99	13.50	13.00	2.00	3.00	2.00	4.00	5.00	4.00	14.10	20.63	7.30	3.67	4.33	3.67
	Low[z] for 10min	26.78	24.49	23.33	3.57	2.99	2.82	6.33	5.67	5.33	19.81	15.52	8.14	7.00	6.00	5.00
	Low for 20min	27.27	24.14	22.40	3.53	4.22	4.10	5.67	5.67	5.00	11.15	19.25	12.20	6.00	6.00	5.67
	Low for 30min	25.87	22.54	20.22	4.17	4.31	13.47	6.00	6.00	5.33	24.07	29.61	20.18	5.33	5.33	5.00
	Medium[y] for 10min	24.41	20.63	20.64	3.65	3.17	2.96	5.67	5.00	4.67	14.11	16.93	10.13	5.67	5.00	4.33
	Medium for 20min	24.22	21.89	20.52	3.45	3.37	2.99	7.67	5.67	5.33	20.53	20.39	12.89	6.33	6.00	5.00
	Medium for 30min	27.40	23.63	20.92	3.52	3.20	2.77	6.00	6.67	5.67	21.50	22.34	14.37	6.67	5.67	5.00
	High[x] for 10min	25.63	20.40	19.92	4.19	4.23	3.96	7.67	8.00	6.67	19.48	22.27	14.28	8.67	9.67	7.67
	High for 20min	28.04	20.37	19.37	3.81	3.50	3.21	6.00	5.00	4.67	19.26	18.20	10.90	6.00	10.33	9.67
	High for 30min	25.23	20.18	18.07	3.25	2.96	2.96	5.67	4.67	4.33	19.64	18.93	11.62	5.33	5.00	4.67
NaN_3	Control	18.99	13.50	13.00	2.00	3.00	2.00	4.00	5.00	4.00	14.10	20.63	7.30	3.67	4.33	3.67
	Low for 10min	21.52	21.48	20.40	2.93	2.73	2.35	5.33	6.33	5.67	15.99	25.12	16.71	6.00	5.67	5.00
	Low for 20min	25.74	23.74	20.18	2.74	3.07	2.55	6.67	6.67	5.67	30.99	25.12	16.57	9.00	7.00	6.00
	Low for 30min	24.91	20.53	19.37	3.26	3.10	2.57	7.67	7.00	6.33	28.55	22.41	14.44	7.33	7.33	6.00
	Medium for 10min	23.85	19.03	18.33	3.20	2.46	2.17	5.33	5.33	5.00	13.28	14.53	8.30	6.00	5.33	5.00
	Medium for 20min	21.85	21.65	20.25	2.47	2.27	1.86	4.33	5.00	4.67	23.57	11.07	6.09	6.00	5.00	4.33
	Medium for 30min	18.55	18.30	18.96	2.17	2.28	1.74	4.33	4.00	4.00	13.56	11.29	6.09	5.00	4.33	4.00
	High for 10min	23.88	17.57	17.77	2.63	2.00	1.73	5.33	4.00	4.00	15.71	14.33	10.62	5.00	5.00	4.67
	High for 20min	19.58	16.22	16.28	2.26	2.06	1.71	4.33	4.00	3.67	15.71	21.31	11.73	4.33	4.00	4.00
	High for 30min	17.71	15.17	15.12	1.93	2.11	1.81	4.67	4.00	3.67	14.05	13.14	10.73	4.67	4.00	4.00
L.S.D. at 0.05			1.30			3.78			0.89			6.95			1.22	

Z:100 µm for DES or 2 µm for NaN_3, Y: 200 µm for DES or 4 µm for NaN_3, X: 400 µm for DES or 8 µm for NaN_3, W:NaCl.

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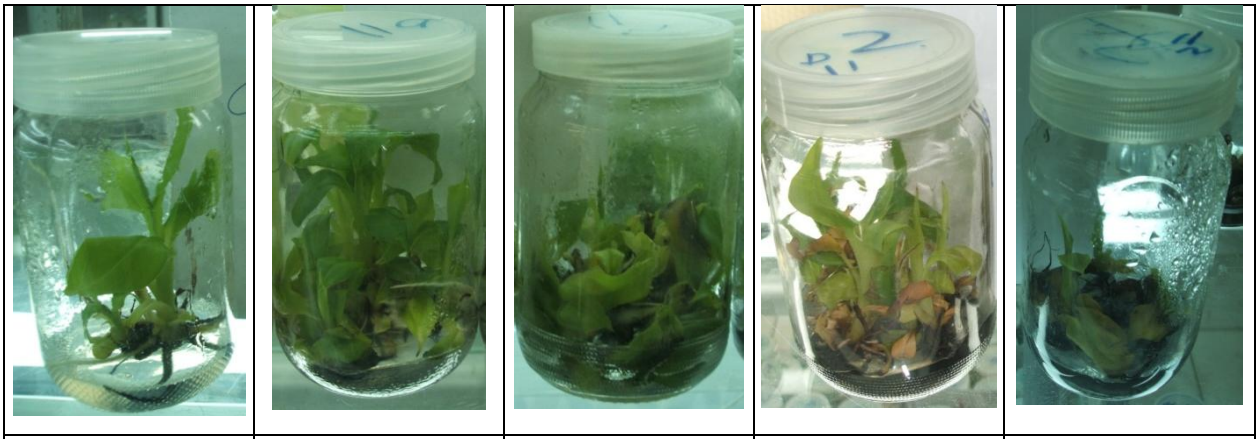
The present study emphasized that there was a great differences between used mutagens and its doses (Figs. 1 and 2), the frequency and saturation of mutations can be regulated by varying the mutagen dose and mutagenic agents can induce different extensions of genomic lesions, ranging from base mutations to larger fragment insertions or deletions Jander *et al*.,[30]Menda *et al.*,[31]MacKenzie *et al*.,[32] and Kim *et al*.,[33]. The

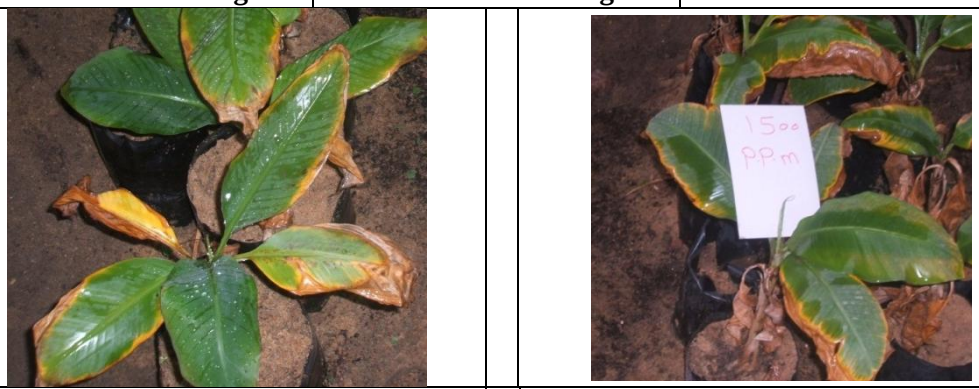
response to physical and chemical mutagens is species-specific and largely unknown for the majority of the species Gilchrist and Haughn[34].



Control plant	400µm DES for 10 min	8µm NaN_3 for 10 min	400µm DES for 10 min +1000 ppm salt stress	8µm NaN_3 for 10 min +1000 ppm salt stress

Fig. 1. Effect of mutagen concentrations plus soaking duration during in vitro culture of Grand Nain banana plant under salinity level.

400µm DES for 10 min +1000 ppm salt stress during acclimatization stage	8µm NaN_3 for 10 min + 1000 ppm salt stress during acclimatization stage	Banana plant under salt stress 500 ppm sea water



Banana plant under salt stress 1000 ppm sea water	Banana plant under salt stress 1500 ppm sea water

Fig. 2. Effect of mutagen concentrations plus soaking duration during ex vitro culture of Grand Nain banana plant under different salinity level.

The negative effects of salinity on growth of crops produced from tissue culture or conventional methods might be attributed to ion toxicity, disturbance of cellular and tissue water status, the increase in ATP for osmatic adjustment, protein synthesis and cell division reduction Gupta[35] Pessarakli and Touchane[36] postulated that mechanism of salt may result in cell division inhibitory and hence, reduces the rate of plant development. On the other hand, the effects of salt stress might be explained as the inadequate photosynthesis caused by stomatal closure and the reduction of carbon assimilation rate under salt stress Ben Ahmed *et al.*[37]All these previously mentioned disorders of salinity could be reversed by using chemical mutants. Such mutants play an important role in the genetic level. In this concern, chemical mutagens induce the formation of O-alkyl adducts of nucleotides leading to mispairing that preferentially cause C/G to T/A transitions. As compared with physical mutagens, chemicals may give rise to relatively more gene mutations rather than chromosomal changes Berenschot*et al*.[38]

2.Minerals and Proline content:

Effect of mutagens concentrations plus soaking duration under salinity level at 1000 ppm on K, Na and proline contents:

A. Potassium (K):

Data in Table (6) and Fig (3) showed the effect of mutagens concentrations plus soaking durations on potassium content of ex vitro Grand Nain banana plants exposed to salinity level at 1000 ppm.

It is shown from the obtained data that treating plants with NaN_3at 4 µM for 30 min significantly produced the highest potassium content (38380.67mg/100g DW).

Untreated plants (control) significantly came in the second position and recorded 25077 mg/100g DW. It could be also noticed that NaN_3at 2 µM for 30 min then for 20 min produced the lowest potassium content with significant difference between them as recorded 4738.67 and 10405.00 mg/100g DW, respectively.

B. Sodium:

Data in Table (6) and Fig (4) showed the effect of mutagens concentrations plus soaking durations on sodium content of ex vitro Grand Nain banana plants exposed to salinity level at 1000 ppm.

It is clear from the obtained data that treating plants with NaN_3 at 4 µM for 10 min significantly produced the highest sodium content (852.67 mg/100g DW). It could be also noticed that NaN_3 at 4 µM for 20 min and untreated plants (control) produced the lowest sodium content with insignificant difference between them as recorded 334.00 and 308.67 mg/100g DW, respectively.

Table (6). Effect of mutagen concentrations plus soaking duration on K, Na and proline contents of ex vitro Grand Nain banana plant under salinity level at 1000 ppm.

(B)Time / **Conc**.(A)	**K %**	**Na %**	**proline** µg/g
1- n3(2µmNaN_3+10min+1000ppm)	14307.67±562.63	554.67±11.26	162.00±2.71
2- n4(2µmNaN_3+20min+1000ppm)	10405.00±26.56	379.00±2.89	116.50±1.39
3- n5(2µmNaN_3+30min+1000ppm)	4738.67±106.52	844.67±19.34	00.00±00.00
4- n7(4µmNaN_3+10min+1000ppm)	15692.67±592.07	852.67±25.12	169.30±1.73
5- n8(4µmNaN_3+20min+1000ppm)	11692.67±355.36	334.00±3.46	85.07±1.88
6- n9(4µmNaN_3+30min+1000ppm)	38380.67±190.24	718.00±0.58	144.40±3.23
9- d9(200µmDES+30min+1000ppm)	12615.67±118.65	381.00±2.89	106.17±3.72
10- d11(200µmDES+10min+1000ppm)	16205.00±1954.04	569.67±2.60	165.00±6.87
11- d12(200µmDES+20min+1000ppm)	21282.00±740.16	405.00±1.73	114.50±1.27
12- c1000 (1000ppm)	24359.00±147.80	546.00±1.73	136.40±0.69
13- cg (control plant)	25077.00±147.80	308.67±5.49	117.70±3.18
L.S.D. at 0.05	1932	27.08	8.40

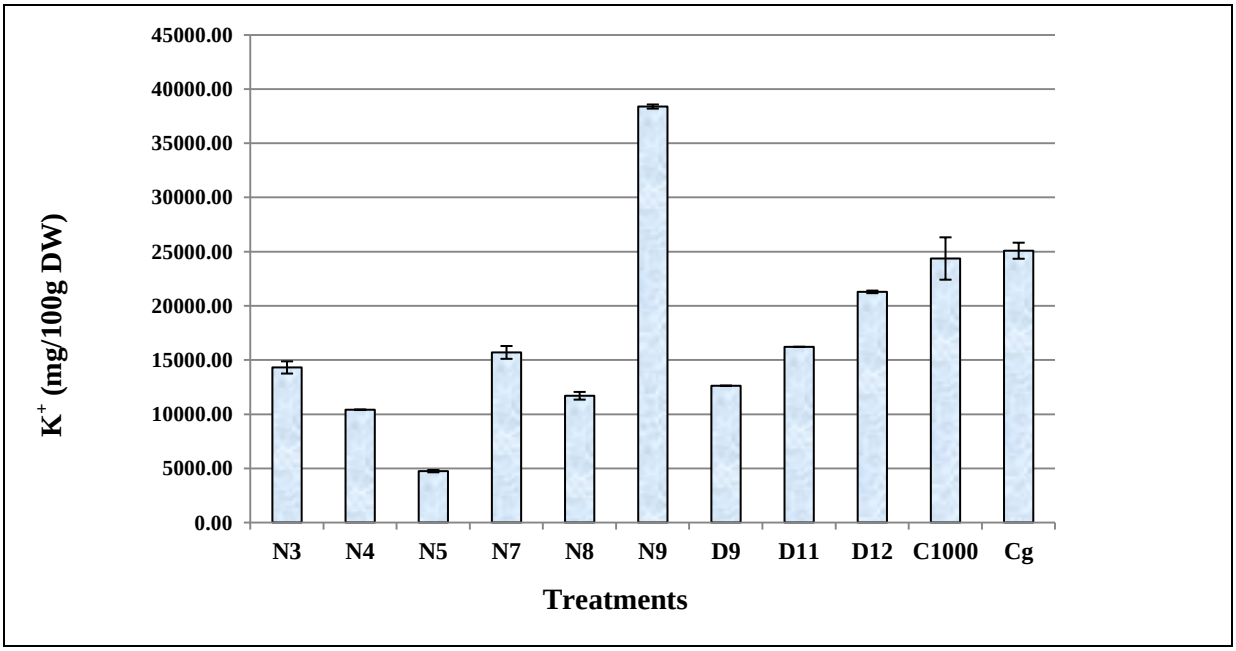


Fig. 3. Effect of mutagen concentrations plus soaking duration on K content of ex vitro Grand Nain banana under salinity level at 1000 ppm.

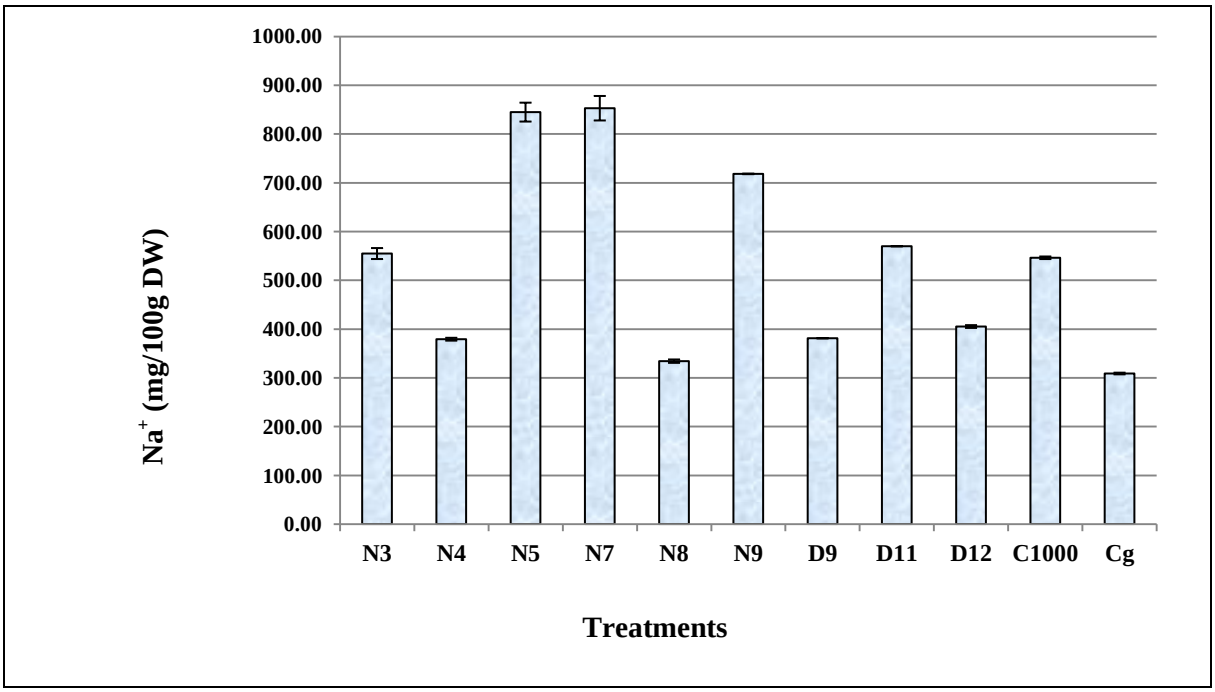


Fig. 4. Effect of mutagen concentrations plus soaking duration on Na content of ex vitro Grand Nain banana plant under salinity level at 1000 ppm.

C. Proline content:

Data in Table (6) and Fig (5) showed the effect of mutagens concentrations plus soaking durations on proline content of ex vitro Grand Nain banana plants exposed to salinity level at 1000 ppm.

It is shown from the obtained data that treating plants with NaN_3 at 4 µM for 10 min significantly produced the highest proline content (169.30µg/g FW.). Plants which produced from 200µm DES+10min insignificantly came in the second position and recorded 165.00 µg/g FW. It could be also noticed that NaN_3at 4 µM for 20 min produced the lowest proline content as recorded 85.07 µg/g FW**.**

Many authors reported the effect of salinity on ions accumulation in plant tissues Khan *et al.*[39]on *Atriplexgriffithii*; Gomes *et al.*[40]on five banana genotypes and Tavakkoli *et al.*[41] on four barley genotypes), such findings were in harmony with previous mentioned results of this study. It well known that increase salt uptake induces specific ion toxicities like that of high Na^+, Cl^-, or sulfate (SO_4^{2-}) that decrease the uptake of essential nutrients like phosphorus (P), potassium (K^+), nitrogen (N), and calcium (Ca^{++}) Zhu[42]. On the other hand Na^+ is the primary ion as it interferes with the uptake of potassium (K^+) ion and disturbs stomatal regulation that ultimately causes water loss while the Cl^- ion disturbs the chlorophyll production and causes chlorotic toxicity Tavakkoli*et al.*[41] .Despite the fact that plants grown under salinity conditions tend to accumulate high amounts of toxic ions (e.g. Na^+) and this in turn led to decrease the essential nutrients (e.g. K^+), this study revealed that using different mutagens reverse this bad effects and increase K^+ accumulation in leaves tissue.

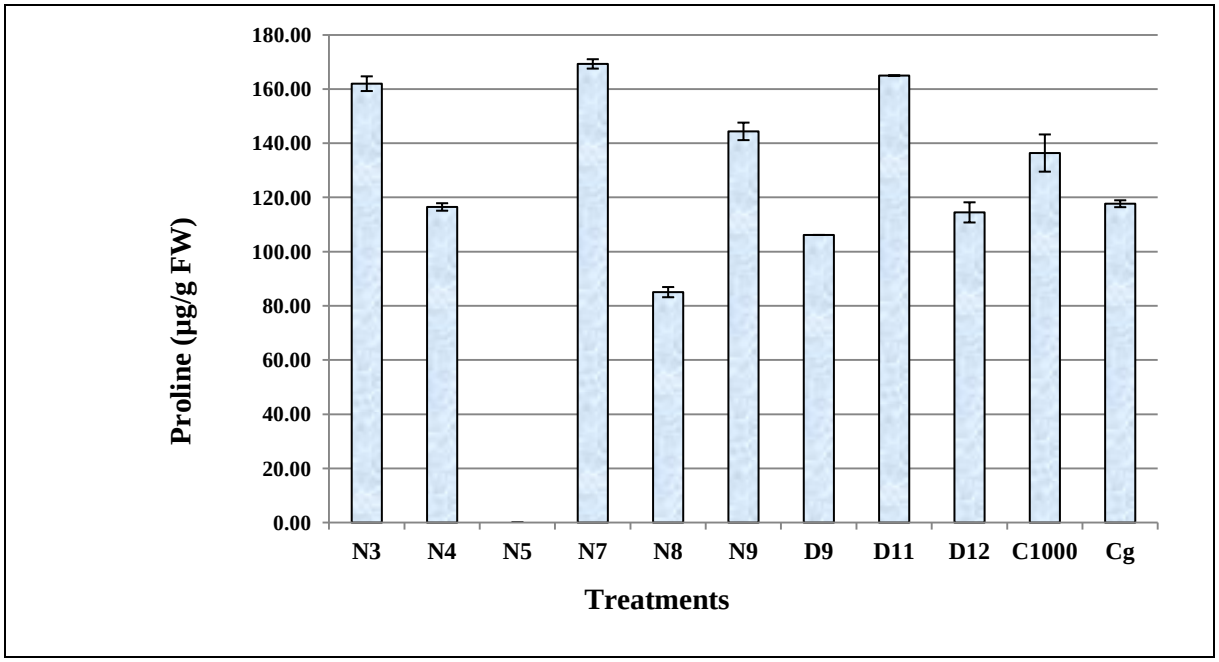


Fig. 5. Effect of mutagen concentrations plus soaking duration on proline content of ex vitro Grand Nain banana plant under salinity level at 1000 ppm.

3. Genetic variations:

A. PCR-ISSR:

Inter simple sequence repeat (ISSR)-PCR is a technique, which involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate multilocus markers. It is a simple and quick method that combines most of the advantages of microsatellites (SSRs) and amplified fragment length polymorphism (AFLP) to the universality of random amplified polymorphic DNA (RAPD). ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology Reddy *et al.*[43]Lamare and Rao[44] reported that ISSR is effective technique for the analysis of substantial genetic variability and the genetic relationship within *M. acuminata*.

In this study samples of Grand Nain banana cv. which have been exposed during *in vitro* multiplication stage to different treatments [C (untreated), N1 (200 µM of DES for 30 min under salinity level at 1000 ppm) and N3 (200 µM of DES for 10 min under salinity level at 1000 ppm)] were amplified with 4 primers (UBC-810, UBC-818, UBC-843 and UBC-850) as shown in Table (7).

Table 7. Selected inter-simple sequence repeats (ISSR) primers used in this study.

Primer	Sequence	
UBC-810	**$(GA)_8$ T**	GAGAGAGAGAGAGAGAT
UBC-818	**$(CA)_8$ G**	CACACACACACACACAG
UBC-843	**$(CT)_8$ RA**	CTCTCTCTCTCTCTCTRA
UBC-850	**$(GT)_8$ CA**	GTGTGTGTGTGTGTGTCA

The number of products generated per primer was found to range from 9 to 12 of different sizes in the range of 0.5 to 7.5 kb (Fig. 6). The primers UBC-810 and UBC-850 exhibited the maximum product and produced 12 bands for each one, where as primer UBC-843 gave the least number of products and recorded only 9 bands. A total of 43 amplified products were produced with an average of 10.75 products per primer, of which 12 (27.91 %) were polymorphic in nature and 31 (72.09 %) products were monomorphic (Table, 8).

The percentage of polymorphic bands ranged from 22.22 % for primers UBC-843, 25.00 % for primer UBC-850, 30.00 % for primer UBC-818 to 33.33 % for primers UBC-810 with an average polymorphism of 27.64% per primer. Such results showed – to some extend – a genetic variation among the studied treatments, all primers proved their effectiveness in showing polymorphism between these treatments.

The polymorphism of the four ISSR primers used to distinguish between the two banana mutants which revealed a high tolerance of NaCl stress (N1 and N3) is very obvious. Data showed that UBC-818 generates a polymorphic band in ≈ 4.5 kb, 850bp and 500bp in N1 sample not present in N3 (Fig 6). Same situation for UBC-850 primers which generate two polymorphic bands for N3 (2kb, 300bp) and only one band specific for N3 1.5kb (Fig 6). On the other hand, other two primers UBC-810 and UBC-843 did not generate any polymorphic bands between the mutants, still those two primers confirm the similarity between them Fig (6). Using ISSR technique alone is not convenient to characterize our selected mutants under salt stress condition. We need more validation experiments to analyzed those mutants specially the chromosome mutation location also identifies the phenotypic and genotypic characterization for N1 and N3.

A lot of studies confirmed that the micropropagation of banana up to the eighth subculture generation without adding any mutagens show low variation, in this regard Borse *et al*[45]reported that on banana (*Musa acuminata*. Grand Nain cv.) both types of ISSR and REMAP markers revealed high degree of monomorphism and very low variation was observed up to the eighth subculture generation with polymorphic bands being low in both ISSR (0.96%) and REMAP (0.95%) markers. But in this study the polymorphic percentage reaches to 33.33% by using DES mutagen.

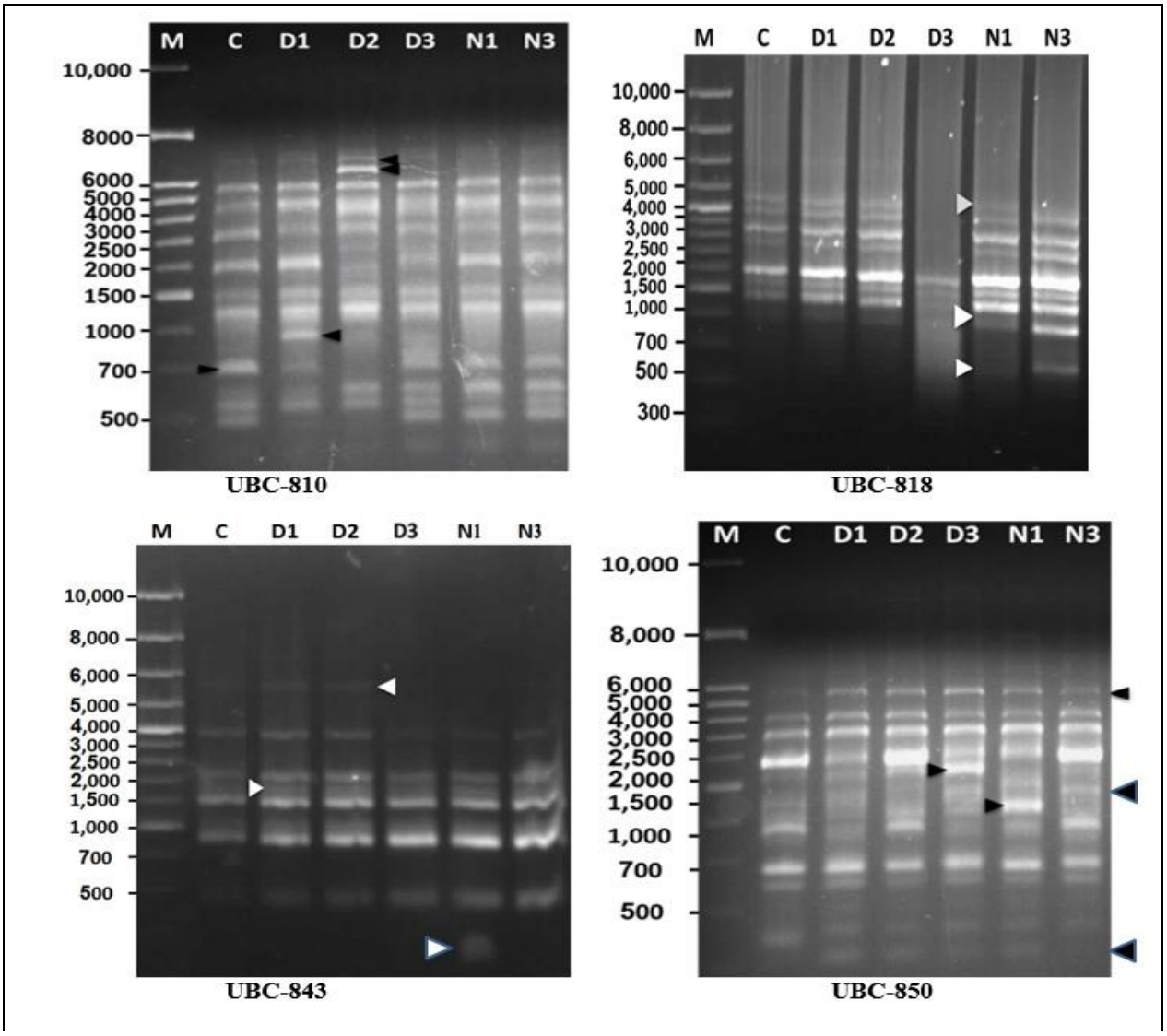


Fig. 6. Inter-simple sequence repeats (ISSR) banding patterns amplification using UBC-810, UBC-818, UBC-843 and UBC-850 primers of mutagenic banana transplants under salt stress; C (untreated) N1 (200 µM of DES for 30 min under salinity level at 1000 ppm) and N3 (200 µM of DES for 10 min under salinity level at 1000 ppm).

Table 8. Selected inter-simple sequence repeats (ISSR) primers used to detect number of polymorphic bands (P.B.), number of monomorphic bands M.B., total number of bands (T.B.) and percentage of polymorphism bands (P.P.B.).

Primer	Sequence	P.B	M.B	T.B	P.P.B (%)
UBC-810	**$(GA)_8$ T**	4	8	12	33.33
UBC-818	**$(CA)_8$ G**	3	7	10	30.00
UBC-843	**$(CT)_8$ RA**	2	7	9	22.22
UBC-850	**$(GT)_8$ CA**	3	9	12	25.00
Total		12	31	43	27.64

B. Protein SDS-PAGE:

Denatured polyacrylamide gel electrophoresis was employed to assess the differences of protein pattern in the experimental samples. In SDS-PAGE analysis, a total of 15 protein bands were determined (Fig. 7). The result of SDS-PAGE analysis revealed that the absence and/or presence of bands were also observed in some treatments.

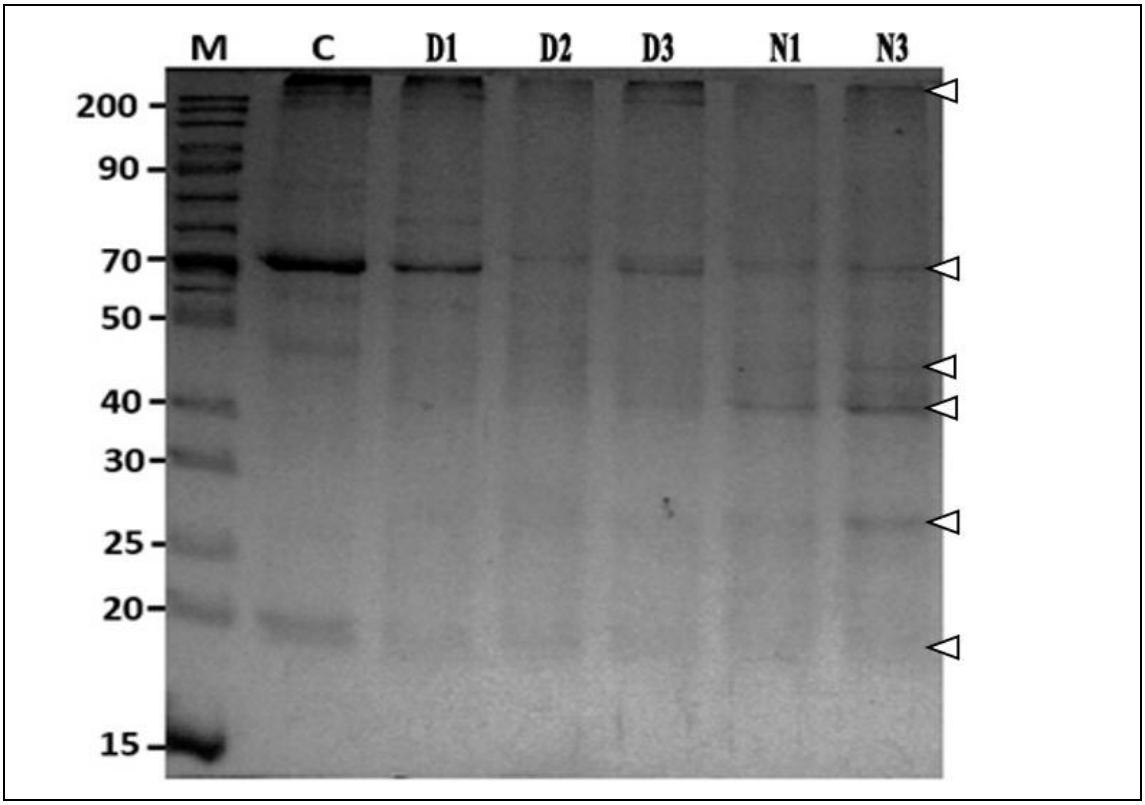


Fig. 7. SDS-PAGE of banana treated with C (control), N1 (200 µM of DES for 30 min under salinity level at 1000 ppm) and N3 (200 µM of DES for 10 min under salinity level at 1000 ppm).

Clear differences pattern in protein changes was seen between treated banana with different mutagens under salinity stress on the polyacrylamide gels from presence or absence bands to varied intensity of expression. A 70 kDa protein band in control plants showed more intensity than other treatments. While A 40 kDa protein band in N3 (200 µM of DES for 10 min under salinity level at 1000 ppm) showed more intensity than other treatments .According to the control sample we can clearly see three bands (≈ 45, 40, 27 kDa) presented in N3 sample and did not detect in the control in spite of salt stress treatment which generally decreased the total soluble proteins in plant Abdul Qados[46]. N1 sample also have the same profile except for 40 kDa band to differ from the un-mutated sample. The low molecular weight protein bands also have been decreased because of the salt stress and their sensitivity for degradation under stress conditions in both samples N1 and N3. SDS profile data for the mutated samples by 200 µM of DES reviled quite difference between them against salt stress those protein bands which distinguish between N1 and N3 total soluble protein profile could be related to photosynthesis, energy metabolism and defense mechanism.

Conclusion

From the result, it can concluded that DES at 100 and 200µM for different durations (10 to 30 min) and at 400 µM for 10 to 20 min only could be used to reduce the negative effects of salinity (1500 ppm) on ex vitro produced banana plants during acclimatization stage, and this study could be a base to use diluted sea water in irrigating banana plants produced from in vitro culture and treated with DES mutagens.

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