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Research Article

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Genotypic of salt stressed sunflower *(Helianthus annuus)*

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ABSTRACT

Sunflower (Helianthus annuus L.), the family Asteraceae and the genus Helianthus, the last few years, production of sunflower has increased many fold due to the expansion of its cultivation in several parts of the world, it is the most important oilseed crop, where salinity is an increasing problem. Sunflower is moderately sensitivity. The physiological results showed that the effect of sodium chloride was significant and highly significant decreased gradually for all varieties and all treatments, in shoots lengths where the highest proportion registered in Sakha 53 which decreased 22.87 cm from the control to 250 mM, and leaves area where the lack reached a peak in Gisa 102 with 28.25 cm^2 from control to 250 mM. Concerning the biochemical effect, the proline contents in leaves were significant and highly significant increased gradually with application of the salt, recorded the highest value in Giza 102 variety with 0.533 mg/g/F weight at 250 mM, A total of seven ISSR primers (UBC-10, UBC-12, UBC-13, UBC-25, UBC-34, UBC-35 and UBC-40) were tested on Sakha 53, Giza 102, Abendsoone and Teddy bear, and showed (62.5%) of polymorphism for untreated varieties, while primer (UBC-40) given (80%), for untreated with treated varieties with different concentrations of sodium chloride (100 mM, 200 mM and 250 mM) diminished (54.05%). Cluster analysis showed higher similarity matrix between the Egyptian varieties Sakha 53 and Giza 102 (84.9%), similar between Abendsoone and Teddy bear (75.6%). The effect of salt were significant with appearance of bands in treated varieties not founded in the control and disappearance of bands from all treated varieties approximately.

***Key words**: Sunflower, Salinity, sodium chloride, ISSR , varieties.*

INTRODUCTION

Plants all over the world are subjected to multitude of stresses throughout its growth period. The major environmental factor that currently reduces plant productivity is salinity[24] . Effects of salinity are more obvious in arid and semi-arid regions where limited rainfall, high evapo-transpiration and high temperature associated with poor water and soil management practices are the major contributing factors[3]. Hence it is considered as an important abiotic stress factor seriously affecting plant productivity and survival in most of the soils[10]. Sunflower is moderately sensitive to soil salinity, where it can tolerate salinity up to EC equal to 1.7 dsm-1. The promotion of sunflower could be successful to increase the domestic production provided proper cultivars are available which are suitable to different soil and climatic conditions[19]. Inter Simple Sequence Repeats (ISSR) based on PCR amplification, have been widely used for population genetic studies of various plant species, comprise short oligonucleotide sequences, two to six bases long, repeated in tandem array, which occur very frequently in eukaryotic genomes[5,21]. ISSR was also found better than RAPD to detect genetic diversity among barley accessions[16]. The present study aim is to screening of salt stress effects on Sunflower *(Helianthus annuus*) varieties and assessing the genetic diversity molecular marker system to study the genetic patterns with providing

data applicable to conservation and breeding uses, also evaluating the genotypic variation of sunflower by analyzing Inter-simple sequence repeat (ISSR) under various salt stress response.

Plant material and salt treatment:

Healthy seeds of six varieties of *Helianthus annuus L* were used in this study: Sakha 53, Giza 102, Abendsonne, Teddy bear, Gigante and White Hassawi, taken from different sources, exposed to five concentrations of sodium chloride: 50 mM ,100 mM, 150 mM,200 mM, 250 mM.

Biochemical substances:

Proline:

Proline was assayed according to the method described by Bates *et al.*[4]. Fresh leaves (0.5 g) were homogenized in 10 ml of 30% aqueous sulphosalicylic acid. The homogenate was centrifuged at 9000 × g for 15 min. A 2 ml aliquot of the supernatant was mixed with an equal volume of acetic acid and acid ninhydrin and incubated for 1 h at 100 °C. The reaction was terminated in an ice bath and extracted with 4 ml of toluene. The extract was vortexed for 20 s and the chromatophore-containing toluene was aspirated from the aqueous phase and absorbance determined photometrically at 520 nm on Spectrophotometer for reading result, using toluene for a blank. Calibration curve was prepared from L-proline of different concentration to calculate the proline content in samples, treated similar to that of aliquots, as described above. The amount of proline was expressed as mg/ g$^{/}$ fresh weight.

DNA analysis:

DNA extraction:

DNA was isolated from seedlings using CTAB by method Doyle and Doyle[9] modified by Wittzell[39]. Leaves were cleaned raised from the soil and impurities and grinded o.5 g very finely in liquid nitrogen with a mortar and a pestle pre-chilled. The young seedlings were ground into 4 ml extraction buffer (10 ml tris buffer (pH 8,25), 1.25 ml EDTA, 11.7 g NaCl, 3g CTAB, 5g PVP, 4 ml mercaptoethanol) completed to 100 with DDW then 300 µl SDS, after 10 mg/ml RNase with order and careful do not mixed them before addition mix well with vortex and the suspension was gently mixed and incubated at 65°C for 20 min with occasional mixing, then cooled to room temperature and an equal volume of chloroform:isoamyl alcohol (24:1) was added. The mixture was centrifuged at 12,000 rpm for 5 min. The clear upper aqueous phase was then transferred to a new tube and 3 ml of ice-cooled isopropanol was added and incubated at -20°C for overnight. After the nucleic acid was collected by centrifugation at 10,000 rpm for 10 min. The resulting pellet was washed twice with 80% ethanol and air-dried under a sterile laminar hood. Finally the nucleic acid was dissolved in TE (10 mM Tris buffer pH 8, 1 mM EDTA) at room temperature and stored at 4°C until used.

PCR amplification by ISSR analysis:

PCR amplification was done with ISSR primers according to the protocol developed by Zeitkiewicz *et al*[40]. Seventeen ISSR primers (UBC10, UBC 11, UBC 12, UBC 13, UBC 18, UBC 22,UBC 23, UBC 24, UBC 25, UBC 30, UBC 34, UBC 35, UBC 36, UBC 40, UBC 41, UBC 44 and UBC 50) were used in this study to amplify the genomic DNA extracted from untreated and treated seedlings. Seven of them gave as a result nucleotide sequencing according to the (Table 1).

PCR reaction was carried out of 1 bead from PCR beads (assay buffer, taq polymerase, dNTP),20 µl of deionized sterile water, 25 ng (2 µl) DNA dilution (5µl DNA extraction + 50µl DDW) and 10 pm (2.5 µl) of primer (6.65µl primer + 93.35 DDW) a total volume 25 µl were added in each tube. Tubes were vortexed and briefly centrifuged after adding template DNA and primer in master mixture. The amplification was done on 96 well plates on a Primus PCR machine, as per the programme: initial denaturation at 94°C for 5 min, followed by 40 cycles for 1min at 94°C, 1min 45°C for 1min, 1.30 min 72°C, a final extension for 7 min at 72°C, and 4°C was performed for amplification.

Agarose gel electrophoresis:

After completion of the amplification process of DNA, separation process was conducted on agarose gel concentration of 1.3 % with (1.3 g Agarose + 50 ml DDW + 900µl TAE 1X) putet into a glass beaker, packaged with aluminium foil flask and placing in the microwave until boiling, then outed and leaved to

cool slightly. Ethidium bromide (2-5 µl) were added before freezing and Shacked manually then asting in a mold (Gel cast) after comb is put in place right and left even hardened gel. Samples processing to take (8 µl) of each sample and added to (2 µl) of the dye solution (bromophenol blue) and injected them into wells formed by comb and filling up electric device thank with (1X TAE) solution. Separation process was conducted using an electric current strongly (80 - 100 V), samples began move from the cathode to the anode until reached the end of the template gel After the passage of time from 2 to 3 hours after, separated the electrical from the horizontal slab and removing the gel, after gel were transferred to the imaging device. Statistical analysis was conducted with MVSP 3.1 (Multi Variate Statistical Package) programme for protein and DNA (http://www.kovcomp.com/mvsp). Bands were scored visually and recorded as presence (1) or absence (0) of bands. Similarity coefficients were used to construct the dendrogram using the unweighted pair group method with arithmetic means (UPGMA)

Table 1: **List of ISSR primers sequences**

N0.	Primer	Primer sequences (5'-3')
1	UBC-10	GAGAGAGAGAGAGAGAT
2	UBC-12	GAGAGAGAGAGAGAGAA
3	UBC-13	CTCTCTCTCTCTCTCTT
4	UBC-25	ACACACACACACACACT
5	UBC-34	AGAGAGAGAGAGAGAGYT
6	UBC-35	AGAGAGAGAGAGAGAGYC
7	UBC-40	GAGAGAGAGAGAGAGAYT

A: Adenine, T: Thymine, G: Guanine, C: Cytosine, Y: (C, T) [34]

RESULTS

Growth measurement:

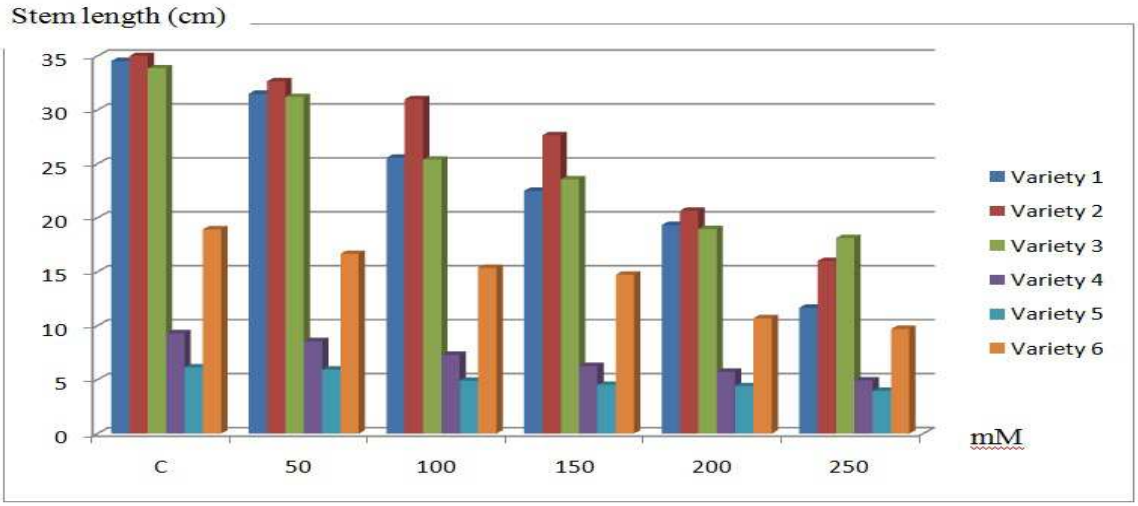
Shoot length:

Sodium chloride induced a slight decrease in the shoot length (Figure 1). Plant shoot at (100 mM, 150 mM, 200 mM, 250 mM) was highly significant in Sakha 53which decreased 22.87 cm from the control to 250 mM of treatment (Table 2), which recorded the highest proportion affected in shoot length compared to the rest of varieties, whereas in Giza 102 significant at (150 mM) and highly significant at (200 mM, 250 mM) decreased 19 cm from the control to the high concentration of salt, in Abendsoone variety it was significant from 100mM to 250 mM while it recorded 33.86 cm in the control, 31. 20 cm at 50 mM, 25.40 cm at 100 mM, 23.56 cm at 150 mM, 18,96 at 200 mM and 18.13 mM at 250 mM decreased 15.73 cm from the control to the last concentrations, for Teddy bear which is a short variety in the general form, we recorded 9.30 cm in the control decreased gradually till it reached 4.93 cm, concentrations 150 mM and 250 mM were significant while 250 mM was highly significant but in Gigante variety did not show a big difference between the control and the last concentrations witch recorded 2.17 cm and it was significant just in 250 mM, in white Hassawi it was significant in al concentrations and at 200 mM and 250 mM was highly significant with difference of 9.23 cm from the control to 250 mM. Figure (3.4.5.6.7.8) showed varieties after treatment .

Table (2): Shoot length (cm)

Treatments Mm	Parameter (Mean ± SD)					
	Variety					
	Sakha 53	Giza 102	Abendsoone	Teddy bear	Gigante	Hassawi
C	34.53 ± 2.837	35.00 ± 1.732	33.86 ± 2.150	9.30 ± 0.300	6.13 ± 1.457	18.93± 1.193
50	31.50 ± 1.322	32.66 ± 1.607	31.20 ± 5.980	8.56 ± 0.351	5.93 ± 1.250	16.66 ± 1.040*
100	25.56 ± 0.404***	31.00 ± 3.464	25.40 ± 2.535*	7.30 ± 1.664	4.90 ± 0.100	15.36 ± 0.808**
150	22.50 ± 1.802***	27.66 ± 0.763**	23.56 ± 4.523*	6.26 ± 0.896**	4.53 ± 0.404	14.73 ± 0.472**
200	19.33 ± 0.577***	20.66 ± 0.577***	18.96 ± 1.193**	5.73 ± 0.321**	4.40 ± 0.264	10.70 ± 0.721***
250	11.66 ± 1.154***	16.00 ± 1.000***	18.13 ± 0.776**	4.93 ± 0.585***	3.96 ± 0.208*	9.70 ± 1.058***
LSD at 5 %	2.802	3.207	3.629	1.494	1.447	1.627
LSD at 1 %	3.928	4.493	5.086	2.095	2.028	2.379

Figure (1): Effect of salt on shoot length



Leaf area:

The data presented in Table (3) and Figure (2) indicated that the used of salt had affected *Helianthus annuus L*. plant leaf area, where it was significant in Sakha53 variety from 100 mM to 250 mM when we recorded 25.65 cm^2 in the control decreased till reached 7.60 cm^2 in the last concentrations with the difference of 18.05 cm^2 while in Giza 102 it was highly significant ($p<0.01$) from 100 mM to 250 mM where the leaf area was in the Control 32.53 cm^2, 26.07 cm^2 at 50 mM, 12.98 cm^2at 100 mM, 8.90 cm^2 at 150 mM, 6.22 cm^2 at 200 mM, 4.28 cm^2 at 250 mM, we noted that it decreased 6.46 cm^2 from the first treatment and 28.25 cm^2 from then control to the last treatment whereas in Abendsoone variety we recorded 28.13 cm^2 in the control decreased gradually until reached 9.73 cm^2 while it was significant at 150 mM to 250 mM, for Teddy bear variety the effect of salt was highly significant at 150 mM, 200 mM, 250 mM and significant at 100mM, the leaves are the largest area compared to the rest of varieties with 35.56 cm^2 registered in the control reached to 15.33 cm^2 in the last treatment but we don't noted the big difference between the control and the 250 mM in Gigante variety which was 2 cm^2 and non significant whereas in White Hassawi it was significant in all treatment while we recorded 10.56 cm^2 of difference

from the control till the last concentrations, Lack of leaf area reached a peak in Gisa 102 with 28.25 cm^2 from control to 250 mM .

Table (3): Leaf area (cm^2)

Treatments Mm	Parameter (Mean ± SD)					
	Variety					
	Sakha 53	Giza 102	Abendsoone	Teddy bear	Gigante	Hassawi
C	25.65 ± 5.690	32.53 ± 3.633	28.13 ± 8.732	35.56 ± 0.513	5.56 ± 1.050	15.76 ± 2.538
50	19.96 ± 4.763	26.07 ± 0.922*	18.70 ± 4.107	29.60 ± 4.103	6.20 ± 1.276	10.53 ± 1.700*
100	12.15 ± 4.448*	12.98 ± 1.644***	22.63 ± 4.481	23.70 ± 1.452*	4.20 ± 1.178	10.06 ±1.365 *
150	10.01 ± 0.971**	8.90 ± 2.482***	15.03 ± 7.219*	15.83 ± 3.803***	3.96 ± 1.401	7.33 ± 1.322*
200	9.96 ± 2.878**	6.22 ± 2.489***	13.56 ± 3.308*	16.10 ± 3.151***	3.03 ± 1.446	5.76 ± 1.850**
250	7.60 ± 2.589**	4.28 ± 0.715***	9.73 ± 0.763*	15.33 ± 5.200***	3.56 ± 1.510	5.20 ± 3.516**
LSD at 5 %	4.031	3.953	9.657	6.105	2.095	3.893
LSD at 1 %	5.651	5.541	13.539	8.557	2.935	5.456

Figure (2): Effect of salt on leaf area

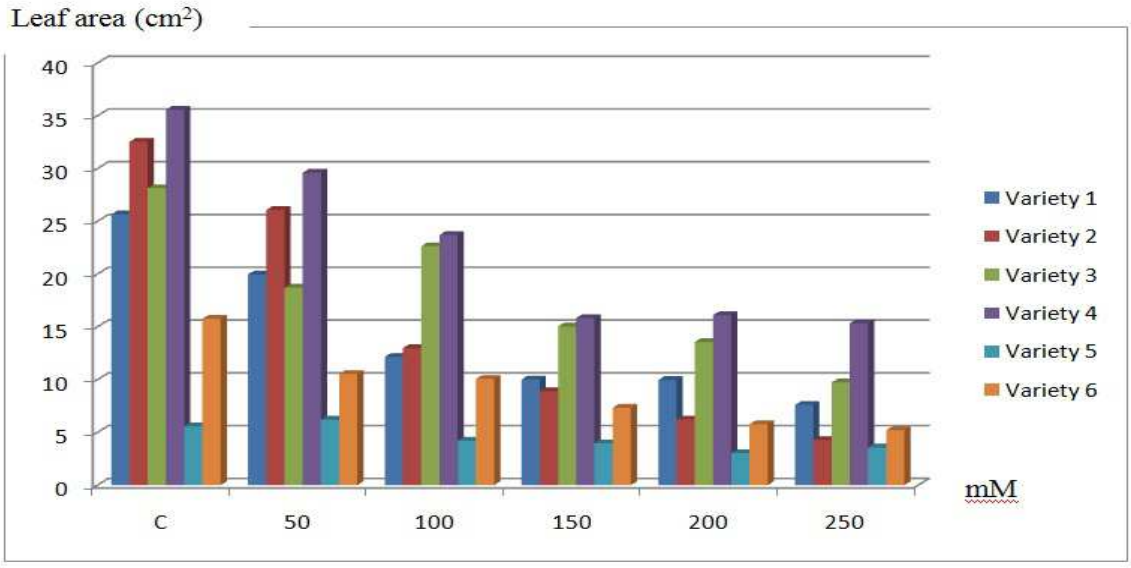


Figure (3): Sakha 53 variety after treatment

1C	**1 (150 mM)**
	
1 (50 mM)	**1 (200 mM)**
	
1(100 mM)	**1 (250 mM)**
	

Figure (4): Giza 102 variety after treatment

2C	**2 (150 mM)**
	
2 (50 mM)	**2 (200 mM)**
	
2(100 mM)	**2(250 mM)**
	

Figure (5): Abendsoone variety after treatment

3C	3 (150 mM)
	
3 (50 mM)	**3 (200 mM)**
	
3(100 mM)	**3(250 mM)**
	

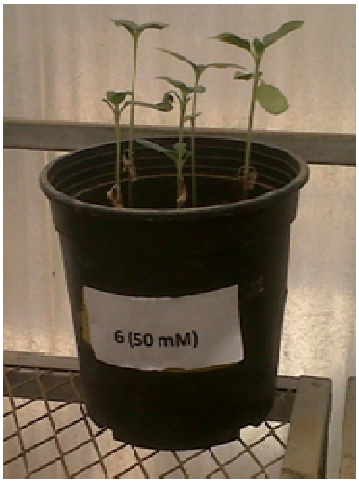
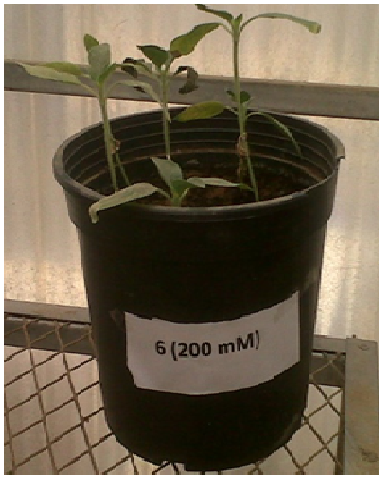
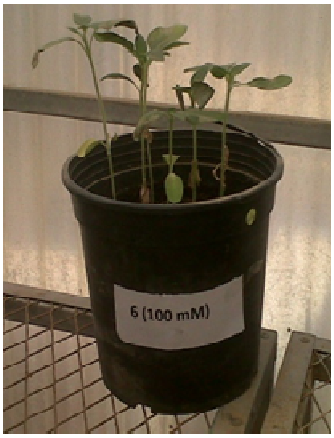
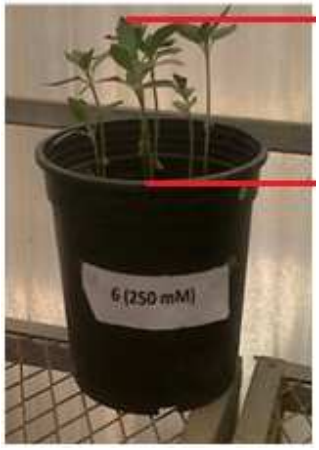
Figure (6) : Teddy bear variety after treatment

4C	**4 (150 mM)**
	
4 (50 mM)	**4 (200 mM)**
	
4(100 mM)	**4 (250 mM)**
	

Figure (7): Gigante variety after treatment

5 C	**5 (150 mM)**
	
5 (50 mM)	**5 (200 mM)**
	
5 (100 mM)	**5 (250 mM)**
	

Figure (8): White Hassawi variety after treatment

6 C	**6 (150 mM)**
	
6 (50 mM)	**6 (200 mM)**
	
6 (100 mM)	**6 (250 mM)**
	

Biochemical results:

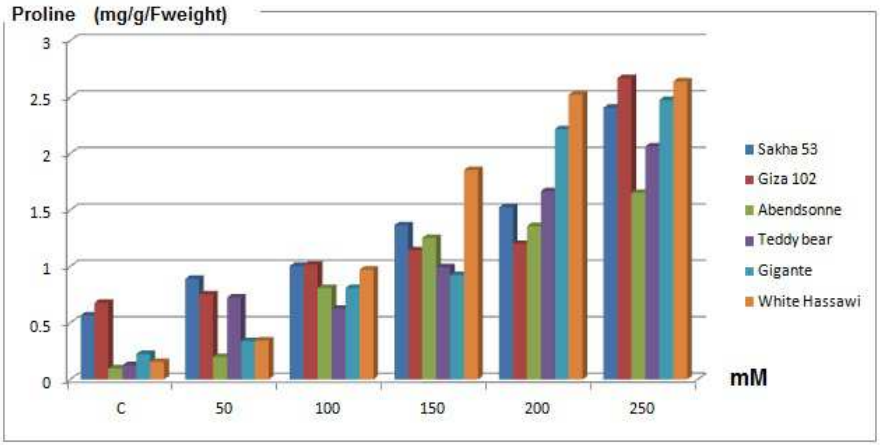
Proline:

It is clear from the results that the contents of proline increased gradually in all varieties from control to the last concentration of salt in leaves (Figure 9), it was significant in Sakha 53 at 150 mM, 200 mM and highly significant in 250 mM increased gradually from the control where recorded 0.567 mg/g/F weight value to the last concentrations which was 2.407 mg/g/F weight, similar in Giza 102 when registered 0.678 mg/g/F weight in the control gradually increased until it reached 2.668 mg/g/F weight at 250 mM which was highly significant value, While in Abendsoone variety the effect of salt on proline content was significant at 100 mM, 150 mM, 200 mM and highly significant at 250 mM when recorded 0.097 mg/g/F weight in the control and 1.652 mg/g/F weight at 250 mM, for Teddy bear variety registered 0.125 mg/g/F weight in the control, 0.725 mg/g/F weight at 50 mM, 0.626 mg/g/F weight at 100 mM, 0.995 mg/g/F weight at 150 mM (significant value), 1.667 mg/g/F weight at 200 mM (significant value) and 2.063 mg/g/F weight at 250 mM which was highly significant, similar in Gigante variety when noted 0.225 mg/g/F weight in the control increased gradually till reached 2.474 mg/g/F weight, values were significant at 150 mM, 200 mM and highly signifant at 250 mM , while in White Hassawi variety it was significant at 100 mM and highly significant at 150 mM, 200 mM and 250 mM Recorded the highest value of proline in Giza 102 variety with 2.668 mg/g/F weight in the last treatment, also al values in al varieties were highly significant at 250 mM (Table 4).

Table (4): Proline content (mg/g/F weight)

Treatments Mm	Parameter (Mean ± SD)					
	Variety					
	Sakha 53	Giza 102	Abendsoone	Teddy bear	Gigante	White Hassawi
C	0.567 ± 0.408	0.678 ± 0.120	0.097 ± 0.054	0.125 ± 0.041	0.225 ± 0.064	0.154 ± 0.169
50	0.890 ± 0.198	0.754 ± 0.423	0.198 ± 0.067	0.725 ± 0.486	0.388 ± 0.275	0.344 ± 0.425
100	1.005 ± 0.407	1.016 ± 0.537	0.810 ± 0.315*	0.626 ± 0.248	0.810 ± 0.622	0.972 ± 0.423*
150	1.366 ± 0.257*	1.144 ± 0.618	1.253 ± 0.321**	0.995 ±0.395*	0.924 ± 0.703*	1.854 ± 0.413***
200	1.524 ± 0.494*	1.200 ± 0.106	1.357 ± 0.338**	1.667 ± 0.532**	2.217 ± 0.446***	2.522 ± 0.094 ***
250	2.407 ± 0.613***	2.668 ± 0.362***	1.652 ± 0.761***	2.063 ± 0.637***	2.474 ± 0.342***	2.639 ± 0.310***
LSD at 5 %	0.748	0.729	0.691	0.777	0.821	0.592
LSD at 1 %	1.047	1.020	0.968	1.087	1.148	0.830

Fig (9): Effect of salt on proline content



Molecular analysis:

The study was limited to just four species which are Sakha 53, Giza 102, Abendsoone and Teddy bear, due to the lack of clarity of packages in two others varieties (Gigante and Wight Hassawi) Figure (12) and (15).

Comparison between untreated varieties:

Figures (10), (11), (12), (13), (14), (15) and (16) showed gel sliced for ISSR primers that gave clear segments, ranging from 300 to 10000 bp in size. Which gave primer UBC10 (8) bands, (11) bands in UBC12 whereas in UBC13 (9) bands, for UBC25 (7) bands, while (6) bands in UBC34, (8) bands in UBC35, finally (10) bands in UBC40. The total number of bands resulting from 7 primers for four varieties 59 bands, matched 22 genetic bands while the rest of the bands varied the number of 37 bands. The percentage of polymorphic bands is 62.50 %. The highest percentage of polymorphic bands (80%) was obtained for primer UBC40 and lowest percentage (50%) for primer UBC35 Table (5).

Evidenced by the percentage of similarity matrix of the results of ISSR (Table 6) that the genetic distance between varieties ranged between (0.462) to (0.849) Table 6. Where the Egyptian genotypes (Sakha 53 and Giza 102) recorded the highest rate of convergence by the similarity of (0.849) however the similarity matrix in (Abendsoone and Teddy bear) were relatively high estimated of (0.756) while the lowest similarity matrix recorded between (Sakha53 and Abendsoone) and between these values estimated other genotypes.

The dendrogram Figure (17) obtained using Jaccard's Coefficient has one main cluster branched to two principal clusters. The first cluster included Sakha 53 and Giza 102 varieties by genetic similarity of (84.9%), the second cluster included Abendsoone and Teddy bear varieties by genetic similarity of (75.6 %).

Total results:

Figures from (10) to (16) showed gel sliced for ISSR primers, which gave primer UBC10 (27) bands, (33) bands in UBC12,whereas in UBC13 (32) bands, for UBC25 (27), similarity in UBC34, while (32) bands in UBC35, finally in UBC40 (28) bands. From this the total number of bands resulting from 7 primers for four variety with treatments are 206 bands, matched 96 genetic bands while the rest of the bands varied the number of 110 bands. The percentage of polymorphic bands were 54.05 %. Table (7) showed the total number of bands, number of polymorphic bands, number of monomorphic bands for each primer, percentage of polymorphic bands.

The total dendrogram Figure (18) obtained using Jaccard's Coefficient has one main cluster. The first cluster included Abendsoone and Teddy bear varieties by genetic similarity of 75.6 % Table 8. The second cluster separated in a single branch by 250 mM concentrations and a cluster with 200 mM treatments in a single branch, while the other branch divided on subcluster included Sakha 53 and Giza 102 varieties by genetic similarity of 84.9% and a single branch by 100 mM concentrations.

Figures from (10) to (16) showed the effect of salt with appearance of bands in treated varieties not founded in the control and disappearance of bands from all treated varieties approximately.

Figure (10) : Patterns of ISSR by primer UBC-10.

Line 4 (variety1): disappearance of band above 600 bp.Line 6-7-8 (variety 2) at 300 bp between 300-600 bp in line 6, at 900 bp. Line 3-4 under 600 bp, under 1200 bp in line 4. : New appearance.

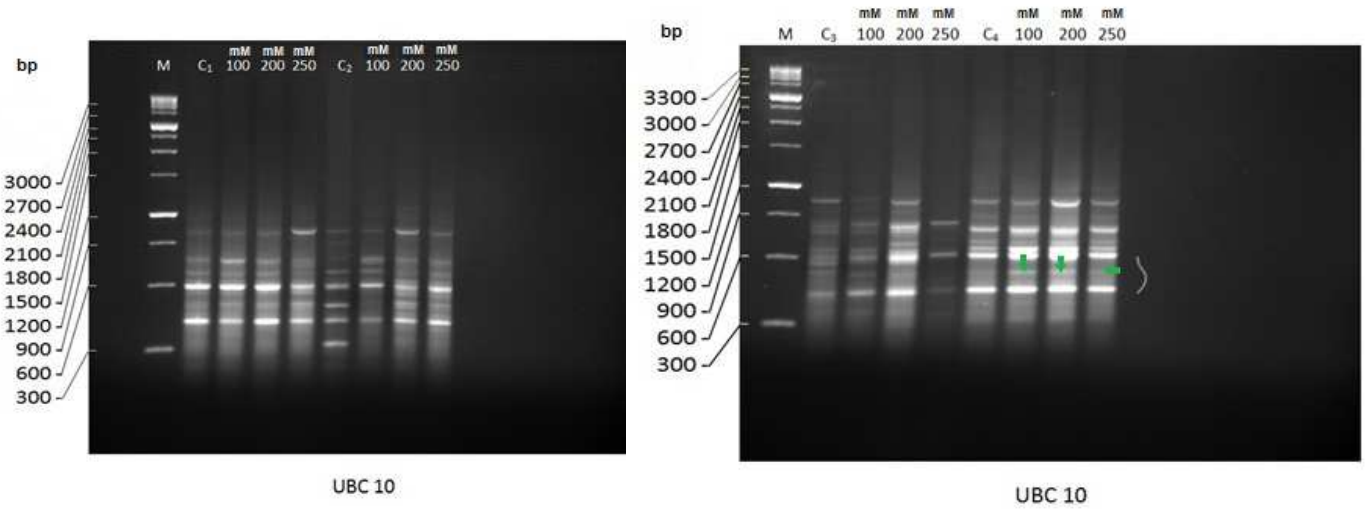


Figure (11): Patterns of ISSR by primer UBC-12.
Line 6-8 (variety 2): disappearence of bands under 1200 bp. Line 2-4 (variety 3): under 1200 bp.Line 8 (variety 4): between 600-1200 bp, ↑: New appearance)

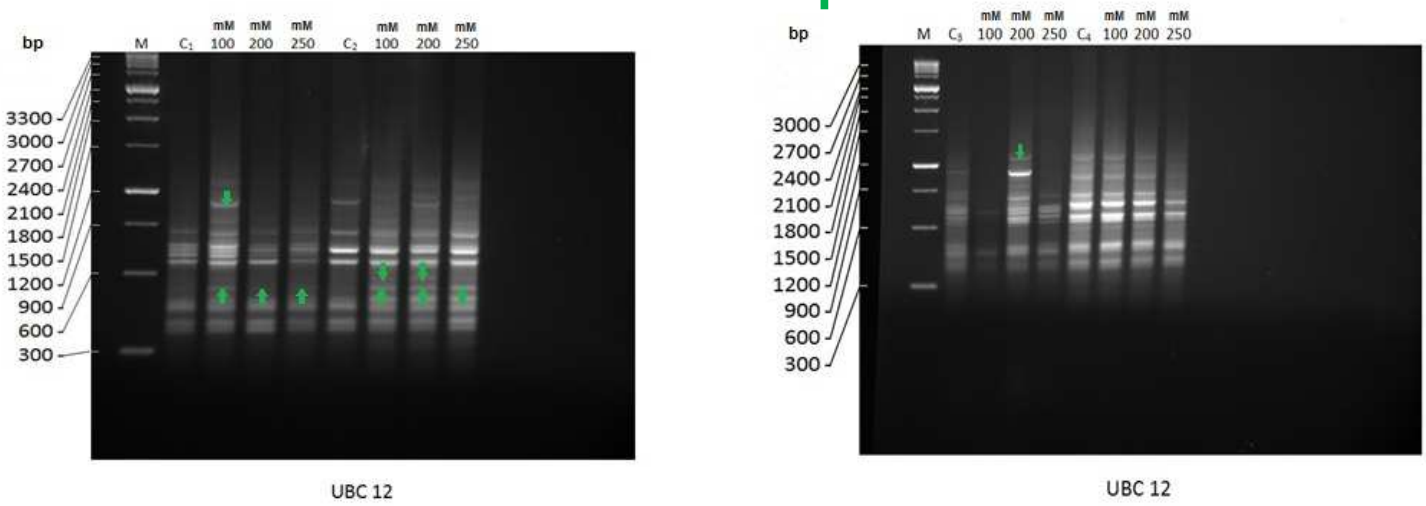


Figure (12): Patterns of ISSR by primer UBC-13.
Line 2 (variety 1):disappearance of band at 600 bp.Line 6 (variety 2): at 600 bp, ↑: New appearance.

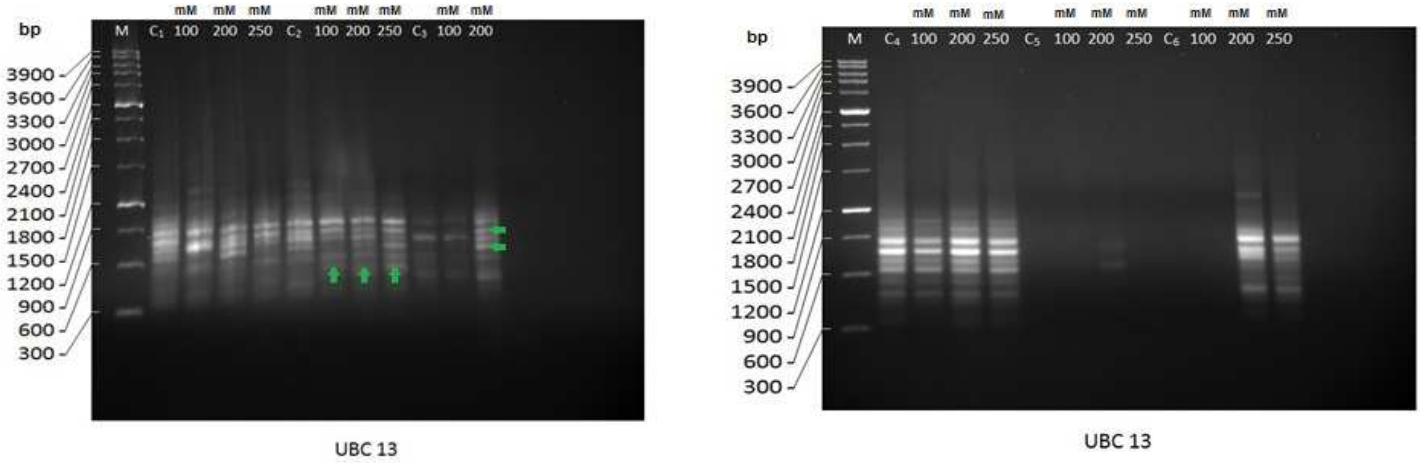


Figure (13): Patterns of ISSR by primer UBC-25.
Line 4 (variety 1):disappearance of bands between 600-1200 bp. Line 7-8 (variety 2): at 900 and 1200 bp.Line 10-11-12 (variety 3): above 600 bp in line 10-11 and 300-900 bp in line 12.

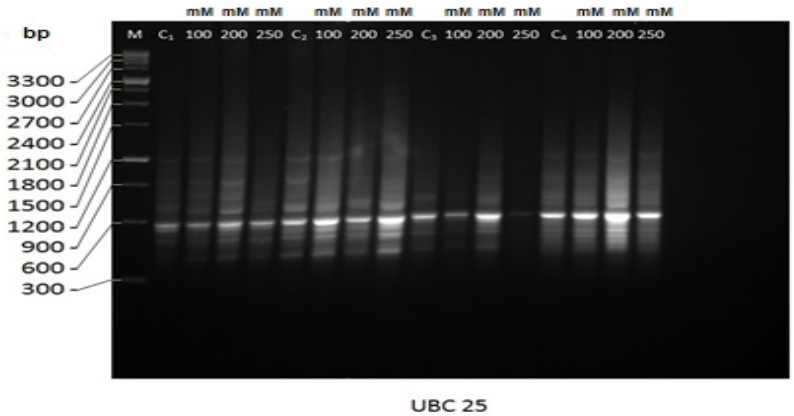


Figure (14): Patterns of ISSR by primer UBC-34.
Line 2 (variety 1): disappearance of band above 300 bp. Line 7-8: at 900 bp. Line 2-3-4 (variety 3): under 900 bp in line 2-3 and between 300-900 in line 4. Line 6 (variety 4): above 600 bp, ↑: New appearance.

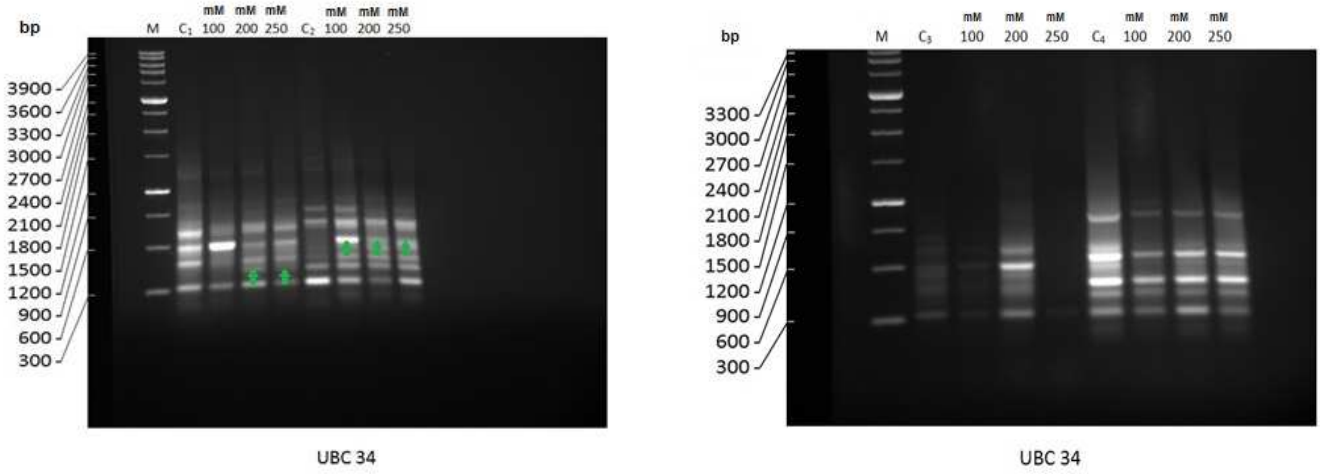


Figure (15): Patterns of ISSR by primer UBC-35.
Line 3-4 (variety 1):disappearance of bands at 900 bp, ↑: New appearance.

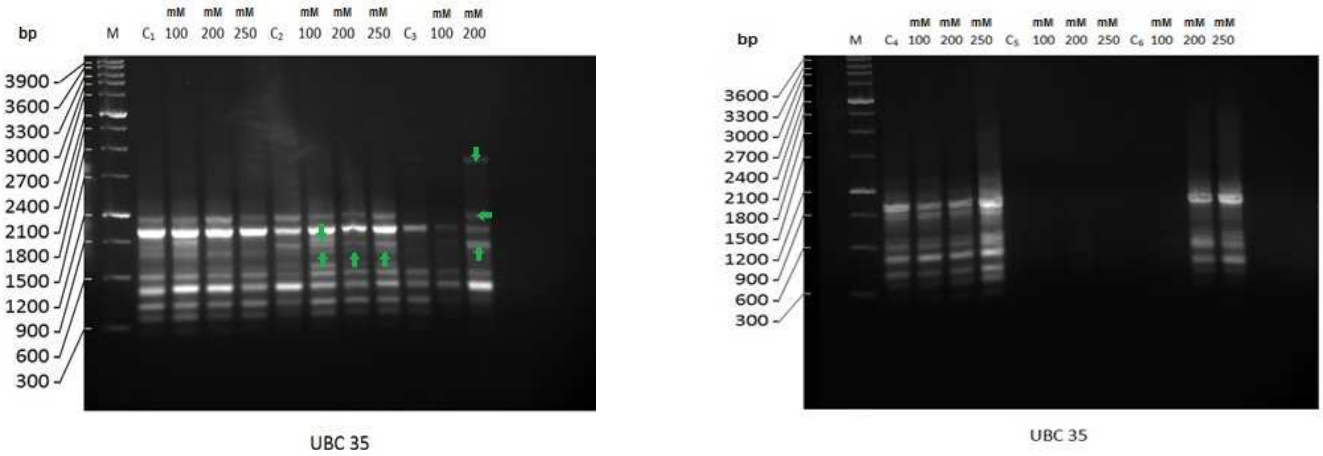


Figure (16): Patterns of ISSR by primer UBC-40.
Line 2-3-4 (variety 1): disappearance of bands between 300-900 bp. Line 6 (variety 2): between 300-600 bp. Line 4 (variety 3):between 600-900 bp, ↑: New appearance.

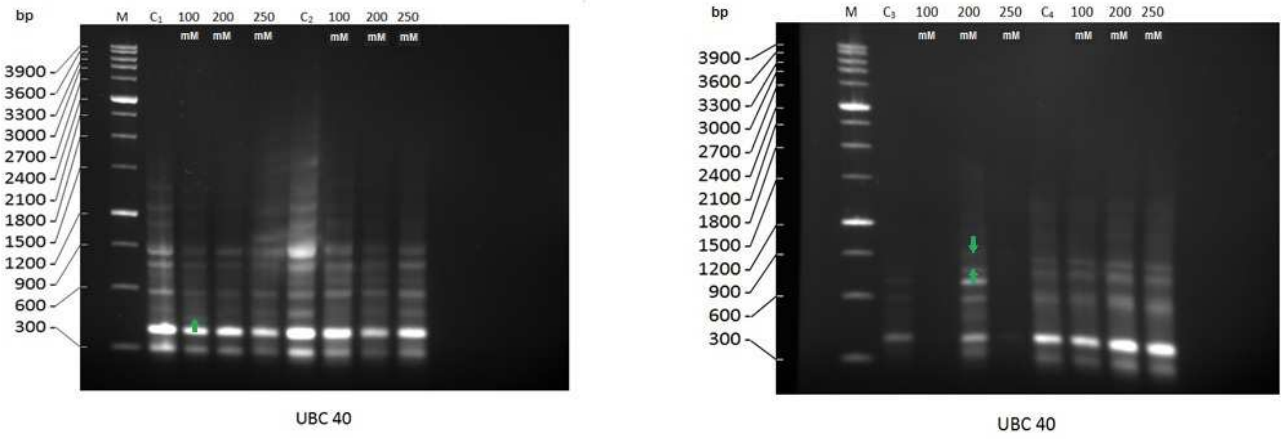


Table (5) : Results of bands from ISSR for untreated varieties

Primer Number	Primer	Total number of bands	Polymorphic Bands	Monomorphic Bands	Polymorphism %
1	UBC 10	8	5	3	62.50
2	UBC 12	11	6	5	54.54
3	UBC 13	9	6	3	66.66
4	UBC 25	7	4	3	57.14
5	UBC 34	6	4	2	66.66
6	UBC 35	8	4	4	50.00
7	UBC 40	10	8	2	80.00
Total		59	37	22	62.50

Table (6): Similarity matrix between untreated varieties (controls)

Similarity matrix	Teddy bear	Abendsoone	Giza 102	Sakha 53
Teddy bear	1			
Abendsoone	0.756	1		
Giza 102	0.525	0.586	1	
Sakha 53	0.579	0.462	0.849	1
	Teddy bear	**Abendsoone**	**Giza 102**	**Sakha 53**

Figure (17): UPGMA dendrogram of *H.annuus* for ISSR analysis (grouped untreated varieties)

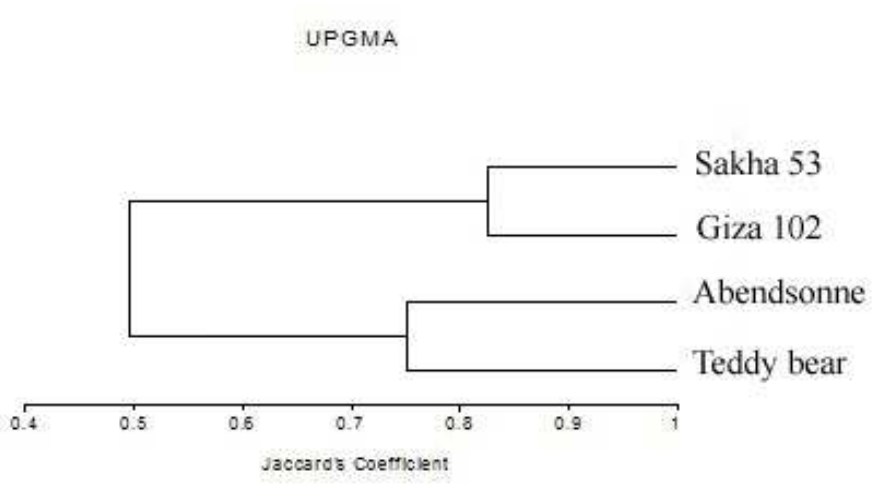


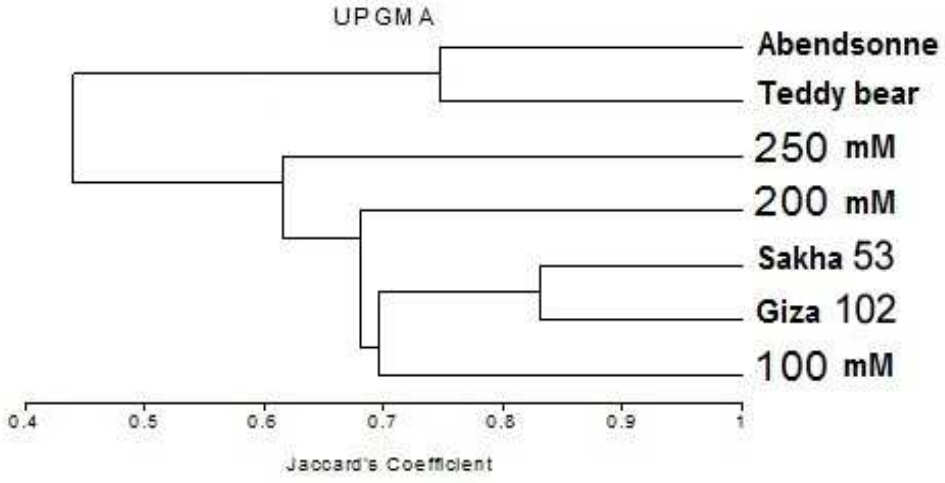
Table (7) : Total results of bands for untreated and treated varieties

Primer Number	Primer	Total number of bands	Polymorphic Bands	Monomorphic Bands	Polymorphism %
1	UBC 10	27	17	10	62.96
2	UBC 12	33	21	12	63.63
3	UBC 13	32	16	16	50
4	UBC 25	27	13	14	48.14
5	UBC 34	27	11	16	40.74
6	UBC 35	32	19	13	59.37
7	UBC 40	28	13	15	53.57
Total		206	110	96	54.05

Table (8): Similarity matrix for all varieties with treatment

Similarity matrix	100 mM	200 mM	250 mM	Teddy bear	Abendsoone	Giza 102	Sakha 53
100 mM	1						
200 mM	0.818	1					
250 mM	0.71	0.806	1				
Teddy bear	0.632	0.561	0.679	1			
Abendsoone	0.519	0.481	0.56	0.756	1		
Giza 102	0.829	0.8	0.727	0.689	0.586	1	
Sakha 53	0.812	0.812	0.8	0.733	0.632	0.849	1
	100 mM	**200 mM**	**250 mM**	**Teddy bear**	**Abendsoone**	**Giza 102**	**Sakha 53**

Figure (18): Total dendrogram for ISSR marker of *Helianthus annuus*



DISCUSSION

The response of *Helianthus annuus L.* plants to different concentrations of NaCl was investigated in the present study. The effect of NaCl were positively in shoot lengths as well as leaf area in comparison with control; decreased with increasing concentrations. Varieties (Sakha 53, Giza 102, Abendsoone and Teddy bear) showed the best results; the difference between the control and the last concentrations (250 mM) were very large, while the two other varieties (Gigante and White Hassawi) results had not the same degree of effect; there was no significant difference between the control and 250 mM focus in addition to the difficulty of distinguishing between different concentrations Figure (5),(6) , it is possible explained by the quality of seeds, also differences in seeds germination.

Results thus indicate that salt can cause ionic stresses, Inside the cell, largely as Na+ (and Cl-) inhibit metabolic processes including protein synthesis. "Na+ can rise to toxic levels in older leaves, causing them to die. This reduces the leaf area Table (2) available for photosynthesis and so the plant cannot sustain growth or crop yield[26]. Reductions in the rate of leaf are probably due to factors associated with water stress rather than a salt-specific effect. The cause of the injury is probably due to the salt load exceeding the ability of the cells to compartmentalize salts in the vacuole. Salts then would rapidly build up in the cytoplasm and inhibit enzyme activity. Alternatively, they might build up in the cell walls and dehydrate the cell[25].

Salt stress reduces the plant's ability to take up water, and this leads to reduction in growth. This is the osmotic or water-deficit effect of salt stress. Both cellular and metabolic processes involved in osmotic stress due to salinity are common to drought. The rate at which new leaves are produced depends largely on the water potential of the soil solution, in the same way as for a drought-stressed plant. Salts themselves do not build up in the growing tissues at concentrations that inhibit growth, as the rapidly elongating cells can accommodate the salt that arrives in the xylem within their expanding vacuoles. So, the salt taken up by the plant does not directly inhibit the growth of new leaves[25].

Shoot length are affected positively by salt stress Table (2); sodium chloride induced a slight decrease in the shoot length, is due to toxic effect of salts as well as inhibition of cytokinesis and cell expansion. Additionally, the decrease in hormones that stimulate the growth and increase in hormones that hinder growth can cause shorter root and shoot lengths [2,12,30,38] .

Most of the literature indicates that plants are particularly susceptible to salinity. Examples are found in Wheat, when Datta *et al* [7] showed that different level of salinity significantly affected the growth attributes by reducing root and shoot length for salinity below 125mM.

Oxidative stress is initiated by reactive oxygen species (ROS) (are chemically reactive molecules containing oxygen) such as superoxide radical (O-2), hydrogen peroxide (H2O2) and hydoxyl (OH-) radical are also produced during salinity stress, and are responsible for the damage to membranes and other essential macromolecules such as photosynthetic pigments, proteins, DNA and lipids, ROS scavenge by various antioxidant enzymes such as catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO), witch increased during salt stressed. Survival under these stressful conditions depends on the plant's ability to perceive the stimulus, generates and transmits signals and instigates biochemical changes that adjust the metabolism accordingly[8.]

Previously Schactman and Munns **[36]** found that salt tolerance was not due to genotypic differences in growth rates. It was independent of overall growth rate (vigor) of the genotypes.

Biochemical effects of sodium chloride (NaCl):

Proline:

With regard to proline content, there is a strong correlation between increased cellular proline levels than the capacity to survive the effects of high environmental salinity. It may also, serve as nitrogen reserve **[35]**, salinity-induced proline accumulation is associated with stress tolerance in some plants. In sunflower plant, proline accumulation was observed in all stressed plants of the six varieties compared with control Table (4). Similar results have been reached by Khattab [18] on different plant species. The higher level of proline content in sunflower leaves may be due to expression of gene encoding key enzymes

(pyrroline-5-carboxylate synthase) of proline synthesis and low activity of the oxidizing enzymes which is controlled by osmotic and salinity stress[1] . Proline also can play a role as protective agent for cytoplasmic enzymes[28] and/or scavenging hydroxyl radicals[15]. Thus, it could be suggested that salt tolerance was manifested via activated proline synthesis and hydrolysis of protein into free amino acids to act as osmoprotectants in the different organs of the tested sunflower plant. This means that the inhibitory effect of salt stress on the tested sunflower plant was alleviated by salt treatments through increasing proline synthesis and/or enhancing the biosynthesis of other amino acids and their incorporation into protein. Finally, proline able to activate multiple responses that are component of the adaptation processes[22].

ISSR analysis:

Genetic fingerprinting results obtained using the ISSR technique, with seven different primers (UBC-10, UBC-12, UBC-13, UBC-25, UBC-34, UBC-35 and UBC-40) were able to distinguish between genotypes well as the percentage of genetic variation between untreated varieties were (62.5%) Table (5) and Figure (17), while primer (UBC-40) given (80%) of polymorphism . In this study, the highest genetic variability between varieties may be due to the historical, eco-geographical and genetic isolation of populations this different environmental conditions consistent with the finding of Kumar and De Britto[20], also the reason for higher genetic diversity revealed by microsattelite markers may be their codominant or multiallelic nature, hypervariability, high information content and amenability to automation[29, 33, 27]. The fast evolutionary rate and the hypervariability of ISSR may suggest that ISSR bands represent neutral markers[11].

This result also confirms that ISSR marker is efficient in detecting polymorphism within and among populations and/or varieties of sunflower. In the present study, it suggested the existence of higher diversity among wild sunflower varieties. Thus, ISSR marker systems will provide a useful tool in the future design of collection strategies for conservation and use of wild sunflower varieties . Moreover, this marker observed to be very useful in detecting genetic diversity and population structure. These results were in agreement with many investigators who studied the genetic diversity of sunflower and other species using molecular markers with morphological traits[32, 23].

Similar investigations have been carried out by Mahmoud and Abdel-Fatah,2012 (44.86%) were polymorphic among 13 sunflower genotypes. Similar reports were reported by Kumar *et al* [20, 31,14].

Molecular tools are also being used for fingerprinting or genetic characterization of varieties of different crops. Molecular characterization of germplasm is basic to the improvement of the species and can be done at the DNA level. By using ISSR primer, the study demonstrated that there was considerable genetic diversity within populations of *H.indicum*, 79.07% of bands were polymorphic in 5 populations. The percentage of polymorphic bands (PPB) in each population ranged from 13.95% to 20.93%. Similar investigations have been carried out by earlier workers. ISSR were used to estimate genetic diversity within and among 10 populations of *Rhodiola chrysanthemifolia* 13. 116 discernible DNA fragments were generated of which 104 (89.7%) were polymorphic, indicating substantial genetic diversity at the species level. The percentage of polymorphic bands ranged from 21.97% to 48.8%. They suggested that the main factor responsible for the high level of differentiation among populations is probably the historical, geographical and genetic isolation of populations in a harsh mountainous environment[20].

The highest similarity between the Egyptian varieties Sakha 53 and Giza 102 (84.9%), similar between Abendsoone and Teddy bear varieties (75.6 %) from the same source; may be explain for their geographic origin.

The salt stress-tolerant varieties are collectively different from untreated varieties used as control, concentrations (100 mM, 200 mM and 250 mM) were branched in a single branch each concentrations alone, with (45.75%) similarity matrix between all controls and 250 mM Table (4.23); this is agree with[37].

The polymorphism in our results for untreated with treated varieties with different concentrations of sodium chloride (100 mM, 200 mM and 250 mM) diminished (54.05%) compared to untreated varieties

where recorded (62.55%); result of treatment with salt and so its impact on gene expression for this varieties witch suggested with reading of sequence. Natural diversity provides a rich source of genetic re-combination and mutations which can be analyzed for salt tolerance[13, 17].

However, molecular marker found in *Helianthus annuus,* showed the positive effect of salinity on varieties suggested by the number of bands and localizations deferent between controls and treatments; means bands were detected in treated varieties with different concentrations of salt and not in varieties untreated and disappearance of bands from treated samples (Figure from 10 to 16) which showed patterns of ISSR with different primers; these bands may be considered as specific markers for sunflower tolerance.

CONCLUSION

In light of the physiological results the effect of salt shoot length and leaf area was significant and highly significant decreased with application of salt treatment with 50 mM, 100 mM, 150 mM, 200 mM and 250 mM in most of varieties (Sakha 53,Giza 102, Abendsoone, Teddy bear, Gigante and white Hassawi).Biochemical analysis results showed that the proline content in leaves was significant and highly significant from 100 mM to the last concentrations (250 mM), increased gradually in all varieties. The ISSR technique used here was found to be quite effective in determining the genetic variation among *Helianthus annuus L* genotypes. By knowing about the diversity of varieties, (62.5%**)** were polymorphic in all primers, while (80%) registred in primer (UBC-40), also the highest similarity recorded between the Egyptian varieties Sakha 53 and Giza 102 (84.9%), similar between Abendsoone and Teddy bear varieties (75.6 %).Whereas the ISSR were efficacy to detect the effect of sodium chloride on molecular changes with the disappearance of bands in varieties treated and appearance of new others and by cluster analysis. Finally it's important to applying DNA sequencing from same species that contribute to the establishment of a genetic map for economic plants.

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