Zagazig J. Agric. Res., Vol. 44 No. (5) 2017 1693-1713

Genetics and Biotechnology

http:/www.journals.zu.edu.eg/journalDisplay.aspx?Journalld=1&queryType=Master

THE USE OF EMBRYO CULTURES FOR STUDYING SALT STRESS TOLERANCE OF FOUR DIFFERENT BREAD WHEAT VARIETIES AND THEIR PROMISING MUTANTS

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Received: 14/06/2017 ; Accepted: 09/08/2017

ABSTRACT: The present study was carried out to determine the genetic response of four different bread wheat varieties and their mutants for callus induction using mature embryo culture and salt stress tolerance and to assess the genetic diversity between them using ISSR markers. The results of the ability of callus induction revealed that differences among genotypes, callus induction media (CI) and their interactions were highly significant for callus induction frequencies (CIF). Also in relation to the callus fresh weight (CFW), the differences between genotypes and their interactions with media were highly significant, whereas insignificant differences were found between media after 14 at 28 days of culture. The best medium for CIF was CIM2 containing 2 mg/l of 2,4-D for all genotypes except Gemiza 9-1 and Sakha 93-3 had the best CIF of CIM3 containing 3 mg/l of 2,4-D. Seds 12-7 had the highest CIF, whereas Sakha 93-3 had the lowest CIF of CIM2. Gemiza 9-1 had the highest CFW, whereas Sakha 93-3 had the lowest CFW after 28 days of culture. The fresh callus produced after month was transferred to salt stress media at 10000ppm NaCl level. Giza 168, Gemiza 9 and Sakha 93 showed increase in CFW, whereas other genotypes strongly decreased. Sakha 93 and Gemiza 9 gave the lowest value of callus necrosis (CN) and the highest value of callus *in vitro* tolerance (CINTOL) at the 10000ppm NaCl level after 28 days on salt stress media. The salt stressed callus was tested for shoot induction ability; callus of Gemiza 9 and Sakha 93 showed the best shoot induction frequencies at 10000 ppm level. Days to shoot induction were decreased with the increase of salt levels compared to normal conditions. Transfer of shooty callus on plant regeneration media to be grown and development failed. The callus turned brown and some of the resulting growths were weak and white. The use of ISSR technique illustrated the genetic similarities between the wheat genotypes, which ranged from 0.638 to 0.979 with an average of 0.809, suggesting a high genetic diversity between the wheat genotypes. Cluster analysis performed to generate dendrogram showed that wheat genotypes had been clustered into two main clusters. From the previous results, we recommended that Gemiza9 and Sakha93 are good materials for salt stress tolerance of bread wheat breeding programs..

Key words: Embryo culture, salt stress tolerance, ISSR, bread wheat.

INTRODUCTION

Wheat is the most important cereals world-wide and is cultivated in many regions (Briggle and Curtis, 1987), which has been described as the 'King of Cereals' because of the area it occupies, its high productivity and its prominent international position in the food grain trade (FAO, 2005). According to NCBI Taxonomy Browser, (*Triticum aestivum* L.) is recorded as belonging to the family Poaceae, subfamily Poodeae and tribe Triticeae. In Egypt, one of the most important cereal crops is wheat, which is considered the most important cereal groups. Wheat represents for about 10% of the total agricultural production value and about 20% of total agricultural imports. Egypt is also the largest importer of wheat in the world. Through

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the General Authority for Supply Commodities (GASC) within the Ministry of Supply and Internal Trade of Egypt (MoSIT). FAO has decided that Egypt is the world's largest wheat buyer. It is therefore clear that wheat is of utmost importance to Egypt, and wheat policy is a priority for the government (FAO, 2015).

A current problem which will become more critical in the future for crop plants are salt stress by salinity in soils caused by over-fertilization, poor irrigation practices and other causes. According to food and agricultural organization statistics FAO (2008), more than 800 million hectares of land worldwide are currently affected by salinity, including saline and sodic soils accounting for more than 6% of the world's total land area. The continuous salinization of arable land is expected to have a huge global impact, resulting in a loss of 30 percent of agricultural land over the next 25 years and a 50 percent loss by 2050. Overall, it has been estimated that the world is losing at least 3ha of arable land every minute due to soil salinity (Kundzewicz *et al.,* 2007). The total area of salt-affected land on a global basis has been estimated to be approximately 76.3 million hectares, of which 41.5 is considered to be seriously degraded (Oldman *et al.,* 1991).

Mature and immature embryos have been used in tissue culture protocols extensively; the mature embryos were a better choice in comparison to immature embryos (Ozgen *et al.,* 1998). Immature embryos are better explant source when regeneration is considered, but they require time and growth facilities (Zale *et al.,* 2004) whereas mature embryos are available throughout the year. Mature embryos can either be dissected (Yu *et al.,* 2008) or used directly (Ozgen *et al.,* 1998).

Plant tissue culture techniques provide a promising and feasible approach to develop salt tolerant plants. Dracup (1991) and Tal (1994) decided on the *in vitro* selection of cell lines salt tolerant for several species. Although conducted research on *in vitro* selection for salt tolerance in wheat useing mainly somaclonal variants (Barakat and Abdel-Latif, 1996; Karadimova and Djambova, 1993). Limited studies have been conducted to the genotypic assessment for callus induction and *in vitro* salt tolerance.

The present study aimed to determine the genetic response of bread wheat varieties and their promising mutants for callus induction using mature embryo culture, salt stress tolerance and evaluate the genetic diversity between them using ISSR markers.

MATERIALS AND METHODS

All Experiments were carried out in the green house and plant tissue culture laboratory, Department of Genetics, Faculty of Agriculture, Zagazig University, Egypt during 2016 – 2017.

Plant Material

Mature seeds of four Egyptian bread wheat varieties and their mutant lines presented in (Table 1) were used as the source of plant Material.

Mature Embryo Culture for Callus Induction

Mature embryos were isolated from mature seeds of bread wheat genotypes under study and used as explants. Mature embryo culture was conducted according to the methods used by Salama *et al.* (2013), Yin *et al.* (2011) and Ashraf and Osama (2004) with some modifications. Mature seeds were immersed in 70% ethanol for 5 min and then seeds were disinfected with commercial bleach (5% Sodium hypochlorite) for 25 min supplemented with 2 drops of Tween 80 under sterile conditions. After that seeds were rinsed with sterile distilled water 4 times in order to remove the excess of the chemicals. Seeds were then soaked in sterile distilled water for overnight at room temperature (22-25ºC) in complete darkness. Mature embryos were isolated from freshly imbibed seeds using sterile scalpels and sharp forceps and cultured with scutellum side up on different callus induction media.

Callus induction media were MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 3% (*W/V*) sucrose, 100 mg l^{-1} casein hydrolysates, solidified with 7.5 g l^{-1} agar and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) at 1.0, 2.0, 3.0 mg l^{-1} for callus induction with three replicates. All media were adjusted to pH 5.7± 0.1 before autoclaved for 20 minutes at 121°C. Ten embryos were cultured in each jar. Jars were incubated in darkness at 25±2ºC for one month and subcultured every 2 weeks.

Table 1. Name, pedigree and source of the four bread wheat varieties and their mutant lines used in the present study

No.	Name	Pedigree	Source	Origin
1	Giza 168	MIL/BUC// Seri CM93046-8M-0Y-0M-2Y-0M.	Wheat Res. Institute, Agricultural Res. Center, Dokki, Egypt	Egypt
2	Gemiza 9	Ald"S" / Huac"S" // CMH 74A.630/ 5x CGM 4583-5GM-1GM-0GM.	Wheat Res. Institute, Agricultural Res. Center, Dokki, Egypt	Egypt
3	Sakha 93	Sakha 92TR81032 S8871-1S-2S-1S-0S.	Wheat Res. Institute, Agricultural Res. Center, Dokki, Egypt	Egypt
4	Seds 12	BUC// 7c/ Ald/5/Maya 74/On/ 1160.147/3/BB/G11/4/ Chat"S" /6/Maya/vu1 // Cmh74A.630/4*sx, SD7096- 4SD-1SD-0SD.	Wheat Res. Institute, Agricultural Res. Center, Dokki, Egypt	Egypt
5	Giza 168-3	New Promising Mutant Line of M7 generation by using EMS 0.5% (GIZA168- EMS)	Developed by wheat salt and drought tolerance programme under supervision of Prof. Dr. Said Soliman at Genet. Dept., Fac. Agric, Zagazig Univ.	Egypt
6	Gemiza 9–1	New Promising Mutant Line of M7 generation by using EMS 0.5% (Gem9-EMS)	Developed by wheat salt and drought tolerance programme under supervision of Prof. Dr. Said Soliman at Genet. Dept., Fac. Agric, Zagazig Univ.	Egypt
7	Sakha 93-3	New Promising Mutant Line of M7 generation by using EMS 0.25% (Sakha93-EMS)	Developed by wheat salt and drought tolerance programme under supervision of Prof. Dr. Said Soliman at Genet. Dept., Fac. Agric, Zagazig Univ.	Egypt
8	Seds 12-7	New Promising Mutant Line of M7 generation by using Gamma rays 300 Gy dose (Seds12-Gamma ray)	Developed by wheat salt and drought tolerance programme under supervision of Prof. Dr. Said Soliman at Genet. Dept., Fac. Agric, Zagazig Univ.	Egypt

Callus fresh weight (CFW) was measured to determine callus growth by weighing fresh callus tissues after 14 and 28 days of culture. Growth was expressed as relative growth (RG) and calculated as following:

RG = W – W0/W0 × 100 (Meredith, 1978 ; Chen *et al.,* 2006). W0 means the initial fresh weight of callus and W means the fresh weight at the end of the culture passage.

Callus induction frequencies (CIF) were also determined after two weeks of culture according to the formula : CIF = number of embryos produced callus/total number of cultured embryos × 100 (Arzani and Mirodjagh, 1999).

Callus Cultures under Salt Stress

One month old callus were used to begin *in vitro* salt tolerance selection experiments. For that choose the best callus induction medium supplemented with four salt levels (0, 4000, 7000, 10000 ppm) of NaCl with three replicates.

Cultures were maintained in complete darkness at 25 ± 2ºC for one month. CFW were measured before and after salt treatment to determine the effects of salt stress on CFW. Callus necrosis (CN) and callus *in vitro* tolerance (INTOL) were also measured according to the formula:

CN = (number of necrotic calli / total number of calli) × 100 (Bouiamrine and Diouri, 2012).

INTOL= RGR $_{treatment}$ / RGR $_{control}$ (Al-Khayri and Al-Bahrany, 2004). Where RGR is relative growth rate and was measured by the formula: CRG = [LnW_2-LnW_1] /GP (Birsin and Ozgen, 2004). Where W_1 and W_2 are the initial and final weight of callus, respectively and GP is the growth period.

Testing of the Tolerant Calli for Shoot Induction and Plant Regeneration

Calli derived from salt treatment were transferred to MS medium supplemented with 1.5 mg l^{-1} BAP, 0.1 mg l^{-1} 2,4-D, 3% (*W/V*) sucrose, 100 mg l^{-1} casein hydrolysate and solidified with 0.7-0.8% (*W/V*) agar for testing to shoot induction. The medium adjusted to pH 5.7±0.1 before autoclaved at 121ºC for 20 min. Cultures were incubated at 25 ± 2ºC under 16/8 hr., light/dark photoperiod for one month, according to Salama *et al.* (2013) with some modifications. Days to regeneration were determined and callus regeneration frequency (CRF) was measured according to the formula:

CRF =(number of callus inducing shoots/total number of callus in the culture) × 100 (Arif *et al.,* 2014).

For plant regeneration and rooting formation, shooty calli were cut into pieces and transferred to four media as following: MS-free hormone, MS + 2 mg l^{-1} BAP, MS + 2 mg l^{-1} BAP + 0.1 mg l^{-1} IAA and MS + 2 mg l^{-1} BAP + 0.5mg l^{-1} IAA. Cultures incubated at same conditions for one month.

Evaluation of Genetic Diversity by ISSR Markers

DNA isolation

Leaves of 40 days and seedlings were used for genomic DNA isolation and the procedure was performed using Qiuagen genomic DNA extraction kit.

ISSR markers

Five ISSR primers presented in Table 2 were used for estimating the genetic diversity of bread wheat genotypes under study. Reaction of amplification was performed according to (Hoisington *et al.,* 1994). Run PCR products on 1.6% agarose gels in Tris-acetic acid- EDTA (TAE) buffer. Ethidium bromide staining was used for detection of amplified bands. Amplified bands were determined and scored for presence (1) or absence (0) of bands. Cluster analysis demonstrating genetic relationships of accessions was measured. Based on the Dice coefficient the similarity matrix was created and create the dendrogram with the UPGMA.

Statistical Analysis

The data were analyzed by IBM SPSS Statistics software. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by least significant difference method (LSD) at levels 5% and 1% (Gomez and Gomez, 1984). Based on the Dice coefficient the similarity matrix was created and create the dendrogram with the UPGMA method of the NTSYS-PC software, Version 2.1 (Rohlf, 2000).

RESULTS AND DISCUSSION

Callus Induction Frequency

Callus formation was observed after 2-3 days of mature embryo culture. Same results obtained by Parmar *et al.* (2012). The highest callus induction frequency (CIF) was obtained of CIM2 medium containing 2 mg l^{-1} 2,4-D for all genotypes except Gemiza 9-l and Sakha 93-3 which had the highest CIF on CIM3 medium containing 3 mg l^{-1} 2,4-D. Seds 12-7 had the highest (CIF) whereas Sakha 93-3 had the lowest CIF on CIM2 (Fig. 1a). ANOVA for CIF showed that all factors (genotypes and media) and their interactions were highly significant (Table 3). Arun *et al.* (1994) found that 90-100% of immature embryos of 1.0-1.5mm were formed calli when cultured on MS medium supplemented with 1.5 or 2.0 mg/l 2, 4-D.

ANOVA for CIF showed that all factors (genotypes and media) and their interactions were highly significant (Table 3). Suggesting a presence of genetic variability, different responses of genotypes to callus induction and possible selection of callus induction in bread wheat using mature embryos of wheat. And then the CIF depends on genotype and media composition which is in agreement with reports of callus induction in durum wheat (Bommineni and Jauhar, 1996; Ozgen *et al.*, 1996) and in bread wheat (Hess and Carman, 1998).

Table 2. Oligonucleotide sequences of ISSR primers used in the present study

Primer name	Primer sequence (5' - 3')	Annealing temp.
ISSR-1	5'- $(AG)_8$YC - 3'	47°C
ISSR-2	5'- $(AC)_8$YT - 3'	51°C
ISSR-3	5'- $(AC)_8$YA - 3'	63°C
ISSR-4	5'- $(AC)_8$YC - 3'	52°C
ISSR-5	5'- HVH$(TCC)_5$ - 3'	60°C

Y = C or T

Table 3. Mean sum of squares (MS) and heritability in broad sense (H^2) for callus fresh weight (CFW) and callus induction frequency (CIF) of four bread wheat varieties and their mutant lines using mature embryo culture on three different callus induction media after 14 and 28 days of culture

SOV	d.f	Mean sum of squares (MS)		
		CFW_1	CFW_2	CIF
		14 days	28 days	28 days
Replications	**2**	0.09061**	0.24005**	2535.76**
Genotypes (G)	**7**	0.06036**	0.22104**	836.51**
Media (M)	**2**	0.00427	0.00383	643.06**
G × M	**14**	0.00579**	0.02212**	201.39**
Error	**46**	0.00144	0.00441	65.470
H^2 (%)		82.8	85.3	65.2

** Highly significant at $p < 0.01$, * Significant at $p < 0.05$

CFW_1 = callus fresh weight after 14 days, CFW_2 = callus fresh weight after 28 days of mature embryo culture

Callus Fresh Weight

The highest CFW was obtained of CIM2. After 14 days of culture, Gemiza 9-1 had the highest callus fresh weight (CFW) whereas Giza168 had the lowest CFW. After 28 days of culture, all genotypes showed increase in CFW except Sakha 93-3 which showed reduction in CFW (Fig. 3). Gemiza 9-1 had the highest CFW, whereas, Sakha 93-3 had the lowest CFW. Results for CFW presented in Tables 4 and 5.

ANOVA for CFW obtained after 14 and 28 days of culture showed that genotypes and their interactions were highly significant and insignificant differences between media (Table 3). This suggests that CFW is also genotype dependent as CIF.

Callus Fresh Relative Growth

Giza168 showed the highest callus fresh relative growth CFRG, whereas Sakha 93-3 showed the lowest CFRG (-19.70) on CIM3 as shown in (Fig. 1b).

Table 4. Means for callus fresh weight (g) of four bread wheat varieties and their promising mutants using mature embryo culture on three different callus induction media after 14 days of culture

Media \ Genotype	CFW_1 (g)								
	Giza 168	Gem 9	Sakha 93	Seds 12	Giza 168-3	Gem 9-1	Sakha 93-3	Seds 12-7	General mean
CIM_1	0.150	0.203	0.397	0.377	0.350	0.417	0.187	0.373	0.307
CIM_2	0.233	0.230	0.297	0.413	0.343	0.427	0.303	0.310	0.320
CIM_3	0.153	0.250	0.317	0.293	0.317	0.410	0.220	0.383	0.293
General mean	0.179	0.228	0.337	0.361	0.337	0.418	0.237	0.356	0.306
LSD 0.05	0.062								
0.01	0.083								

Table 5. Means for callus fresh weight (g) of four different bread wheat varieties and their promising mutants using mature embryo culture on three different callus induction media after 28 days of culture

Media \ Genotype	CFW_2 (g)								
	Giza 168	Gem 9	Sakha 93	Seds 12	Giza 168-3	Gem 9-1	Sakha 93-3	Seds 12-7	General mean
CIM_1	0.483	0.303	0.623	0.317	0.527	0.630	0.130	0.620	0.454
CIM_2	0.440	0.547	0.497	0.357	0.563	0.690	0.200	0.527	0.478
CIM_3	0.277	0.633	0.510	0.413	0.533	0.677	0.177	0.593	0.477
General mean	0.400	0.494	0.543	0.362	0.541	0.666	0.169	0.580	0.469
LSD 0.05	0.109								
0.01	0.146								

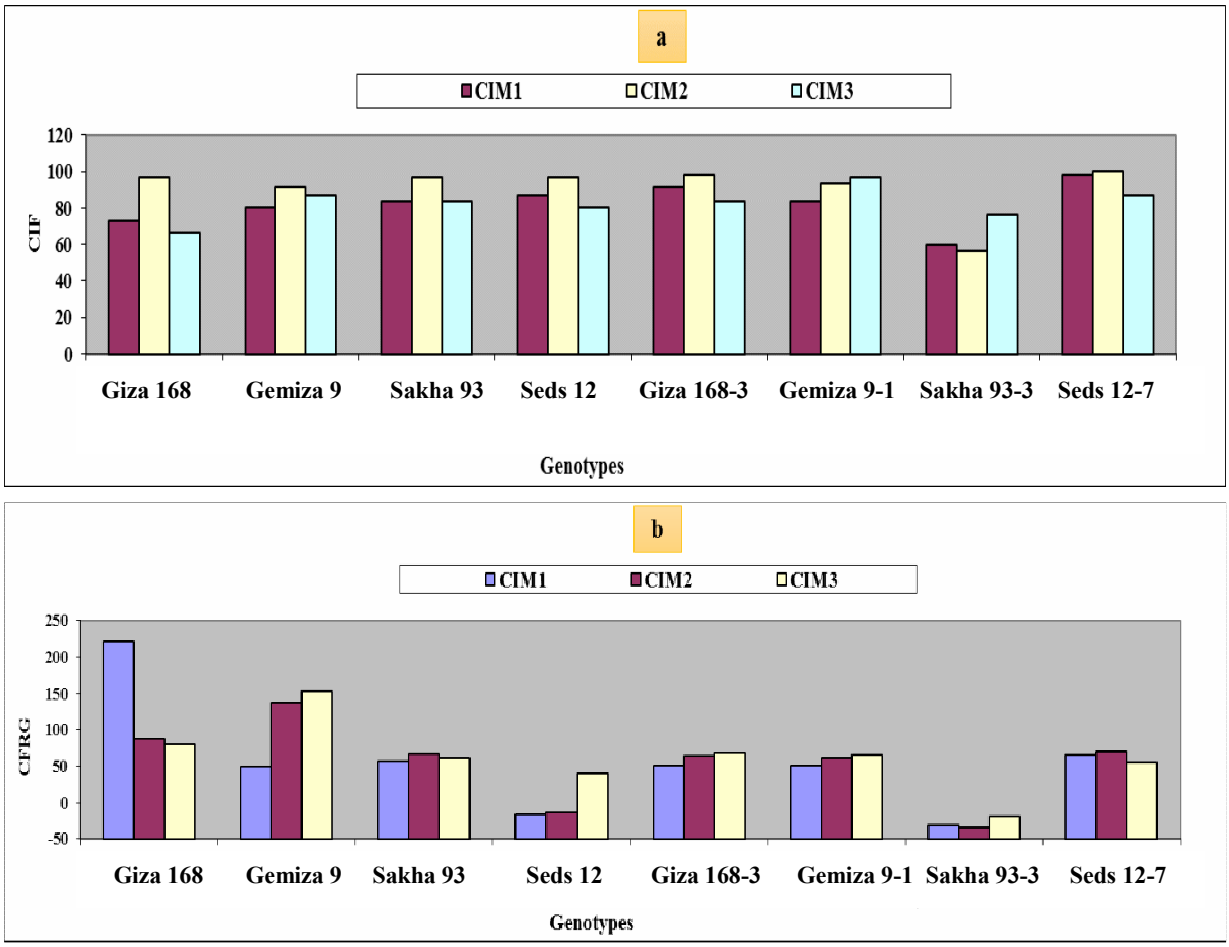

Fig. 1. Interactions between bread wheat genotypes and callus induction media (1.0, 2.0, 3.0 mg/l 2,4-D) for a. callus induction frequency and b. callus fresh relative growth after 28 days of culture

Callus Cultures under Salt Stress Levels

Callus fresh weight

After 28 days under different salt stress levels, all genotypes showed increase in CFW under control. Sakha 93-3 and Seds 12 showed reduction in CFW under the three salt stress levels. All genotypes showed increase in CFW at 4000 and 7000 ppm NaCl levels except Seds 12 and Sakha 93-3. However, Giza168, Gemiza 9, Sakha 93 and Gem9-1 showed increase in CFW at 10000ppm NaCl level, whereas, Seds12, Giza 168-3, Sakha 93-3 and Seds12-7 showed reduction in CFW at the same NaCl level (Fig. 2a). This suggests that the two genotypes Sakha93-3 and Seds12 are very sensitive for salt stress levels 4000, 7000 and 10000ppm NaCl and not recommended for salt stress tolerance breeding programs. However, the genotypes Giza 168-3 and Seds 12-7 are tolerant for salt stress levels 4000 and 7000 ppm NaCl, but sensitive for the salt stress level 10000 ppm. While genotypes Sakha 93, Gem 9, Giza 168 and Gem 9-1 showed increase in callus growth on all salt stress levels suggesting its ability to tolerate salt stress up to 10000 ppm and maybe a good material for *in vitro* and field salt stress tolerance breeding programs. Similarly, Omran *et al.* (2013) found that the genotype Latifia gained more callus fresh weight under higher levels of salt stress, thus it is a promising genotype since it responded better than Hashimia and Tamooz genotypes for selection in gaining more callus fresh weights. Abdel-Hady (2006) reported that the tolerance to high salt levels may depend on genotypic differences.

Sodium chloride (NaCl) caused a significant reduction in callus fresh weight. The higher NaCl concentration in the culture medium caused the higher reduction in callus fresh weight. This is in agreement with the results reported by several investigators (Mith and McComb, 1983; Binzel *et al.*, 1985; Singh *et al.*, 1985). The reduction in callus fresh weight may be a result of water availability reduction in the culture medium as result increased NaCl concentrations (Omar *et al.*, 1993).

ANOVA for CFW data under salt stress showed that genotypes and salt levels were highly significant, but insignificant differences between their interactions (Table 6). Similar results were reported by Kintzios *et al.* (1996) who observed significant differences among salt levels.

Callus fresh relative growth

Seds 12-7 had the highest callus fresh relative growth (CFRG) at control whereas; Sakha93-3 had the lowest CFRG at the same level. Seds 12 and Sakha 93-3 had the lowest CFRG at the three salt stress levels, respectively with negative values. This suggests that these genotypes are very sensitive to salt stress levels. At level 4000 ppm NaCl, Sakha 93 had the highest CFRG whereas; Seds 12-7 had the highest CFRG at 7000 ppm NaCl. Giza 168 had the highest CFRG at 10000ppm NaCl followed by Gemiza 9 and Sakha 93 (Fig. 2b) predicting to be tolerant genotypes. Fazelienasab *et al.* (2004) reported that at callus level, cultivars show their ability of stress resistance at initial treatment and this observed in parameters such as callus induction, callus volume and callus relative growth of sensitive cultivars. CFRG is a more efficient factor for assess of salt tolerance in callus level (Yue *et al.*, 2001). In control treatment, all genotypes showed the best CFRG. Similar results obtained by Bahman *et al.* (2013).

Callus *in vitro* tolerance

Based on callus *in vitro* tolerance **(**CINTOL), Sakha 93 had the highest CINTOL at 4000 ppm NaCl, whereas Gemiza 9 had the highest CINTOL at 7000 and 10000 ppm NaCl level. Seds 12 and Sakha 93-3 had the lowest CINTOL and were strongly affected by all salt stress levels (Fig. 2c). Suggesting that Sakha 93 and Gemiza 9 are more tolerant to salt stress.

Callus necrosis

Sakha 93-3 had the highest callus necrosis (CN) 91.25% at control and salt stress levels, whereas Sakha93 had the lowest CN (7.5%) at the same levels. Based on percentages of CN, the best genotypes were Sakha93 and Gemiza9 which showed high tolerance to CN, whereas Sakha93-3 and Seds12 were high sensitive to all salt stress levels (Table 7). Bahman *et al.* (2013) concluded that tolerant cultivars have more mechanisms to keep their K^+ levels at favorable concentration in order to grow efficiently. Callus necrosis (CN) was increased in response to the increase of salt stress level. Quesada *et al.* (2000) and Yue *et al.* (2001) found that with increase in Na^+/K^+ or decrease of K^+ levels, cells will not be able to grow well and soon will show necrosis state.

Regeneration of Tolerant Selected Callus

Shoot induction frequency

One regeneration medium was used to test the selected tolerant callus for the regeneration ability. The best genotypes for shoot induction were Gemiza 9 (Fig. 4) and Sakha 93 (Fig. 5) at the three salt stress levels. Sakha 93-3 and Seds 12 had no shoot induction at control and all salt stress levels. This may due to non-embryogenic calli which were found not to be regenerable (Malik *et al.*, 2004). Callus derived from 4000 ppm NaCl level showed the highest shoot induction rate for all genotypes except Seds12 and Sakha93-3. While, callus derived from 7000 and 10000 ppm NaCl level showed lower shoot induction rate (Table 8).

Kavi Kishor (1999) found that dehydration of calli of the rice cultivars Tellahamsa and Bala reduced the relative water content of callus to 64% in 3 hours and it dropped to 48% in 48 hours. This callus when placed on regeneration medium failed to invoke any plantlet regeneration. Regeneration potential of many plants declines with time in culture (Barba and Nickell, 1969; Fridborg, 1971). This may be due to polyploidization and related chromosomal abnormalities (Sheridan, 1975).

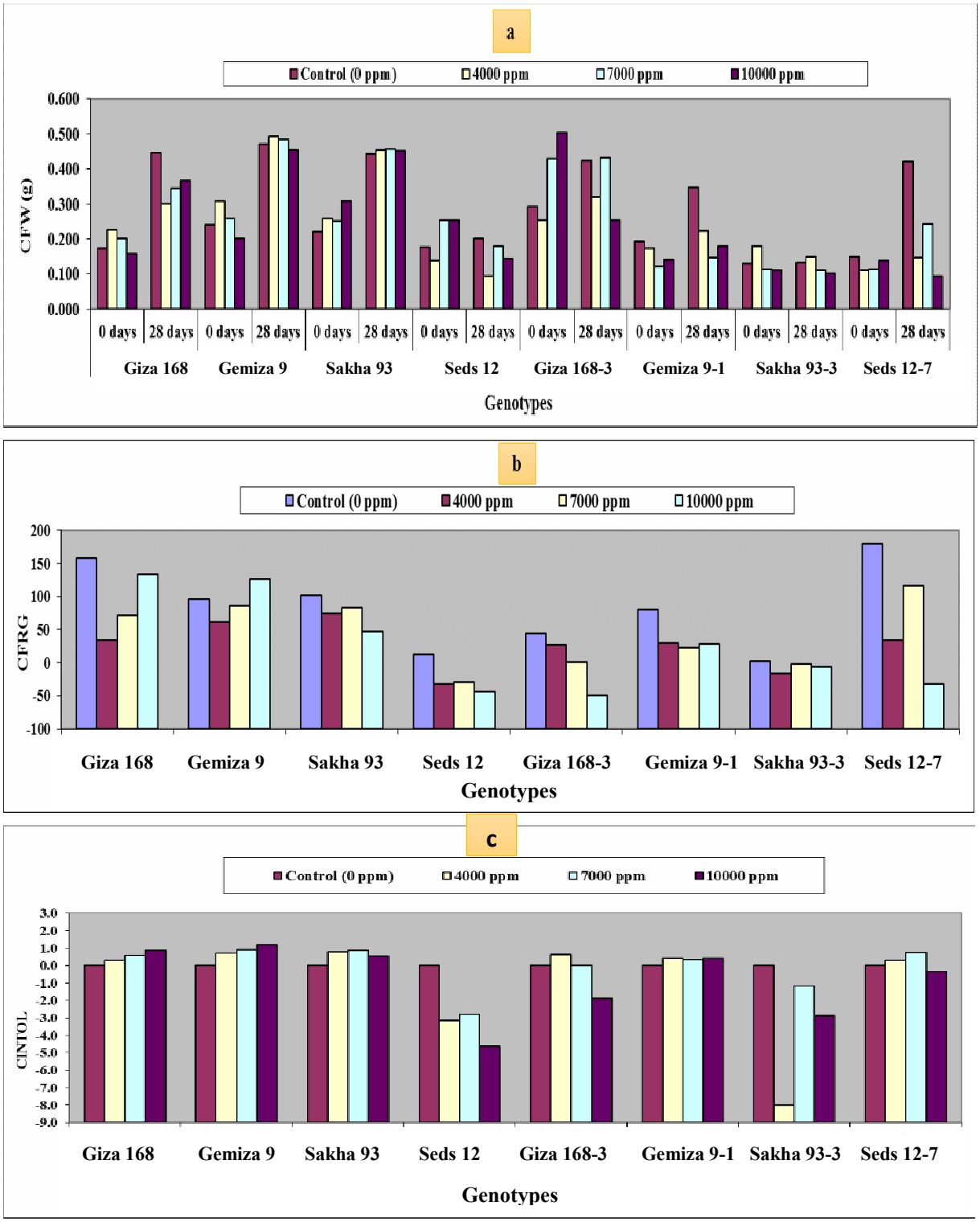

Fig. 2. Interaction between bread wheat genotypes and salt stress levels (0, 4000, 7000, 10000 ppm NaCl) for a. callus fresh weight (CFW), b. callus fresh relative growth (CFRG) and c. callus *in vitro* tolerance (CINTOL) after 28 days of culture

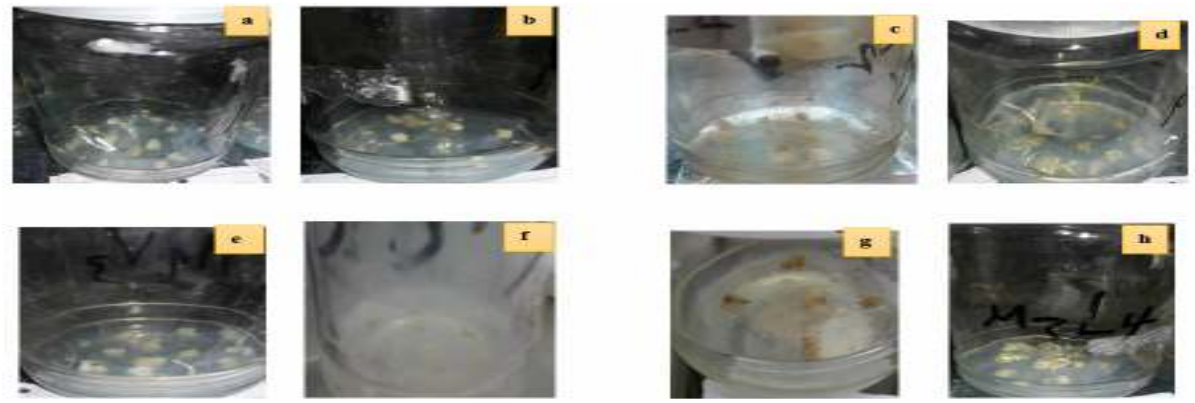

Fig. 3. Callus induction response for bread wheat genotypes using mature embryo explant after 28 days of culture on 2.0 mg/l 2,4-D medium. a. Giza 168, b. Giza 168-3, c. Gemiza 9, d. Gemiza 9-1, e. Sakha 93, f. Sakha 93-3, g. Seds12, h. Seds 12-7

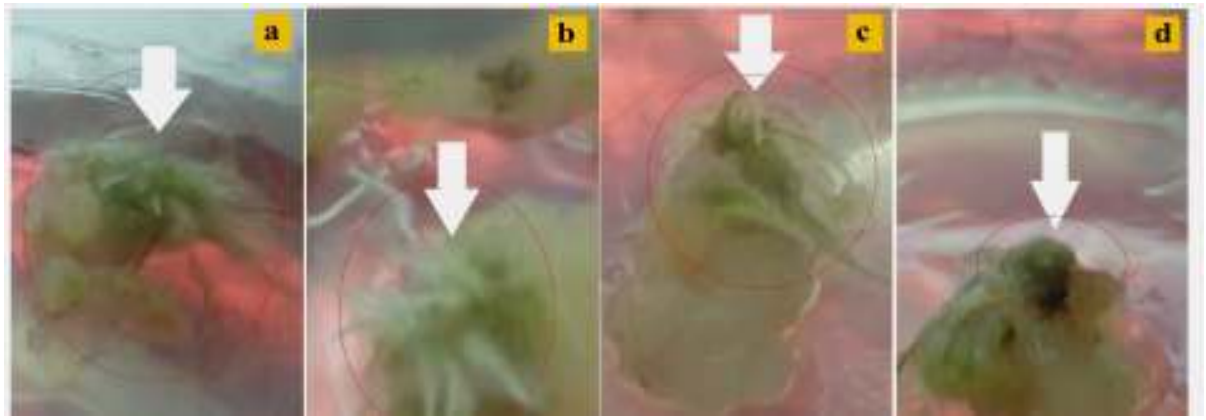

Fig. 4. The response of Gemiza9 salt-tolerant calli for shoot induction after 6 weeks of culture. a. non- salt stressed calli (0 ppm NaCl), b. 4000 ppm NaCl stressed Calli, c. 7000 ppm NaCl stressed calli, d. 10000 ppm NaCl stressed calli

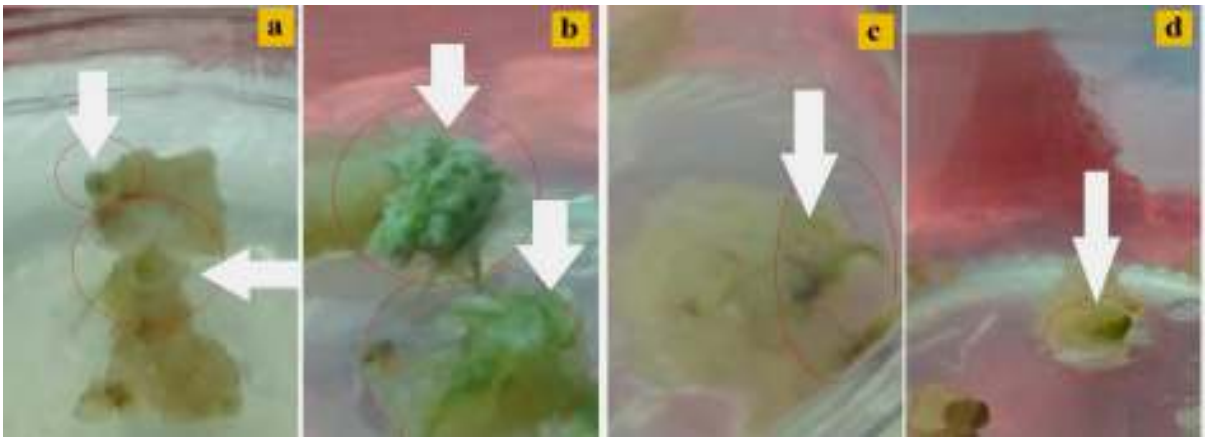

Fig. 5. The response of Sakha93 salt-tolerant calli for shoot induction after 6 weeks of culture. a. non-salt stressed calli (0 ppm NaCl), b. 4000 ppm NaCl stressed Calli, c. 7000 ppm NaCl stressed calli, d. 10000 ppm NaCl stressed calli

Table 6. Mean sum of squares (MS) and heritability in broad sense (H^2) for callus fresh weight (CFW) of four bread wheat varieties and their mutant lines under salt stress conditions (0, 4000, 7000, 10000 ppm NaCl) after 28 days of culture

SOV	d.f	Mean sum of squares (MS)
		CFW
		28 days
Replications	**2**	0.30465**
Genotypes (G)	**7**	0.18726**
Salt Levels (S)	**3**	0.06638**
G x S	**21**	0.01442
Error	**62**	0.01
H^2 (%)		61.77

** Highly significant at $p < 0.01$ * Significant at $p < 0.05$

Table 7. Average means for callus necrosis percentage (CN%) of four bread wheat varieties and their mutant lines under salt stress conditions (0, 4000, 7000, 10000 ppm NaCl) after 28 days of culture

Genotype / Media	CN (%)								
	Giza 168	Gemiza 9	Sakha 93	Seds 12	Giza 168-3	Gemiza 9-1	Sakha 93-3	Seds 12-7	General mean
Control (0 ppm)	25	15	0	60	33.33	42.8	75	0	31.391
4000 ppm	33.33	10	0	85	42	62.5	95	50	47.229
7000 ppm	33.33	15	10	85	66	85	95	75	58.041
10000 ppm	90	25	20	90	85	100	100	100	76.25
General mean	45.415	16.25	7.5	80	56.583	72.575	91.25	56.25	53.228

Table 8. Average means for callus regeneration frequency (CRF) of callus derived from mature embryo culture of four different bread wheat varieties and their promising mutants under salt stress conditions (0, 4000, 7000, 10000 ppm NaCl) after 6 weeks

Genotype / Media	Callus regeneration frequency (CRF)								
	Giza 168	Gemiza 9	Sakha 93	Seds 12	Giza 168-3	Gemiza 9-1	Sakha 93-3	Seds 12-7	General mean
0 ppm	33.3	66.7	33.3	0.0	33.3	42.8	0.0	66.7	34.51
4000 ppm	66.0	83.3	95.0	0.0	33.0	37.5	0.0	40.0	44.35
7000 ppm	66.7	80.0	75.8	0.0	40.0	20.0	0.0	0.0	35.31
10000 ppm	0.0	75.0	60.0	0.0	0.0	0.0	0.0	0.0	16.88
General mean	41.50	76.25	66.03	0.00	26.58	25.08	0.00	26.67	32.76

Days to shoot induction were recorded after 30: 40 days of culture as shown in (Table 9). The days to shoot induction were decreased in response to salt stressed-callus type *i.e.* callus derived from 10000ppm NaCl level showed the lower number of days to shoot induction than 7000 and 4000ppm NaCl level, respectively compared with control. Rahman *et al.* (2008) reported that shoots formed after 23 days from calli when casein hydrolysate added to the regeneration media with the concentration of 200 mg l^{-1}. While Khokhar *et al.* (2016) found that shoots developed even more rapidly from callus, only within 21 days, although this response may be strongly dependent on the genotype.

Shoot regeneration

Shooty calli cut into small pieces and transferred to four shoot regeneration media. All genotypes showed weak growth, shoot necrosis and failure to maintain on the regeneration media (Fig. 6). This may due to the inability of small pieces calli to survive then became necrotic. Torres (1989) mentioned in his book 'Tissue Culture Techniques for Horticultural Crops' that "Small healthy-looking pieces should be transferred, but the pieces should not be too small, as they may not survive the transfer. Failure to subdivide and transfer the callus leads to necrosis followed by death of the callus". Also, shoot necrosis may be caused by the deficiency of boron (Chandler *et al.*, 1933; Mason and Gutteridge, 1974) or calcium (Sha *et al.*, 1985; McCown and Sellmer, 1987). A comprehensive review of pistachio micropropagation by Barghchi and Alderson (1989) suggested that deficiencies of boron or calcium are most likely to be the causes of shoot-tip necrosis. Benkirane *et al.* (2000) found that *in vitro* immature embryos gave the high frequencies of regenerated plants. Ozgen *et al.* (1996) reported that tissue culture which include regeneration capacity and callus induction of wheat are influenced by the genotypes, explant source, the culture medium, physiological case of the donor plants, geographical origin and the interactions between them.

ISSR Markers for Genetic Diversity

In this study, ISSR markers were used to evaluate the genetic diversity between four bread wheat varieties and their mutants presented in Table 2. The total number of amplified bands, number of unique bands, number of monomorphic bands, number of polymorphic bands and the percentage of polymorphism obtained by each ISSR primers were shown in Table 10. Five ISSR primers amplified a total 47 scorable fragments with an average 9: 4 bands per primer. In these fragments, 17 (36.17%) bands were observed as polymorphic. The number of the polymorphic bands yielded by ISSR primers ranged from 1 (ISSR4) to 6 (ISSR1). The percentage of polymorphism across the bread wheat varieties and their mutant lines ranged from 07.70% (ISSR4) to 66.70% (ISSR1) with an average 39.00% per primer. The total mean percentage of ISSR polymorphism among the eight bread wheat genotypes achieved in our study was lower than that reported by Carvalho *et al.* (2009), probably because different ISSR primers and bread wheat genotypes were used. Tonk *et al.* (2014) reported that the percentage of polymorphism across the triticale varieties and lines ranged from 11.11% to 85.71%. Sarla *et al.* (2005) also obtained high percentage of polymorphism for ISSR in rice, as they examined highly diverse material containing varieties, landraces, ancestral landraces and wild accessions. Primer ISSR1 produced the highest percentage of polymorphism (66.60%), whereas primer ISSR4 produced the lowest percentage of polymorphism (07.70%). An average of 3.4 bands per primer was amplified and 39% were polymorphic Results presented in Table 10 and Table 11 showed that presence (1) and absence (0) of amplified bands for each primer. Only primer ISSR3 and ISSR4 produced unique bands. ISSR3 produced one unique band for Sakha93-3, whereas primer ISSR4 produced eight unique bands, one band for Sakha93 and seven bands for Gemiza9 as shown in Fig. 7.

Genetic similarity of Dice Coefficients based on ISSR markers (Table 2) among the bread wheat genotypes (Table 1) are shown in Table 12. The genetic similarities ranged from 0.638 to 0.979 with a mean of 0.809 which suggests the presence of high genetic variability among the wheat genotypes. These results were agreed with the findings of Carvalho *et al.* (2009) who reported that the genetic similarity coefficient between the bread wheat cultivars (Group I)

Table 9. Average means for days to shoot induction of callus derived from mature embryo culture of four different bread wheat varieties and their promising mutants under salt stress conditions (0, 4000, 7000, 10000 ppm NaCl) after 6 weeks

Media \ Genotype	Days to shoot induction								
	Giza 168	Gem 9	Sakha 93	Seds 12	Giza 168-3	Gem 9-1	Sakha 93-3	Seds 12-7	General mean
0 ppm	40	40	38	0	35	38	0	36	28.4
4000 ppm	30	38	36	0	37	38	0	32	26.4
7000 ppm	34	36	34	0	36	37	0	0	22.1
10000 ppm	0	34	31	0	0	0	0	0	8.1
General mean	26	37	34.8	0	27	28.3	0	17	21.3

Table 10. Number of total bands, unique, monomorphic, polymorphic bands and percent of polymorphism per each primer for four bread wheat varieties and their mutant lines

Primer	Total bands	Unique bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)
ISSR1	9	0	3	6	66.7
ISSR2	9	0	6	3	33.3
ISSR3	9	1	4	4	44.4
ISSR4	13	8	4	1	7.7
ISSR5	7	0	4	3	42.9
Average	9.4	1.8	4.2	3.4	39.0
Total	47	9	21	17	–

Table 11. Presence (1) and absence (0) of amplified bands derived from ISSR-PCR technique for four Egyptian bread wheat varieties and their mutant lines under study

Primer	MW	Seds 12	Seds 12-7	Gemiza 9	Gemiza 9-1	Sakha 93	Sakha 93-3	Giza 168	Giza 168-3	Polymorphism
ISSR1	31.18	0	1	1	0	0	0	1	1	Polymorphic
	24.15	1	1	1	1	1	0	1	1	Polymorphic
	17.03	1	1	1	1	1	1	1	1	Monomorphic
	8.95	1	1	1	1	1	1	0	1	Polymorphic
	6.68	1	1	0	1	1	1	1	1	Polymorphic
	4.48	1	1	1	1	1	1	1	1	Monomorphic
	3.28	1	1	1	1	1	1	1	1	Monomorphic
	1.20	1	1	0	0	1	1	1	0	Polymorphic
	0.45	1	1	0	1	1	0	0	0	Polymorphic
ISSR2	21.49	1	1	1	1	1	1	1	1	Monomorphic
	13.71	1	1	1	1	1	1	1	1	Monomorphic
	9.66	1	1	1	1	1	1	1	1	Monomorphic
	6.80	1	1	1	0	1	1	1	1	Polymorphic
	4.17	1	1	1	1	1	1	1	1	Monomorphic
	3.63	1	1	1	0	1	1	1	1	Polymorphic
	3.03	1	1	1	0	1	1	1	1	Polymorphic
	1.24	1	1	1	1	1	1	1	1	Monomorphic
	1.03	1	1	1	1	1	1	1	1	Monomorphic
ISSR3	0.65	1	1	1	1	1	0	1	1	Polymorphic
	0.75	1	1	1	1	1	1	1	1	Monomorphic
	0.87	1	1	1	1	1	1	1	1	Monomorphic
	1.00	1	1	1	1	1	1	1	1	Monomorphic
	1.24	1	1	0	0	1	1	1	1	Polymorphic
	1.09	1	1	1	1	1	1	1	0	Polymorphic
	0.63	0	0	0	0	0	1	0	0	Unique
	0.54	1	1	1	1	1	1	1	0	Polymorphic
	0.41	1	1	1	1	1	1	1	1	Monomorphic
ISSR4	6.68	0	0	1	0	0	0	0	0	Unique
	5.61	0	0	1	0	0	0	0	0	Unique
	4.79	0	0	1	0	0	0	0	0	Unique
	3.79	0	0	1	0	0	0	0	0	Unique
	3.08	0	0	1	0	0	0	0	0	Unique
	2.24	0	0	1	0	0	0	0	0	Unique
	1.67	0	0	0	0	1	0	0	0	Unique
	0.86	1	1	1	1	0	1	1	1	Polymorphic
	0.76	1	1	1	1	1	1	1	1	Monomorphic
	0.52	1	1	1	1	1	1	1	1	Monomorphic
	0.48	1	1	1	1	1	1	1	1	Monomorphic
	0.34	1	1	1	1	1	1	1	1	Monomorphic
	0.16	0	0	1	0	0	0	0	0	Unique
ISSR5	2.05	1	1	1	1	0	0	0	1	Polymorphic
	1.88	1	1	0	0	1	1	1	1	Polymorphic
	1.61	1	1	1	1	1	1	1	1	Monomorphic
	1.36	1	1	1	1	1	1	1	1	Monomorphic
	1.25	1	1	1	1	1	0	1	1	Polymorphic
	1.04	1	1	1	1	1	1	1	1	Monomorphic
	0.93	1	1	1	1	1	1	1	1	Monomorphic

Table 12. Genetic similarities (Dice Coefficients) of the four bread wheat varieties and their mutants based on ISSR banding patterns

Genotype	Seds 12	Seds 12-7	Gemiza 9	Gemiza 9-1	Sakha 93	Sakha 93-3	Giza 168	Giza 168-3
Seds 12	1.000							
Seds 12-7	0.979	1.000						
Gemiza 9	0.723	0.745	1.000					
Gemiza 9-1	0.872	0.851	0.723	1.000				
Sakha 93	0.936	0.915	0.660	0.809	1.000			
Sakha 93-3	0.872	0.851	0.638	0.745	0.851	1.000		
Giza 168	0.915	0.936	0.723	0.787	0.894	0.872	1.000	
Giza 168-3	0.894	0.915	0.745	0.809	0.830	0.809	0.894	1.000

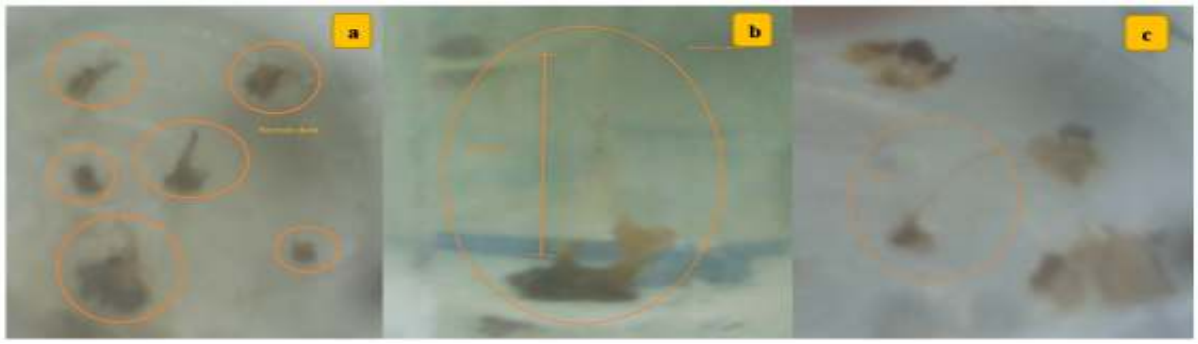

Fig. 6. Shoot regeneration response of shooty callus after 40 days of culture. a. necrotic shoot, b. and c. albino shoot

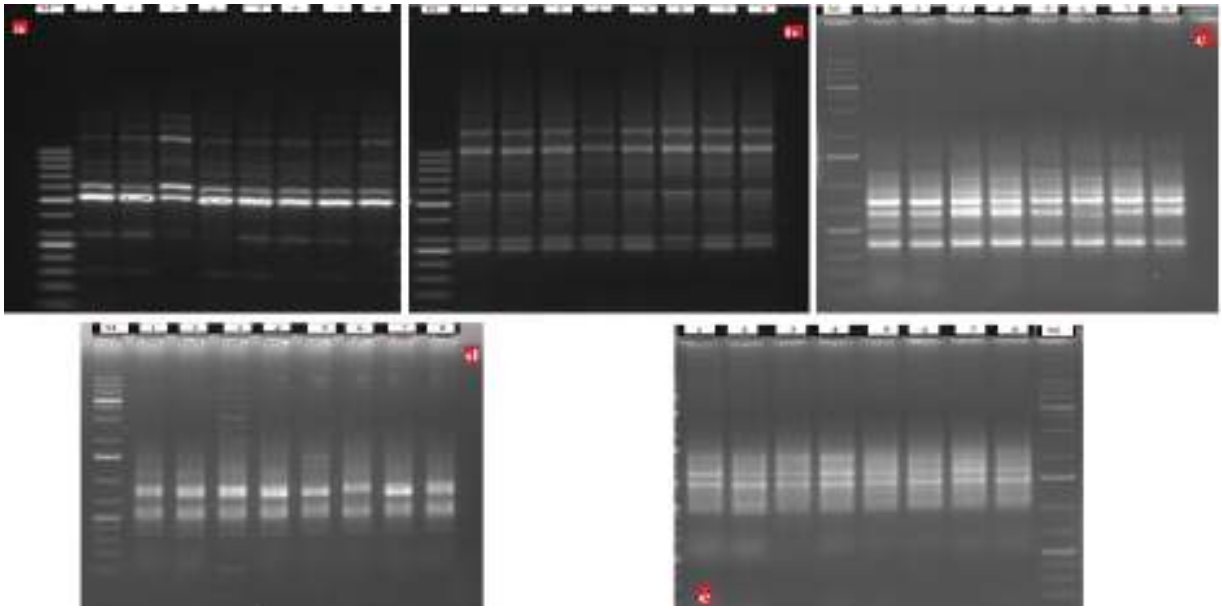

Fig. 7. ISSR-PCR banding patterns of four bread wheat genotypes and their mutant lines. M. ISSR marker, 1. Seds 12, 2. Seds 12-7, 3. Gemiza 9, 4. Gemiza 9-1, 5. Sakha 93, 6. Sakha 93-3, 7. Giza 168, 8. Giza 168-3 using five ISSR primers a. primer ISSR1, b. primer ISSR2, c. primer ISSR3, d. primer ISSR4, e. primer ISSR5

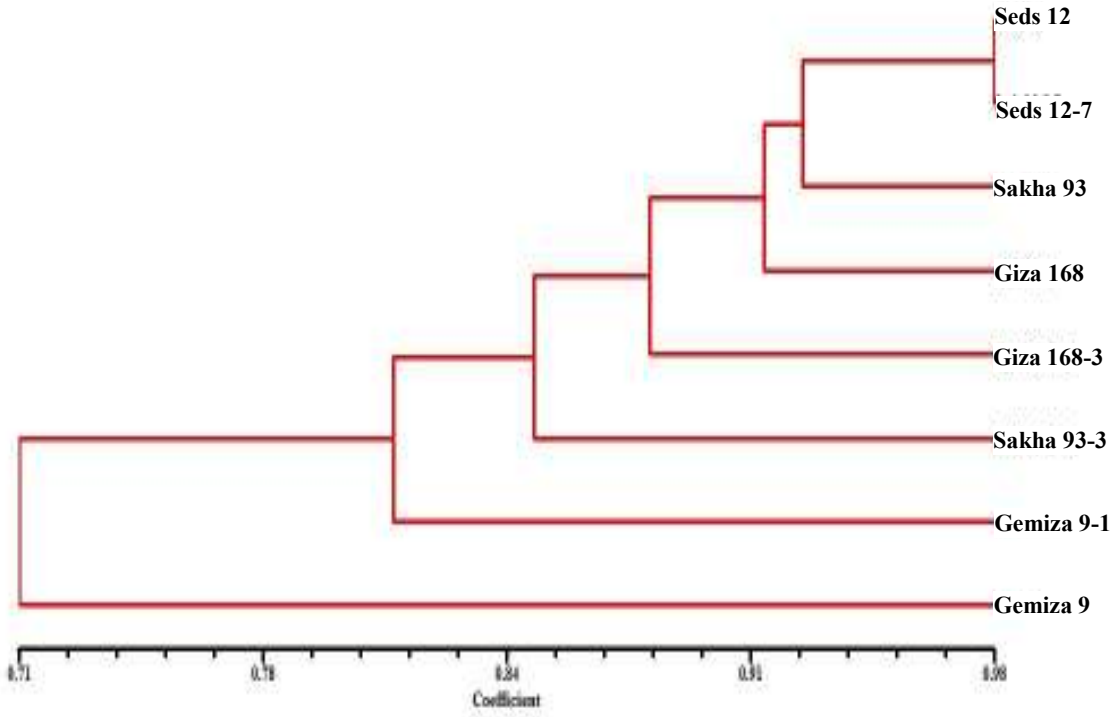

Fig. 8. UPGMA dendrogram representing the genetic relationship among four bread wheat and their mutant lines using UPGMA cluster analysis of dice similarity coefficient generated from ISSR markers

ranged from 0.36 to 0.85, suggesting high genetic diversity among them. The highest genetic similarity value was 0.979 among Seds12 and its promising mutant Seds12-7, whereas the lowest similarity value was 0.638 among Gemiza9 and the mutant Sakha93-3. Based on results achieved by ISSR technique, cluster analysis performed to generate dendrogram (Fig. 8) showed that wheat genotypes were clustered into two main clusters. The first cluster is composed of Gemiza9 only, the second cluster consists of two subclusters, the first subcluster included Gemiza9-1 only and the second subcluster included the remains genotypes. We found that the genetic distances between the genotypes were high, revealing high levels of genetic diversity among the eight bread wheat genotypes.

Najaphy *et al.* (2012) found that inter-simple sequence repeat (ISSR) provided enough polymorphism and fingerprinting profiles which reproducible for evaluating genetic diversity of wheat genotypes.

Sofalian *et al.* (2009) revealed that ISSR markers might be used to evaluate genetic variation in the wheat germplasm efficiently. ISSR analysis include the PCR amplification of regions among inversely oriented microsatellites (Zietkiewicz *et al.,* 1994 ; Blair *et al.,* 1999) can easily distinguish through different individuals (Zietkiewicz *et al.,* 1994; Fang and Roose, 1997; Wolfe *et al.,* 1998).

Conclusion

From the previous results, it could be concluded that: The four bread wheat varieties obviously are different from their mutant lines in their genetic responses for callus induction, *in vitro* salt stress tolerance and shoot induction ability under control and salt stress conditions. Giza 168-3 and Gemiza 9-1 were the best mutant lines for salt stress tolerance up to 7000 ppm NaCl whereas Seds 12-7 was found to be tolerant to salt stress up to 4000 ppm NaCl. Giza 168 was tolerant to salt stress up to 7000 ppm NaCl. Sakha 93 and Gemiza 9 were the

best genotypes for salt stress tolerance and shoot induction ability up to 10000ppm NaCl whereas Seds 12 and Sakha 93-3 were strongly affected by salt stress levels. Derivative calli from mature embryo culture had weak response for plant regeneration according to Ozias-Akins and Vasil (1983). ISSR markers are good tools for estimate intra-specific genetic diversity in wheat and these could distinguish the obtained local varieties (Sofalian *et al.,* 2009). So recommended that these genotypes are good materials for salt stress tolerance of bread wheat breeding progrmas.

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استخدام مزارع الأجنة لدراسة تحمل الإجهاد الملحي لأربعة أصناف مختلفة من قمح الخبز وطفراتها المبشرة

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أجريت الدراسة الحالية لتحديد الإستجابة الوراثية لأربعه أنواع مختلفة من قمح الخبز وطفراتها لإنتاج الكالس بإستخدام مزارع الأجنة الناضجة وتحملها الأجهاد الملحى، وتقييم التنوع الوراثى بينهم بإستخدام واسمات ISSR، أظهرت نتائج قدرة إنتاج الكالس أن الاختلافات بين التراكيب الوراثية وبيئات إنتاج الكالس والتفاعلات بينها كانت معنوية جدا لمعدلات إنتاج الكالس CIF. أيضا فيما يتعلق بوزن الكالس الطازج CFW، كانت الاختلافات بين التراكيب الوراثية وتفاعلاتها مع البيئات معنوية جدا، فى حين وجدت أختلافات غير معنويه بين البيئات بعد ١٤ و٢٨ يوم من الزراعة، كانت أفضل بيئة لإنتاج الكالس هي CIM2 المحتوية على ٢ملليجرام/لتر من هرمون 2,4-D لكل التراكيب الوراثية ماعدا سلالة جميزه ٩-١ وسلالة سخا ٩٣-٣ كانت أفضل بيئة إنتاج كالس لهم CIM3 المحتوية على ٣ ملليجرام/لتر من هرمون 2,4-D، السلالة سدس١٢-٧ أعطت أعلى نسبة CIF لإنتاج الكالس بينما السلالة سخا ٩٣-٣ أعطت اقل نسبة لإنتاج الكالس على بيئة CIM2. السلالة جميزة ٩-١ امتلكت أعلى قيمة لوزن الكالس الطازج CFW بينما السلالة سخا ٩٣-٣ كانت لها أقل قيمة لوزن الكالس الطازج بعد ٢٨ يوم من الزراعة، تم نقل الكالس الطازج الناتج عمر شهر إلى بيئات الإجهاد الملحي على تركيز ١٠٠٠٠ جزء في المليون من كلوريد الصوديم، أظهرت الأصناف جيزه ١٦٨ و جميزة ٩ و سخا ٩٣ زيادة في وزن الكالس الطازج CFW في حين انخفضت التراكيب الوراثية الأخرى بشدة. الصنف سخا ٩٣ وجميزة ٩ أعطى أقل قيمة لصفة callus necrosis (CN) و أعلى قيمة لصفة callus *in vitro* tolerance (CINTOL) عند مستوى ملوحة ١٠٠٠٠ جزء في المليون بعد ٢٨ يوم من الزراعة على بيئة الإجهاد الملحى، تم اختبار قدرة الكالس المعرض للإجهاد الملحي على إنتاج النموات الخضرية الجديدة، وأظهرت النتائج أن كالس الصنف جميزه ٩ والصنف سخا ٩٣ حققا أفضل نسبة لإنتاج النموات الخضرية عند مستوى ملوحة ١٠٠٠٠ جزء في المليون، ولوحظ انخفاض عدد الأيام اللازمة لإنتاج النموات الخضرية بزيادة المستوى الملحي وذلك مقارنة بالظروف العادية، نقل الكالس إلى بيئة التكشف لم تنجح فى النمو والتطور، وتحول الكالس إلى اللون البني وكانت بعض النموات الناتجة ضعيفة ولونها ابيض. وأظهر استخدام تكنيك ISSR أوجه التشابه الوراثى بين التراكيب الوراثية للقمح، والتي تراوحت بين ٠,٦٣٨ و ٠,٩٧٩ بمتوسط ٠,٨٠٩، مما يشير إلى وجود تنوع وراثى كبير بين التراكيب الوراثية للقمح، وأظهر تحليل درجه التقارب dendrogram أن التراكيب الوراثيه للقمح قد تم تجميعها في مجموعتين رئيسيتين، وبناء على النتائج السابقة نوصي بأن كلا من الصنف سخا ٩٣ والصنف جميزا ٩ مادة جيدة لبرامج التربية لتحمل الملوحة في قمح الخبز.

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