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Molecular Markers Associated with Salt-Tolerance of Different Soybean (*Glycine max* L.) Cultivars under Salt Stress

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Abstract

The impact of two concentrations (75 mM and 150 mM) of NaCl salt stress on six soybean cultivars (Giza 21, Giza 22, Giza 35, Giza 82, Giza 111 and Crawford) was studied at molecular level using RAPD, ISSR, SDS-PAGE and peroxidase (POD) isozyme techniques. RAPD analysis using six random primers showed that 61 of 69 total amplified bands were polymorphic with 87.74 % polymorphism under 75 and 150 mM NaCl. The six soybean cultivars exhibited 41 RAPD specific positive bands for salinity tolerance with 56.18 % polymorphism, whereas Giza 35 and Giza 21 had the highest number of salinity – stress specific positive markers at both NaCl concentrations. ISSR analysis using four primers revealed 36 polymorphic fragments with 91.57 % polymorphism from a total of 39 amplified fragments under salinity stress. The six soybean cultivars revealed 30 ISSR specific positive fragments for salinity tolerance with 74.83% polymorphism, whereas Giza 82 and Giza 35 had the highest number of salinity specific markers at both NaCl concentrations. NaCl salt stress caused variations in number of electrophoretic protein bands for all the studied cultivars, whereas Giza 82 showed the highest number of a new protein bands (3 bands) and Giza 111 appeared the lowest number of a new protein bands (1 band) under both levels of NaCl treatment. Native polyacrylamide gel electrophoresis showed presence of seven bands of peroxidase (POD) isozyme under salinity stress. The number and intensity of isozyme bands was correlated to NaCl concentrations and soybean cultivar, whereas Giza 22 possessed the great numbers of positive POD bands at both NaCl concentrations. According to our results, RAPD, ISSR, SDS-PAGE and POD isozyme techniques are useful methods to detect specific markers for salinity tolerance and could be used in soybean breeding programs to select the most tolerant cultivars.

Keywords: Salt stress, Soybean, protein electrophoresis, RAPD, ISSR, Molecular markers.

1. Introduction

Soybean is an important legume crop that is popular for its protein and edible oil. Its protein has great potential as a major source of dietary protein. The oil produced from soybean is highly digestible and contains no cholesterol (**Essa and Al-ani 2001**). Salinity is one of the environmental factors which is limiting the cultivation and production of soybean crop. Legumes are one of the most sensitive plants to salinity (**Fatma *et al.*, 2012**).

Abiotic stresses such as drought, salinity, alkalinity, temperature and mineral toxicity frequently affect plants in agricultural systems and represent major limitations to the yield and quality of many crops. Salinity in the soil is one of the major abiotic stress affecting crops in Egypt and throughout the world. More than 800 million hectares of land are salt affected globally, accounting for more than 6% of the total land area (**Munns and Tester, 2008**). Egypt is one of the countries that suffer severe salinity

problems; over 33% of the cultivated land which comprises only 3% of total land area in Egypt is already saline (**Ghassemi *et al*., 1995**). One way of increasing the yield and productivity of crop plants in in salinized lands is to breed them to become more tolerant to this stress (**Flowers *et al.* 2000)**. However, many challenges faced the breeding programs for tolerance because mechanism of tolerance to stress is controlled by many genes and their simultaneous selection procedures particularly under field conditions are difficult **(Flowers *et al*., 2004**).

Molecular markers have been proved to be valuable tools in detection of genetic variability between plant species, populations, and verities and to find marker related to specific trait (s). It was known that different markers reveal different classes of variation (**Powell *et al*., 1996; Russell *et al*., 1997**). It is dependent on the genome fraction screened by each kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay (**D´avila *et al*., 1999**). Molecular markers such as randomly amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and isozymes have recently shown excellent potentiality to assist selection of quantitative trait loci (QTLs) associated with these traits (**Xue *et al*., 2009**). Both of RAPD and ISSR markers do not require any specific knowledge of the DNA sequence of the target organism. ISSR markers are more feasible, reproducible and highly informative than RAPD amplification (**Bornet *et al.* 2002**).

Association between RAPD markers and salt tolerance was investigated by many workers the last decade. **Bahieldin and Ahmed (1994)** tested six barley cultivars with 29 RAPD primers and they are concluded that RAPD markers can be used as genetic markers for salt tolerance. **Yang *et al.* (2005)** developed many RAPD markers linked to salt tolerance in an F**2** population of alfalfa derived from crossing salt tolerant and sensitive alfalfa cultivars. They reported that RAPD system was useful to determine many markers. **Rao *et al.* (2007)** identified distinct RAPD patterns for salinity tolerance with the presence of specific amplified products in some sorghum accessions. **Khan *et al.* (2013)** used twenty RAPD primers to study the genetic variation among ten soybean cultivars. They are concluded that closer soybean cultivars in the cluster behaved similarity in their response to salinity tolerance. **Abdel-Hamid (2014)** screened the powerful of four barley cultivars to tolerate the salt-stress using six RAPD primers**.** He recorded presence of some RAPD molecular genetic markers were associated with salt tolerance in barley cultivars.

ISSR has been used for detection of genetic polymorphism and discrimination between soybean cultivars (**Yang *et al.*, 1996**). It has been used to identify markers associated with salt tolerance in wheat (**Lang *et al.* 2001**), rice (**Kaushik *et al.* 2003**), barley **(Khatab and Samah, 2013),** Sorghum **(Khalil, 2013)** and sugarcane **(Markad et al., 2014).** Two of five ISSR markers were identified as markers associated with salinity tolerance in alfalfa (*Medicago sativa* L.) genotypes (**Abdel-Tawab *et al*., 2011).** More recently, **Metwali et al. (2016**) identified one drought tolerant tomato cultivar using ISSR markers after screening 15 different tomato cultivars grown under drought stress.

SDS-PAGE has been used to identify and discriminate between the soybean cultivars (**Rashed *et al*., 1997; Xue *et al*., 2009**). Characterizations of salt-tolerance soybean cultivars are considered an important task for many purposes of soybean breeding (**Thomposon *et al*., 1998; Fahmy & Salama, 2002**). Analysis of proteomic profiles of soybean in response to abiotic stress have been investigated in many studies (**Zhen *et al*., 2007; Aghaei *et al*., 2008; Toorchi *et al*., 2009; Cheng *et al*., 2010**). They have been found that expression of the majority of defense proteins either over expressed or down expressed during stress. These investigations showed that a proteomic approach is useful for analyzing the functions of stress-induced proteins and provide a detailed network of salt adaptation mechanisms in many important crops.

Electrophoretically separable and analysis of variant isozymes can provide a precise tool to characterize plant species and cultivars under salinity stress. Isozymes are mostly co-dominant with a simple Mendelian inheritance in most loci and it can be resolved for most plant species regardless of habitat, size or longevity (**Veasey *et al*., 2008)**. Peroxidases (EC 1.11.1.7) are involved in responses to biotic and abiotic stresses, the biosynthesis of lignin (**Lagrimini *et al*., 1987**) and subrin (**Espiele *et al*., 1986**), scavenging of ROS, chlorophyll degradation and senescence (**Yamaguchi and Watada, 1991**). The expression of superoxide dismutase (SOD) isozyme was studied in four soybean cultivars in response to 100, 200 and 300 mM NaCl salinity stress (**Ramana *et al*., 2012**). Their investigation revealed that the expression of two SOD isozymes (Mn-SOD and Cu/Zn-SOD) was enhanced with increasing NaCl

concentrations. **Saad-Allah (2015)** study the influence of three levels (0, 8 and 16 mS/cm^2) of sea salt on electrophoretic banding patterns of peroxidase isozymes in six soybean cultivars. He observed that the number and intensity of peroxidase isozyme bands were changed in respect to sea salt salinity.

The development of molecular markers for screening salt tolerant cultivars has been attempted in several crop plants but there are so limited reports of differentiating salt tolerant soybean genotypes using the molecular markers. Thus, the present study has been undertaken to evaluate the impact of two NaCl salt stress levels (75 and 150 mM) on six soybean cultivars commonly grown in Egypt through evaluating the RAPD, ISSR, protein and isozyme markers associated with the plants under salinity stress in order to identify the soybean cultivars with a better performance even in saline soils.

2. Materials and Methods

2.1. Plant material and growth condition

Seeds of six soybean (*Glycine max* L. Merrill) cultivars were kindly provided by Agriculture Research Center (ARC) El-Dokki, Giza, Egypt (**Table 1**). The seeds were surface sterilized with 0.5% sodium hypochlorite for 5 minutes and washed three times with tap water then rinsed with deionized water. The seeds were sown in plastic pots of 45 cm diameter and 25 cm depth containing 8 kg clay sandy soil (2:1 w/w) and irrigated with tap water. The pots were arranged in spilt plot design in a randomized complete blocks arrangement with three replications. The plants were left to grow under the natural day/night conditions (14h light/ 10h dark) at 28±2°C in the greenhouse of Botany and Microbiology Department, Faculty of Science, Al-Azhar University, during the summer season of 2014. The pots were irrigated whenever needed with the tap water to the end of the growth period (30 days). Thirty days old plants with uniform growth were irrigated with (0, 75 and 150 mM) of NaCl tap water for 15 days. After 7 days of the treatment, fresh leave samples were harvested for the RAPD, ISSR, SDS-PAGE and peroxidase isozyme analysis.

Table 1: Genotype name and pedigree of soybean cultivars.

Cultivar's No.	Cultivar's Name	Pedigree
1	Giza 21	Crawford × Celest
2	Giza 22	Forrest × Crawford
3	Giza 35	Crawford × Celest
4	Giza 82	Crawford × Maplepresto
5	Giza 111	Crawford × Celest
6	Crawford	Williams × Columbus

2.2. RAPD and ISSR Molecular analysis

2.2.1. Genomic DNA extraction

Approximately 500 mg of young and freshly leaves from each cultivar of five-week old plants of the six soybean cultivars were collected and the bulked DNA extraction was performed using DNeasy plant Mini Kit (Qiagen Hilden, Germany) according to the manufacturer manual.

2.2.2. PCR conditions and electrophoresis

Six out of 12 primers for RAPD and four out of 12 primers for ISSR synthesized by (Operon Technologies, USA) were used in this study (**Table 2**). PCR for both analyses was performed in 25 µl volume containing 2.5 mM MgCl2, 0.2 mM dNTPs, 20 µM primer, 50 ng genomic DNA and 1 unit Taq DNA polymerase (Fermentas, Canada). All reactions were performed in DNA thermal cycler (Techno 512). RAPD Program was performed as 1 cycle of 94°C for 4 min and 40 cycles of 94 °C for 1 min, 35°C for 1 min, and 72°C for 2 min. ISSR program was performed as 1 cycle of 94°C for 4 min and 35 cycles of 94°C per 30 sec, 44°C for 45 sec, 72°C for 1.5 min. Then, a final extension step of 72°C for 10 min was done for both analyses. To visualize the PCR products, 15 µl of each reaction was loaded on 1.2 % agarose gel. The gel was run at 90V for 1 h and visualized with UV Transilluminator and photographed using gel documentation system (Bio-Rad, USA). The synthetic

DNA, ladder 100 bp (Pharmacia) was employed as molecular markers for bands molecular weight. Each amplified band profile was defined by the presence or absence of bands at particular positions on the gel. Fragments were scored as (+) if present or (-) if absent based on standard marker using using AlphaEaseFC version 4.0. (Alpha Innotech Corp., San Leandro, CA) software.

2.3. Evaluation of protein banding pattern by SDS-PAGE

Fresh and young Leaves were harvested from control and NaCl treated plants from each soybean cultivar for analysis of total soluble proteins by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Soybean leaves (0.5 g) were homogenized with 2 ml of a buffer containing 50 mm Tris(hydroxymethyl) aminomethane (Tris)-Glycine (pH 8.3), 0.5 m sucrose, 50 mm EDTA, 0.1 m KCl, 2 mm PMSF and 0.1% (v/v) 2-mercaptoethanol in a chilled pestle and mortar at 4 °C. The homogenate was centrifuged in a refrigerated centrifuge (Sigma, 2K15, Germany) at 14,000 × *g* for 10 min. Protein concentration in the supernatant samples was estimated according to the method of **Bradford (1976)**. The supernatants were stored in small aliquots at - 40 °C for SDS-PAGE.

Table 2: Names and their sequences of RAPD and ISSR primers.

RAPD Primers	Sequences	ISSR Primers	Sequences
OPAA-02	5'- GAGACCAGAC-3'	**14A**	5' - GAG AGA GAG AGA CC -3'
OPAA-10	5'- TGGTCGGGTG -3'	**44B**	5' - CTC TCT CTC TCT CTC TTG -3'
OPAA-14	5'- AACGGGCCAA -3'	**HB -11**	5' - GTG TGT GTG TGT TGT CC -3'
OPAA-15	5'- ACGGAAGCCC-3'	**HB -12**	5' - CAC CAC CAC GC- 3'
G2	5'- TGCGTGCTTG -3'		
G3	5'- GACGGATCAG-3'		

Supernatant samples (40 μg protein) were mixed with equal volumes of solubilizing buffer [62.5 mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.01% bromophenol blue] and heated for 4 min at 95 °C, and then it cooled on ice. Polypeptide pattern was analyzed on 12% SDS polyacrylamide gels according to the method of **Laemmli (1970).** The gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma) in 50% (v/v) methanol and 10% (v/v) acetic acid for 2 h and destained with 50% (v/v) methanol and 10% (v/v) acetic acid until the background was clear. The gels were photographed and scanned using a densitometer (GS- 710, Bio-Rad, USA) and analyzed with Quantity one software from Bio-Rad. The protein molecular weight marker from Bangalore Genei, India was used.

2.4. Detection of peroxidase (POD) isozyme

Fresh and young leaves from each soybean cultivar were used for analysis of isoforms of POD isozyme using native polyacrylamide gel electrophoresis (PAGE) system (**Laemmli's, 1970).** Isozymes extraction from the different soybean cultivars homogenizing 0.5 g fresh leaves samples in 1 ml extraction buffer (10% glycrol) using a mortar and pestle. The extract was then transferred into clean eppendorf tubes and centrifuged at 10000 rpm for 5 minutes. The supernatant was transferred to new clean eppendorf tubes and kept at 20 °C until use for electrophoretic analysis. A volume of 40 µl extract of each sample was mixed with 20 µl sucarose and 10 µl bromophenol blue, then a volume of 50 µl from this mixture was applied to each well. The run was performed at 150 volt until the bromophenol blue dye has reached the separating gel and then the voltage was increased to 200 volt. Electrophoresis apparatus was placed inside a refrigerator during running duration. After electrophoresis, the gels were stained according to their enzyme systems with the appropriate substrate and chemical solutions then incubated at room temperature in dark for complete staining. In most cases incubation for about 1 to 2 hours is enough. For peroxidase isozyme, the gel placed into 50 ml solution containing 0.125 gm benzideine di HCL, 2 ml gelecial acetic acid and then 5 drops of hydrogen peroxide was added. The gel was incubated at room temperature until bands appear **(Larsen and Benson, 1970).**

3. Results and Discussion

3.1. RAPD molecular markers related to salinity stress

A total of 69 scorable amplified DNA fragments ranging in size from 115 to 1370 base pairs were observed using the six primers (**Table 2**), whereas 61 fragments were polymorphic and the other amplified fragments were commonly detected among the six soybean cultivars at 75 and 150 mM NaCl as well as the control plants. The six primers showed 87.74 % mean polymorphism, whereas the polymorphism of primer G3 was the highest (100 %), however, the polymorphism of primer OPAA-10 was the lowest (66.67 %) (**Table 3**).

Primer OPAA-02 revealed 13 fragments with sizes ranged from 120 to 1225 bp (**Table 3, Fig. 1**), whereas 12 fragments were polymorphic (92.31 %). Soybean cultivars treated with 75 and 150 mM NaCl exposed five induced amplified bands with 950, 785, 550, 350 and 220 bp which did not exist in the control. Three induced bands (950, 785 and 350 bp) were displayed in Giza 21, one induced band (220 bp) was observed in Giza 35, two induced bands (550 and 350 bp) were detected in Giza 82, one induced band (350 bp) was observed in Giza 111 and two induced bands (350 and 220 bp) were detected in Crawford. Four induced bands were observed at each of the two NaCl levels among the four soybean cultivars. The first band (950 bp) was detected in Crawford at 75 mM NaCl, the second band (785 bp) was observed in Giza 111 at 150 mM NaCl, the third band (220 bp) was characterized in Giza 82 at 150 mM NaCl and Giza 111 at 75 mM NaCl. The forth band (200 bp) was identified in Giza 35 at 150 mM NaCl and in Crawford at 75 mM NaCl. Analysis by this primer appeared Three unique bands (250, 145 and 120 bp) distributed between two soybean cultivars (Giza 111 and Giza 35), respectively at 150 mM NaCl were observed by analysis using this primer.

Primer OPAA-10 exhibited 12 bands ranged in size from 255 to 1370 bp (**Table 3, Fig. 1**), whereas 8 fragments were polymorphic (66.67 %). Giza 111 genotype treated with 75 and 150 mM NaCl exposed tow induced amplified bands with 510 and 340 bp at both of the two NaCl concentrations, which did not appear in the control. Giza 22 displayed two bands (555 and 255 bp) at treated with 75 mM NaCl, while three amplified bands (510, 340 and 255 bp) were detected in Giza 82 at 150 mM NaCl. In addition, two different bands (420 and 555 bp) were observed in Giza 35 when treated with 75 and 150 mM NaCl, respectively.

Six fragments only were detected using OPAA-14 primer ranging in size from 240 to 1100 bp (**Table 3, Fig. 1**), among them five were polymorphic (83.33 %). Both of Giza 35 and Giza 82 displayed one fragment with molecular size of 600 bp at 75 and 150 mM NaCl, respectively. Giza 21cultivar appeared one band (1100 bp) at both of the two NaCl concentrations that did not exist in the control plants.

Primer OPAA-15 exhibited 17 fragments with sizes ranging from 155 to 800 bp (**Table 3, Fig. 1**), whereas 16 fragments were polymorphic (94.12 %). The analysis shows some amplified bands that were newly synthesized in treated plants with salinity and not produced in control plants. For example, seven amplified bands with molecular sizes of 615, 535, 490, 470, 280, 265 and 240 bp were induced at 150 mM NaCl in the five soybean cultivars except Giza 21, which produced one band (220 bp) at treated with 75 mM NaCl. Moreover, four bands (615, 535, 490 and 320) were observed at both 75 and 150 mM NaCl in Giza 21, while two bands (185 and 165 bp) were detected in Giza 22 and Giza 35, respectively. Two unique bands (400 and 155 bp) appeared in Giza 35 cultivar.

Primer G2 appeared 10 fragments ranged in size from 230 to 800 bp (**Table 3, Fig. 1**), whereas nine fragments were polymorphic (90 %). Five induced bands with molecular size of 800, 670, 450, 350 and 250 bp were displayed at both of the two NaCl concentrations in Giza 21, Giza 22, Giza 82 and Crawford cultivars, respectively, which did not exist in the control plants. Three induced bands (400, 380 and 350 bp) were observed in Giza 21, Giza 82 and Giza 111 cultivars treated with 75 mM NaCl, while one induced band (800 bp) was appeared in Giza 22 cultivar after their treatment with 150 mM NaCl. Primer G-2 produced one unique band (230 bp) in Giza 35 cultivar treated with 150 mM NaCl.

Primer G3 produced 11 fragments with sizes ranged from 115 to 710 bp **(Table 3, Fig. 1**), whereas eleven fragments were polymorphic (100 %). Two induced bands with molecular size of 250 and 175 bp were displayed at both of the two NaCl concentrations in Giza 82 and Giza 21 cultivars, respectively, which did not exist in the control plants. One induced band (116 bp) was induced in Giza 21 cultivar at 75 mM NaCl. One unique band (550 bp) was found in Giza 35 cultivar treated with 150 mM NaCl.

Table 3: RAPD analysis of polymorphic bands for six soybean cultivars under 0, 75 and 150 mM NaCl using six random primers (OPAA-02, OPAA-10, OPAA-14, OPAA-15, G2 and G3). +: present of amplified bands, P %: polymorphic percentage, Total: total number of amplified bands. Black boxes refer to the positive bands that are present at 75 and / or 150 mM NaCl only and absent from the control. * (92.31+66.67+83.33+94.12+90+100= 526.43), then Average = 526.43/6= 87.74 %.

Primer name	P %	Base pair (bp)	Giza - 21			Giza - 22			Giza - 35			Giza - 82			Giza - 111			Crawford		
			0	75	150	0	75	150	0	75	150	0	75	150	0	75	150	0	75	150
OPAA-02	92.31	1225				+	+	+	+	+	+	+			+	+	+	+	+	
		950		+	+	+	+	+	+	+	+				+	+	+		+	
		785		+	+												+	+		+
		615	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
		550											+	+	+		+	+	+	
		470	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
		350		+	+	+	+	+	+	+	+		+	+		+	+		+	+
		250															+			
		220												+		+			+	+
		200									+				+	+			+	
		145									+									
		120									+									
Polymorphic bands	12		2	5	5	5	5	5	5	5	8	3	4	5	6	7	8	4	6	6
Total bands	13		3	6	6	6	6	6	6	6	9	4	5	6	7	8	9	5	7	7
OPAA-10	66.67	680													+					
		555					+				+	+	+	+	+	+	+	+	+	+
		525				+	+		+											
		510												+		+	+	+	+	+
		420	+	+	+	+	+	+		+		+			+					
		340	+	+	+	+	+	+	+	+	+			+		+	+	+		+
		300							+	+	+									
		255					+		+	+	+			+						
Polymorphic bands	8		2	2	2	3	5	2	4	4	4	2	1	4	2	3	3	3	2	3
Total bands	12		6	6	6	7	9	6	8	8	8	6	5	8	6	7	7	7	6	7

Table 3. Continued.

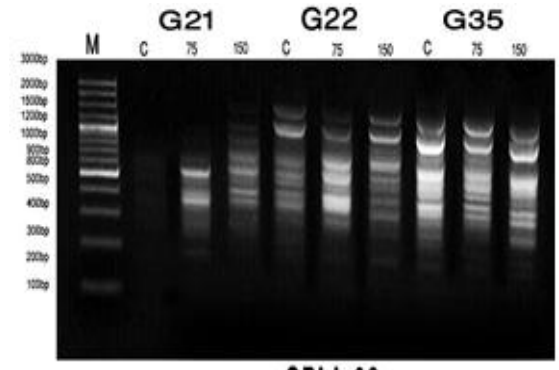
Primer name	P%	Base pair (bp)	Giza - 21			Giza - 22			Giza - 35			Giza - 82			Giza - 111			Crawford		
			0	75	150	0	75	150	0	75	150	0	75	150	0	75	150	0	75	150
OPAA-14	83.33	1100		+	+	+	+		+	+	+									
		600	+	+	+	+	+	+		+				+	+	+	+	+		
		510	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
		320	+	+	+	+			+		+	+	+	+	+	+	+	+	+	+
		240							+	+	+									
Polymorphic bands	5		3	4	4	4	3	1	4	4	4	2	2	3	3	3	3	3	3	2
Total bands	6		4	5	5	5	4	2	5	5	5	3	3	4	4	4	4	4	4	3
OPAA-15	94.12	800							+	+	+									
		615		+	+	+	+	+	+	+				+			+	+	+	+
		535		+	+	+	+					+	+	+			+	+	+	
		490		+	+	+	+	+	+	+	+			+			+	+	+	+
		470						+												+
		440							+	+	+									
		400									+									
		320		+	+	+	+											+		
		280	+	+	+	+	+	+	+	+	+						+	+	+	+
		265						+	+											
		255																+		+
		240				+	+				+							+		
		220		+																
		185					+	+												
		165								+	+									
		155									+									
Polymorphic bands	16		1	6	5	6	7	6	6	6	8	1	1	3	0	0	4	7	4	5
Total bands	17		2	7	6	7	8	7	7	7	9	2	2	4	1	1	5	8	5	6

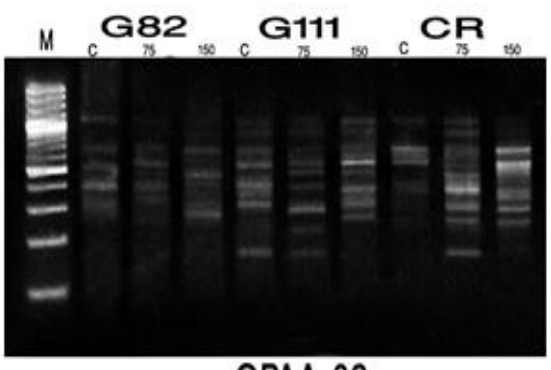
Table 3. Continued.

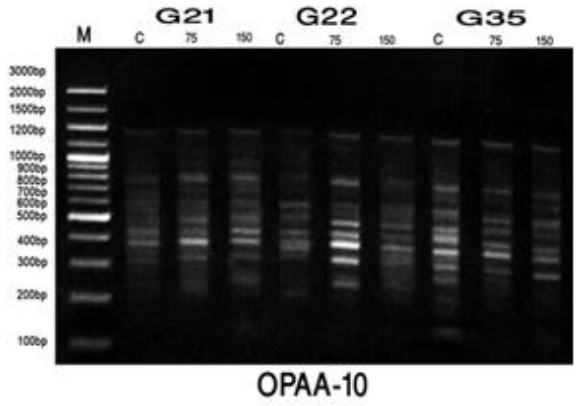
Primer name	P%	Base pair (bp)	Giza - 21			Giza - 22			Giza - 35			Giza - 82			Giza - 111			Crawford		
			0	75	150	0	75	150	0	75	150	0	75	150	0	75	150	0	75	150
G2	90.00	800		+	+			+	+	+	+	+	+	+	+	+	+	+		+
		670	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
		450	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
		400		+		+	+	+	+		+									
		380											+					+		+
		350	+	+	+		+	+	+		+		+	+		+		+	+	+
		280	+	+		+	+	+	+		+	+	+	+	+	+		+	+	
		250										+	+						+	+
		230									+									
Polymorphic bands	9		4	6	4	2	5	6	6	3	7	5	7	5	4	5	3	6	5	6
Total bands	10		5	7	5	3	6	7	7	4	8	6	8	6	5	6	4	7	6	7
G3	100.00	710				+														
		630							+											
		550									+									
		440	+	+	+	+	+	+	+				+	+	+	+	+	+	+	+
		350	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+
		280							+	+	+									
		250	+		+	+		+					+	+				+		+
		220							+	+	+									
		200	+	+	+	+	+	+	+	+	+	+	+	+	+			+		
		175		+	+															
		115		+		+	+	+							+					
Polymorphic bands	11		4	5	5	6	4	5	6	4	5	2	4	4	4	1	1	4	2	3
Total bands	11		4	5	5	6	4	5	6	4	5	2	4	4	4	1	1	4	2	3

Total polymorphic bands = 61

Overall total bands = 69 **Mean polymorphic percentage of the six primers = 87.74***


G21
G22
G35
M
C
75
150
OPAA-02


G82
G111
CR
M
C
75
150
OPAA-02


G21
G22
G35
M
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75
150
3000bp
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1200bp
1000bp
900bp
800bp
700bp
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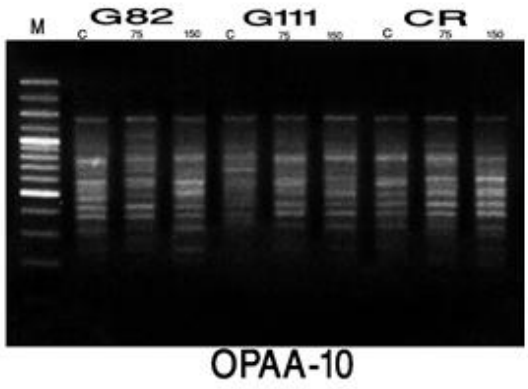
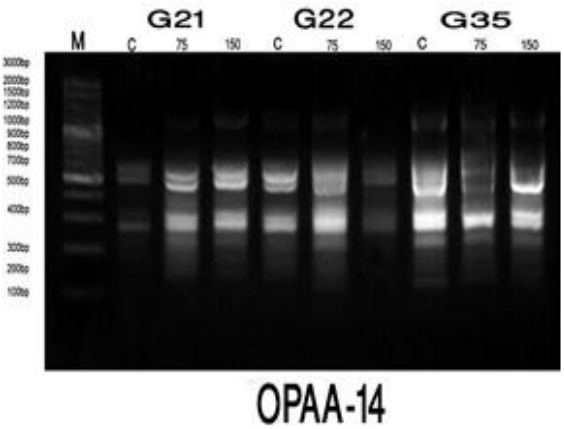
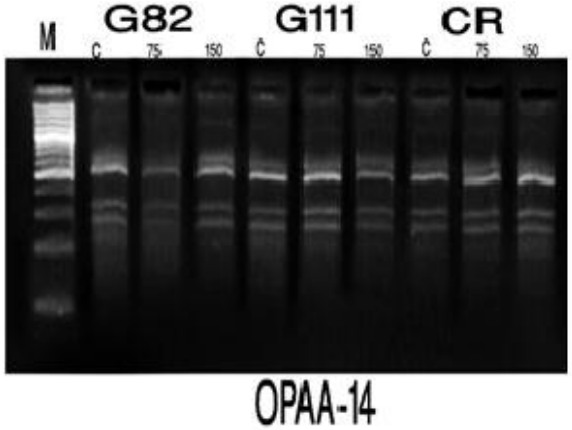
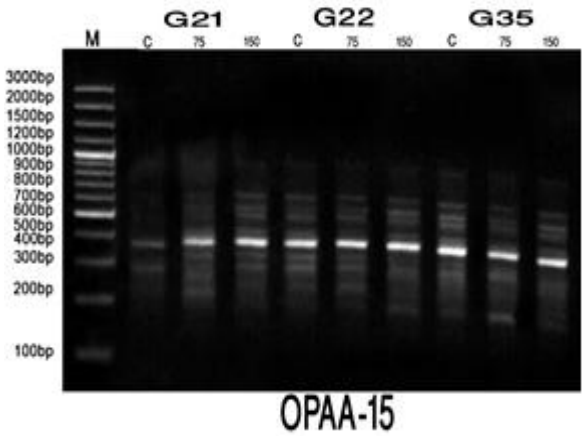
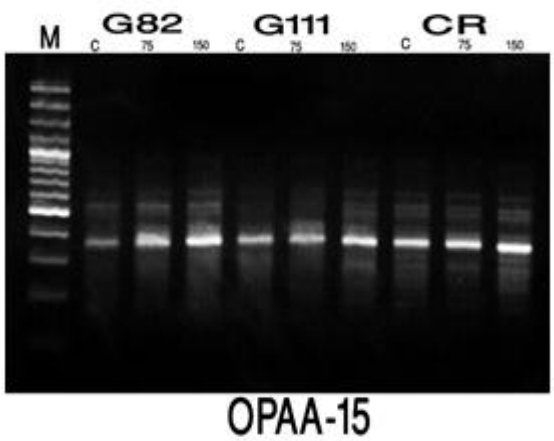
500bp
400bp
300bp
200bp
100bp
OPAA-10


G82
G111
CR
M
C
75
150
OPAA-10


G21
G22
G35
M
C
75
150
OPAA-14


G82
G111
CR
M
C
75
150
OPAA-14


G21
G22
G35
M
C
75
150
3000bp
2000bp
1500bp
1200bp
1000bp
900bp
800bp
700bp
600bp
500bp
400bp
300bp
200bp
100bp
OPAA-15


G82
G111
CR
M
C
75
150
OPAA-15

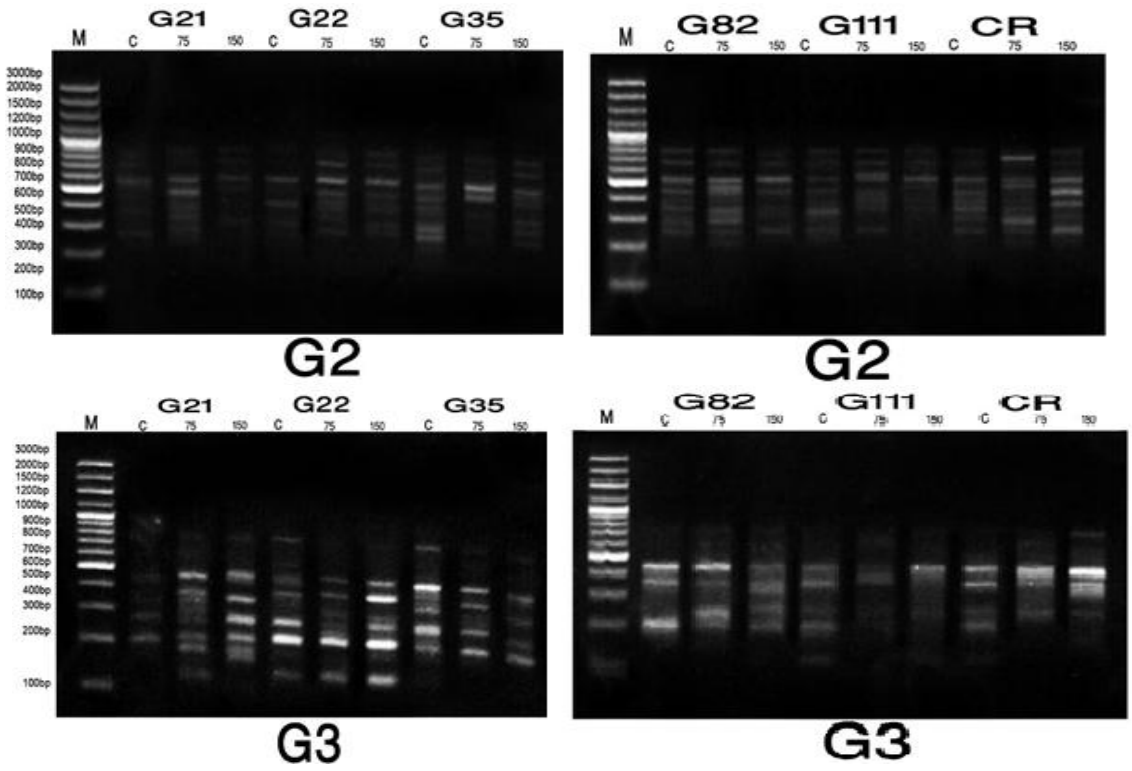


Figure 1: RAPD fingerprinting produced by six primers (OPAA-02, OPAA-10, OPAA-14, OPAA-15, G2 and G3) of six soybean cultivars treated with two levels (75 and 150 mM) of NaCl stress. (C): Control plants (treated with 0 mM NaCl). Soybean cultivars included Giza 21 (G21), Giza 22 (G22), Giza 35 (G35), Giza 82 (G82), Giza 111 (G111) and Crawford (CR). M: DNA Marker.

The results obtained from RAPD analysis in the present study are in agreement with those of **Dogan *et al*. (2012)**, who reported that genetic instability induced by NaCI treated seedlings of cotton was reflected by changes in RAPD profiles through the decrease or the increase in band intensity, disappearance of bands, and appearance of new bands occurred in the profiles in comparison to the controls. It can be concluded that a relatively high number of polymorphic bands were found and could be used as markers.

Among the 69 amplified bands, 61 were polymorphic and among them 41 bands were specific markers for salinity tolerance either at 75 or at 150 mM NaCl with a total average percentage polymorphism of 56.18 % (**Table 4**). It is interesting to note that the six soybean cultivars varied considerably in their salinity tolerant markers using the six primers, whereas at 75 mM NaCl Giza 21 revealed the highest number (13) of markers, followed by Giza 22 and Giza 82 with six and Giza 111 revealed five salinity markers. However, Giza 35 showed the lowest total number (4) of marker bands at 75 mM NaCl. Moreover, at 150 mM NaCl, Giza 82 revealed the highest number with 13 markers, followed by Giza 35 with 11 markers and Giza 21 with 10 markers at 150 mM NaCl. However, Crawford showed the lowest total number of marker bands at 150 mM NaCl. In addition, Giza 21 and Giza 82 revealed the highest total number of marker bands at both NaCl concentrations with 23 and 19 markers, respectively followed by Giza 35 with a total of 15 markers and Giza 111 with 14 markers (**Table 4**). On the other hand, RAPD primer G2 showed the highest marker percentage (80 %), followed by primers OPAA-15 and OPAA-02with 76.47 and 69.23 %, respectively while primer OPAA-14 displayed the lowest salinity marker percentage with 33.33 % compared with the other five RAPD primers, as shown in **Table 4**. Similar result was also reported by **Iqbal *et al.* (2007)** who emphasized that RAPD analysis provides a rich source of specific markers that can be used for characterizing and grouping wheat genotypes, which will be helpful in wheat breeding programs.

Table 4: Specific positive bands related to salinity tolerance in six soybean cultivars at 75 and 150 mM NaCl compared to control as revealed by RAPD analysis.

Primer name	% P	Number of markers with size (bp)	Giza 21		Giza 22		Giza 35		Giza 82		Giza 111		Crawford	
			75	150	75	150	75	150	75	150	75	150	75	150
OPAA-02	69.23	950	+	+									+	
		785	+	+								+		
		550							+	+				
		350	+	+					+	+	+	+	+	+
		250										+		
		220								+	+		+	+
		200						+					+	
		145						+						
		120						+						
Positive bands	**9**		**3**	**3**	**0**	**0**	**0**	**3**	**2**	**3**	**2**	**3**	**4**	**2**
Total	**13**													
OPAA-10	41.67	555			+			+						
		510								+	+	+		
		420						+						
		340								+	+	+		
		255			+					+				
Positive bands	**5**		**0**	**0**	**2**	**0**	**0**	**2**	**0**	**3**	**2**	**2**	**0**	**0**
Total	**12**													
OPAA-14	33.33	1100	+	+										
		600					+			+				
Positive bands	**2**		**1**	**1**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**
Total	**6**													
OPAA-15	76.47	615	+	+						+		+		
		535	+	+								+		
		490	+	+						+		+		
		470				+								+
		400						+						
		320	+	+										
		280										+		
		265				+								
		240						+						
		220	+											
		185			+	+								
		165					+	+						
		155						+						
Positive bands	**13**		**5**	**4**	**1**	**3**	**1**	**4**	**0**	**2**	**0**	**4**	**0**	**1**
Total	**17**													

Table 4: Continued.

G2	**80.00**	800	+	+		+								
		670			+	+								
		450			+	+								
		400	+											
		380							+					
		350							+	+	+			
		250											+	+
		230						+						
Positive bands	**8**		**2**	**1**	**2**	**3**	**0**	**1**	**2**	**1**	**1**	**0**	**1**	**1**
Total	**10**													
G3	**36.36**	550						+						
		250							+	+				
		175	+	+										
		115	+											
Positive bands	**4**		**2**	**1**	**0**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**0**
Total	**11**													
Total positive bands = 41														
Mean polymorphism (%) = 56.18														

Using RAPD technique to detect specific molecular markers for economically important traits is important to distinguish between different cultivars by comparing polymorphism in genomic fingerprints (**Echt *et al.*, 1992; Bahieldin *et al*., 1994**).The results of the present study are in accordance with those **Fahmy *et al.* (1997)** who used RAPD technique to differentiate between drought tolerant and drought sensitive genotypes of berseem clover (*Trifolium alexandrinum* L.) and obtained two positive molecular markers under stress. Also, **Wenzel (1992)** emphasized the potential for DNA markers- based diagnosis of abiotic stress tolerance in plants. The results of the present investigation are in parallel with those of **Abdel-Tawab *et al*. (2001)**, who detected two positive and two negative molecular markers for salt tolerance in maize using bulked sergeant's analysis.

Many other successful attempts to detect RAPD markers for salt or drought tolerance were reported. For example, in tomato, **Foolad and Chen (1998)** identified RAPD markers associated with quantitative trait loci (QTLs) conferring salt tolerance during germination. **Lee *et al.* (2003)** identified two RAPD markers in salt-tolerant rice lines and the transcript involved in the marker showed a higher expression in the salt-tolerant lines than the sensitive lines. **Rania *et al.* (2007)** developed four RAPD markers for salt tolerance in sorghum and **Abdel-Tawab *et al.* (1997 and 1998)** detected one RAPD marker for salt tolerance and three for drought tolerance in maize. **Pakniyat *et al*. (2004)** introduced markers linked to salt tolerance in cultivated and wild barley using RAPD-PCR. **Abdel-Bary *et al*. (2005)** recorded eight positive and negative RAPD markers for salinity tolerance in maize. Also, **Nazari and Pakniyat (2008)** detected markers associated with drought tolerance in wild and cultivated barley genotypes using RAPD markers. **Khan *et al.* (2013)** used twenty RAPD primers to study the genetic variation among ten soybean genotypes. They are concluded that closer soybean cultivars in the cluster behaved similarity in their response to salinity tolerance.

3.2. ISSR molecular markers related to salinity stress

A total of 39 scorable amplified DNA fragments ranging in size from 235 to 1700 base pairs were observed using the four primers (**Table 2**), whereas 36 fragments were polymorphic and the other amplified fragments were commonly detected among the six soybean cultivars at 75 and 150 mM NaCl as well as the untreated plants. The four primers showed 91.57 % mean polymorphism whereas the polymorphism of primer 44-B was the highest (100 %), however, the polymorphism of primer 14-A was the lowest (85.71 %) (**Table 5**).

Primer 14A revealed 7 fragments with sizes ranged from 390 to 1410 bp **(Table 5, Fig. 2**), whereas 6 fragments were polymorphic (85.71 %). Giza 82 soybean cultivar treated with 75 and 150 mM NaCl appeared one induced amplified band with 960 bp at both of the two NaCl concentrations, which did not exist in the control plant. One induced band (960 bp) was displayed at 150 mM NaCl concentration in Giza 35 genotype. Three unique bands with molecular sizes of 870, 765 and 390 bp were observed in Giza 21, Giza 22 and Giza 82 cultivars, respectively.

Primer 44B revealed 11 fragments with sizes ranged from 320 to 1090 bp **(Table 5, Fig. 2**), all of them were polymorphic (100 %). ISSR analysis shows some amplified bands that were newly synthesized in salinity stressed plants and that did not produced in control plants. Giza 21 genotype treated with 75 and 150 mM NaCl exposed one induced amplified bands with 730 bp at 150 mM NaCl concentration, which did not exist in the control. Giza 22 appeared three bands (1090, 695 and 600 bp) at treated with 150 mM NaCl and one band (820 bp) at both75 and 150 mM NaCl. However, two amplified bands (420 and 320 bp) were detected in Giza 35 at both NaCl concentrations compared with control plants. At the same orientation, two different bands (820 and 600 bp) were observed in Giza 111 and Crawford genotypes when treated with 75 and 150 mM NaCl, respectively. Crawford genotype exhibited two amplified bands (730 and 525 bp) at treated with 150 mM NaCl

ISSR analysis using primer HB-11 showed the highest numbers of amplified fragments compared with the other three ISSR primers. Primer HB-11 revealed 12 detectable amplified fragments with molecular sizes ranging from 235 to 1700 bp **(Table 5, Fig. 2**). Eleven fragments were polymorphic (91.67 %). Two induced bands with molecular size of 1050 and 300 bp were displayed among Crawford and Giza 82, respectively under salinity stress that had not existed in the control. Five induced amplified bands were existed at 75 mM NaCl among four soybean cultivars, where Giza 21 showed three induced bands (1700, 890 and 235 bp) and it was shared with Crawford cultivar in the first band (1700 bp). The last two bands (1375 and 1135 bp) were observed among Giza 22 and Giza 82 respectively. Four induced bands were appeared at 150 mM NaCl among three soybean cultivars, for instance, Giza 35 showed two induced bands with molecular sizes of 1290 and 1050 bp and Giza 111 cultivar shared the last band with Giza 35. At the same treatment, Giza 82 cultivar displayed two induced bands (910 and 550 bp). One unique band with molecular size of 510 bp appeared in Giza 21 cultivar treated with 150 mM NaCl.

Primer HB-12 revealed 9 fragments with sizes ranged from 290 to 1275 bp **(Table 5, Fig. 2**), whereas eight fragments were polymorphic (88.89 %). Four induced bands with molecular size of 980, 460, 390 and 290 bp were displayed at both of the two NaCl concentrations in four soybean cultivars and which did not exist in the control plants. The first band was observed in Giza 35, while the second band was detected in Giza 82. The third band was noted in both of Giza 22 and Crawford cultivars, however, the fourth band appeared in Giza 82 cultivar. Two induced bands (800 and 710 bp) were observed in Giza 35, Giza 111 and Crawford cultivars treated with 75 mM NaCl, while the same two bands were appeared in Giza 82 and Giza 21 cultivars at treated with 150 mM NaCl. It was observed presence of one unique band (580 bp) in Giza 35 treated with 150 mM NaCl. Our results are in harmony with the findings of **Yang *et al*. (1996)**, who used the ISSR and microsatellites in soybean cultivars discrimination and reported that microsatellites represent an excellent technique to study the genetic polymorphism. It can be concluded that a relatively high number of polymorphic bands were found and could be used as markers.

Among the 36 polymorphic bands, 30 were newly induced and considered as positive markers for salinity tolerance either at 75 or at 150 mM NaCl with 74.83 % percentage polymorphism (**Table 6**). The six soybean genotypes varied considerably in their salt tolerance markers, whereas at 75 mM NaCl Giza 82, Giza 21 and Crawford revealed the highest number (five) of markers, followed by Giza 35 with four and Giza 111 revealed three salinity markers. Moreover, at 150 mM NaCl, Giza 82 revealed the highest number (eight marker bands), followed by Giza 35 with 7 markers and then Giza 22 with six (**Table 6**). On the other hand, ISSR primer HB-11 showed the highest marker percentage (91.67%), followed by primers HB-12 and 44-B with 77.78 and 72.73 %, respectively while primer 14-A displayed the lowest salinity marker percentage with 57.14 % compared with the other three ISSR primers, as shown in **Table 6**.

Table 5: ISSR analysis of polymorphic bands for six soybean cultivars under 75 and 150 mM NaCl using four primers (14 A, 44 B, HB-11 and HB-12). +: present of amplified bands, P %: polymorphic percentage, Total: total number of amplified bands. Black boxes refer to the positive bands that are present only at 75 and / or 150 mM NaCl and absent from the control. * (85.71+100.00+91.67+88.89= 366.27), then Average = 366.27/4= 91.57 %.

Primer name	P %	Base pair (bp)	Giza - 21			Giza - 22			Giza - 35			Giza - 82			Giza - 111			Crawford		
			0	75	150	0	75	150	0	75	150	0	75	150	0	75	150	0	75	150
14 A	85.71	1410				+														
		960									+		+	+						
		870		+																
		765						+												
		750	+		+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
		390												+						
Polymorphic bands	6		1	1	1	2	1	2	1	1	2	1	2	2	1	1	1	1	1	1
Total bands	7		2	2	2	3	2	3	2	2	3	2	3	3	2	2	2	2	2	2
44 B	100.00	1090						+							+					
		860	+	+	+													+		+
		820					+	+				+	+	+		+	+			
		780	+	+					+		+							+	+	
		730			+				+	+										+
		695						+	+											
		600	+	+	+			+											+	+
		525				+											+			+
		490																+	+	+
		420								+	+									
		320								+	+									
Polymorphic bands	11		3	3	3	1	1	4	3	3	3	1	1	1	1	1	2	3	3	5
Total bands	11		3	3	3	1	1	4	3	3	3	1	1	1	1	1	2	3	3	5

Table 5. Continued.

HB-11	91.67	**1700**		+									+						+	
		1375	+	+			+						+	+		+	+	+		
		1290									+									
		1135												+					+	+	+
		1050	+	+								+	+		+			+		+	+
		910							+		+				+						
		890		+		+	+	+					+			+	+	+			
		550													+						
		510		+																	
		300	+	+	+	+		+	+					+	+	+	+		+	+	+
		235		+									+								
Polymorphic bands	11		3	7	1	2	2	2	2	0	3	5	3	4	4	3	3	2	4	3	
Total bands	12		4	8	2	3	3	3	3	1	4	6	4	5	5	4	4	3	5	4	
HB-12	88.89	**1275**										+									
		890								+	+										
		800								+				+							
		710			+	+										+			+		
		580									+										
		460	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
		390	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+		+	+
		290	+						+	+	+		+	+	+	+	+				
Polymorphic bands	8		3	2	3	2	2	2	3	5	5	2	3	4	3	4	3	1	3	2	
Total bands	9		4	3	4	3	3	3	4	6	6	3	4	5	4	5	4	2	4	3	
Total variable bands = 36																					
Overall total bands = 39															**Mean polymorphic percentage of the six primers = 91.57***						

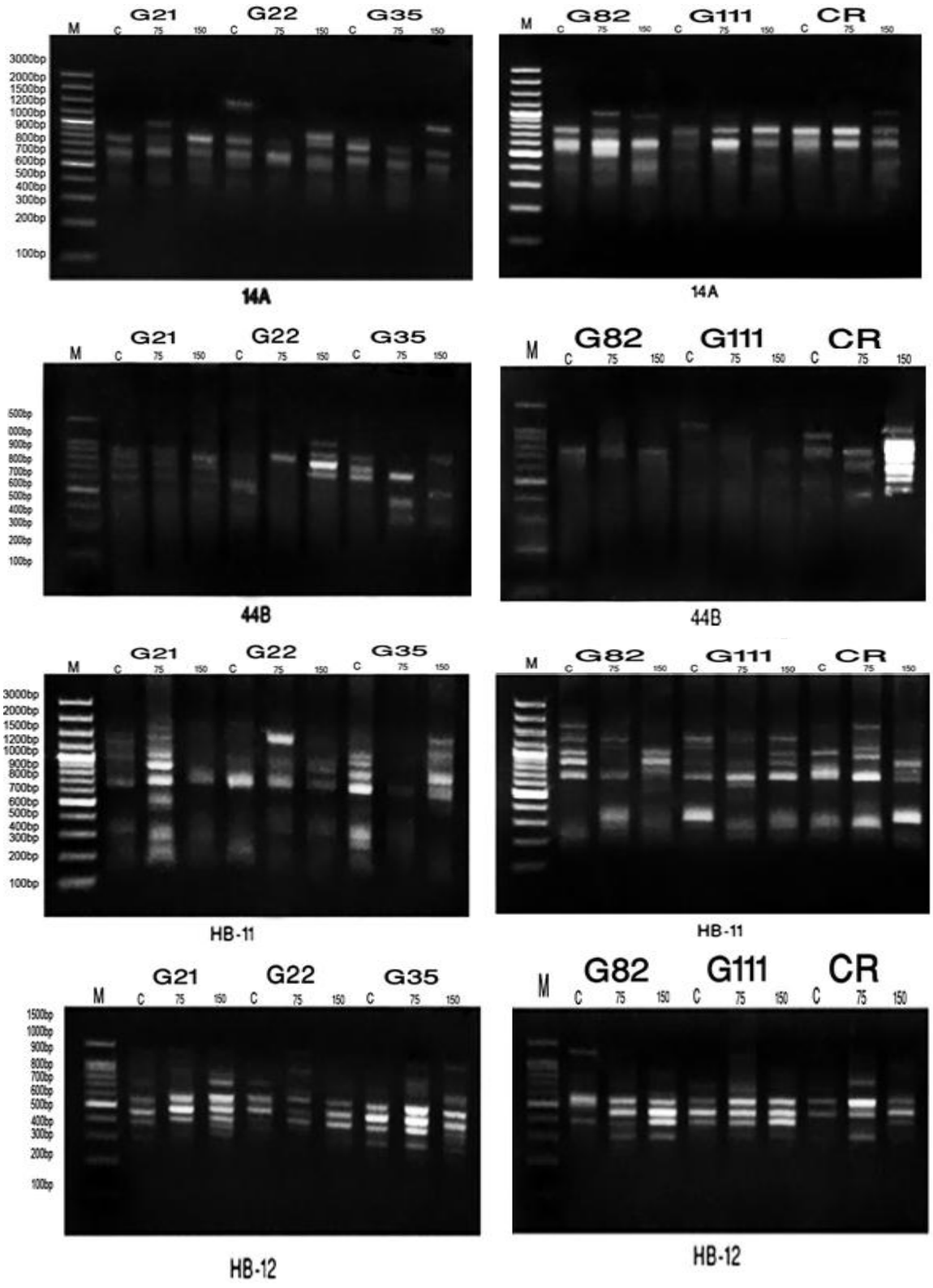


Figure 2: ISSR fingerprinting produced by four primers (14A, 44B, HB-11 and HB-12) of six soybean cultivars treated with two levels (75 and 150 mM) of NaCl salt stress. C: Control plants (treated with 0 mM NaCl). Soybean cultivars included Giza 21 (G21), Giza 22 (G22), Giza 35 (G35), Giza 82 (G82), Giza 111 (G111) and Crawford (CR). M: DNA Marker.

Table 6: Specific positive bands related to salinity tolerance in six soybean cultivars at 75 and 150 mM NaCl compared to control as revealed by ISSR analysis.

Primer Name	P%	Number of markers with size (bp)	Giza 21		Giza 22		Giza 35		Giza 82		Giza 111		Crawford	
			75	150	75	150	75	150	75	150	75	150	75	150
14 A	57.14%	960						+	+	+				
		870	+											
		765				+								
		390								+				
Positive bands	4		1	0	0	1	0	1	1	2	0	0	0	0
Total	7													
44 B	72.73%	1090				+								
		820			+	+					+	+		
		730		+										+
		695				+								
		600				+								
		525										+		+
		420					+	+						
		320					+	+						
Positive bands	8		0	1	1	4	2	2	0	0	1	2	0	2
Total	11													
HB-11	91.67%	1700	+										+	
		1375			+									
		1290						+						
		1135							+					
		1050						+				+	+	+
		910								+				
		890	+											
		550								+				
		510	+											
		300							+	+				
		235	+											
Positive bands	11		4	0	1	0	0	2	2	3	0	1	2	1
Total	12													
HB-12	77.78%	890					+	+						
		800					+			+				
		710		+							+		+	
		580						+						
		460							+	+				
		390			+	+							+	+
		290							+	+				
Positive bands	7		0	1	1	1	2	2	2	3	1	0	2	1
Total	9													
Total marker bands = 30														
Mean polymorphism (%) = 74.83														

Association between salinity tolerance and ISSR markers based molecular polymorphism were investigated by many researchers. For example, **Kaushik *et al.* (2003)** obtained four ISSR polymorphic bands in some of the CSR10 × HBC19 F3 plants the F3 plants raised from a crossed between salt-tolerant *indica* rice variety CSR10 and salt-susceptible Basmati rice variety Basmati HBC19. Such polymorphic bands stand greater chances of having a linkage with the genes/quantitative trait loci (QTLs) for salinity tolerance. **Rania *et al.* (2007)** identified some ISSR and RAPD genetic markers associated with salt tolerance for two studied inbred lines of grain sorghum growing at the field. **Reddy *et al.* (2009)** used ISSR markers to study genetic relationship among nine rice varieties tolerant to drought, flood and salinity. They are found that salt-tolerant varieties were closest to two flood-tolerant varieties, and together they were distinct from the drought-tolerant varieties. **El-Nahas *et al.* (2011)** used ISSR technique to detect some molecular markers associated with drought tolerance in six local and exotic lentil genotypes. Out of five ISSR primers, they were recognized six bands considered as molecular markers associated with drought tolerant genotypes.

The results in the present study confirmed that ISSR was the most useful method to detect molecular markers for salinity tolerance in soybean. This was confirmed by **Hou *et al.* (2005)** who studied the genetic diversity in barley from west China and reported that the ISSR markers were superior to RAPD markers in the capacity of revealing more informative bands in a single amplification. Similar ISSR results were detected in barley (**Fernandez *et al.*, 2002** and **Tanyolac, 2003).** The difference is perhaps explained by the difference in the DNA segments targeted by the two methods, and is consistent with some previous studies which reported that ISSR markers are more polymorphic than RAPD markers (**Godwin *et al.*, 1997; Youssef *et al.*, 2010**).

The variation in the DNA banding profile of soybean cultivars exposed to salinity stress may be attributed to the activation of the defense responsive genes whose transcripts and expression are controlled under salinity stress. Supporting this view, **Muramoto *et al.* (1999)** who isolated *Bnuc1* gene, encoding a nuclease I, from leaves of salt stressed barley and they noticed that the transcript of *Bnuc1* gene increased dramatically in barley leaves under salt stress. They reported that the salt-inducible nuclease activity possibly corresponds to this gene. In addition, both transcriptional and post-transcriptional regulation is assumed to be responsible for developmental changes in gene expression during stress (**Fukuda and Tanaka 2006**).

It was observed that response of soybean cultivars to NaCl treatment at 75 mM concentration was variable, where four cultivars (Giza 82, Giza 35, Giza 22 and Giza 21) appeared positive response, while Giza111 and Crawford exhibited negative response toward treatment with 75 mM NaCl salt stress. Giza 82 and Giza 35 cultivars seemed to have the greatest number of positive markers at 150 mM NaCl, suggested that these two cultivars exerted an effective degree of salt-tolerance which consequently enables such cultivars to survive under conditions of their growth in salty soil. The apparent differential responses of such cultivars might be related to the impact of salinity stress on alteration in DNA structure implicating RNA transcription and gene expression. Supporting this view, **Kantar *et al* (2010)** who demonstrated presence of a positive correlation between levels of mRNA expression and suppression of their target mRNA transcripts in dehydration-stress-treated barley. Such manner of change could be reflected in a modulation in DNA, fingerprints of salt-stressed soybean plants which ultimately determine their capacity to tolerate their growth under salt stress conditions.

3.3. Protein markers related to salinity stress

The total soluble proteins were separated electrophoretically using SDS-PAGE technique in order to find out genotypic variation in specific accumulation salt induced protein upon the salt stress condition **(Table 7, Fig. 3)**. The major variations are expressed as changes in appearance or disappearance of some bands. A total of 21 bands were detected with different molecular weights ranging from 235.6 kDa to 10.5 KD. These protein bands has distributed into 12 monomorphic bands (57.2%) and 9 polymorphic bands (42.8%). Giza 35 soybean cultivar showed the highest number of protein bands (19 bands) and Crawford soybean cultivar appeared the lowest number of protein bands (13 bands) at 150 mM NaCl. The unique bands appeared only in G35 (39.1 KDa) and G22 (11.8 KDa) at 150 mM NaCl. Giza 22 cultivar showed protein band (75.8 kDa) in both of treated and non-treated plants and not found in other five soybean cultivars (**Table 7)**.

Table 7: Effect of two concentrations (75 and 150 mM) of NaCl salt stress on leaf protein banding patterns for six soybean cultivars. Soybean cultivars involved (Giza 21, Giza 22, Giza 35, Giza 82, Giza 111 and Crawford. (C) Control plants (treated with 0 mM NaCl); (+) presence of band; (-) absence of band; M.W= Molecular weight. Color sign refer to the positive bands.

Band No.	M.W (KD)	Giza 21			Giza 22			Giza 35			Giza 82			Giza 111			Crawford		
		C	75	150	C	75	150	C	75	150	C	75	150	C	75	150	C	75	150
1	235.6	+	+	+	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-
2	195.1	+	-	-	+	-	-	+	+	+	-	+	+	+	+	+	-	-	+
3	169.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	137.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	117.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	102.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	87.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	75.8	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
9	65.1	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-
10	54.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	43.2	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	-	-
12	39.1	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
13	33.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	30.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	26.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	21.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	16.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	13.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	11.8	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
20	11.6	-	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-
21	10.5	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	-	-
Total	21	16	17	17	17	17	18	16	17	19	13	15	17	16	16	17	13	12	13
Positive bands			2	2		1	2		2	3		3	4		1	1			1

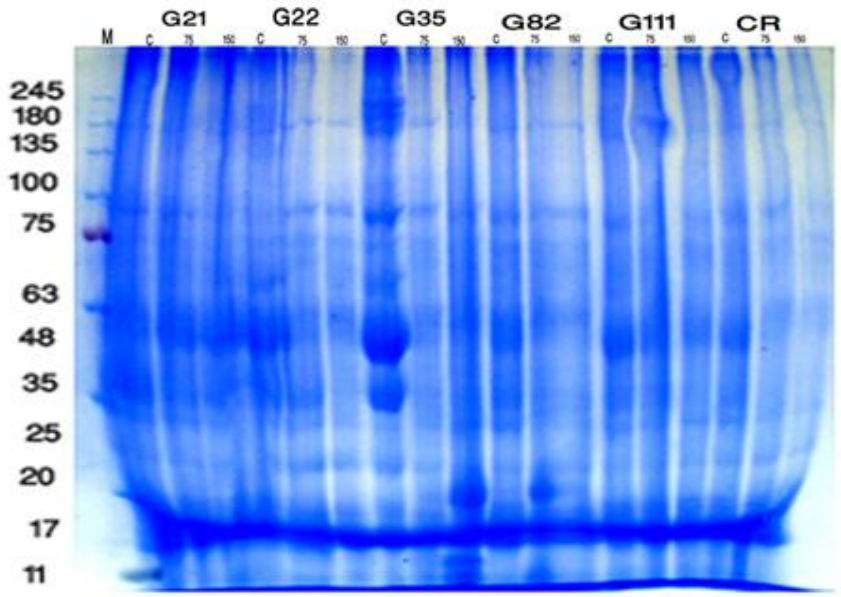


Figure 3: SDS-PAGE protein banding pattern from leaves of six soybean cultivars treated with 75 and 150 mM NaCl. Soybean cultivars involved Giza 21 (G21), Giza 22 (G22), Giza 35 (G35), Giza 82 (G82), Giza 111 (G111) and Crawford (CR). M= Protein marker and C= Control plants (treated with 0 mM NaCl).

In Giza 21, it was observed absence of a protein band (195.1 kDa) and presence of two new protein bands (65.1 and 11.6 kDa) under the two levels of NaCl compared with control plants. In Giza 22, one protein band with molecular weight of 195.1 kDa was disappeared at the two levels of NaCl compared with control plants. Moreover, two new protein bands (one with molecular weight of 11.6 kDa at both NaCl concentrations, and one with molecular weight of 11.8 kDa at 150 mM NaCl) were observed compared with control plants. Two new protein bands with molecular weights of 43.2 and 10.5 kDa appeared under both levels of NaCl concentrations in Giza 35 and Giza 82 as compared with the non-treated plants. In addition to a newly protein bands with molecular weight of 195.1 kDa was produced under both levels of NaCl treatments for Giza 82 soybean cultivar. In Giza111, one protein band with molecular weight of 10.5 kDa was newly formed under both levels of NaCl treatment at compared with non-treated plants. In Crawford, one protein band with molecular weight of 235.6 kDa was absent under both levels of NaCl at compared with non-treated plants. In general, Giza 82 showed the highest number of a new protein bands (3 bands) and Giza 111 appeared the lowest number of a new protein bands (1 band) under both levels of NaCl treatment indicated that induction of synthesis a new proteins may play an important role in soybean response to salt stress.

The changes in protein profile could reveal information about tolerance mechanism of plants growing under salt stress. The tolerance reaction might be resulted from rapid synthesis or less degradation of responsive proteins to salinity stress especially for the proteins that possess a higher molecular weight (Arefian *et al*., 2014**)**. One possible explanation for appearance of some proteins under salt stress is that the gene (s) responsible for certain proteins had been completely enhanced as a result of stress **(Amal *et al*., 2010)**. It is also possible that the genes had not been completely suppressed but inhibited as the result of stress and complete recovery of the inhibition was not achieved **(Amal, 2005)**. The specifically synthesized protein under salt stress appears to have a role in providing adaptation to plants. **El-Farash et al. (1993)** studied the expression of 12 different proteins bands, which were induced in salt stressed tomato plants. They reported that the expression of these proteins was genetically regulated, depending on the salt concentration. **Hassanein (1999)** reported that NaCl treatment of peanut plants (*Arachis hypogaea*) promoted induction (127 and 52 kDa) or repression (260 and 38 kDa) in the synthesis of few polypeptides. Variation in protein pattern via the appearance of new bands and disappearance of the others, of different soybean cultivars under salt stress would indicate either enhancement or repression of gene expression in these cultivars. This might alter the produced proteins in response to salt stress either on the transcription or post-transcription levels of gene expression. Similar conclusions were recorded **(Jonathan et al., 1990; Fahmy et al., 1992; Rashed et al., 2006; Khalil, 2013).** Our results are parallel with those of several authors, who used some total protein pattern electrophoresis variations to evaluate some cultivars under different salt stress to be used as biochemical genetic markers for early evaluation (**Rashed et al., 2006**; **Sayed and gabr, 2013**). Therefore, soybean plants growing under salinity stress might be adapted to such stress through promoted an acceptable variation in the protein banding patterns. Supporting this view the result that obtained by **Sobhanian *et al.* (2010),** who reported that 19, 22 and 14 out of 340, 330 and 235 bands extracted from soybean leave, hypocotyl and roots, respectively were up and down regulated by treatment with 40 mM NaCl for one week. Their results revealed that metabolism related proteins were mainly down-regulated due to NaCl treatment suggested that these proteins play a role in each organ in adaptation to saline condition.

Several newly synthesized proteins in response to abiotic stresses were reported as stress-proteins (**Patharkar and Cushman, 2000**). **Yen *et al*. (1997)** reported that five polypeptides with molecular weights of 40, 34, 32, 29 and 14 kD were accumulated in the callus of *Mesembryanthemum crystallinum* under salt stress. Also, **Tamas *et al*. (2001)** reported that salinity induced accumulation of four polypeptides in maize roots with molecular weights of 61, 51, 39 and 29 kD. **Rangan and Swaminathan (2002)** recorded that out of 58 bands detected by Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in *Potresia coarctata*, 35 are affected by salt stress. **Barakat (2003)** concluded that the proteins with molecular weights of 68, 52, 46, 43, 35, 33, 18 and 11 KD could play an important role in sever salinity tolerance. Changes in protein synthesis under salt stress may be due to changes in the efficiency of mRNA translation or the regulation of RNA transcription, transport and constancy (**Barakat, 2003; Sobhanian *et al.*, 2010**). However, **Sohrabi *et al*. (2011)** attributed the decrease in protein content by salinity to the decreased rate of protein synthesis, the increased activities of hydrolyzing enzymes, the decreased availability of amino acids, or the

denaturation of the enzymes concerned with amino acids and protein synthesis.

3.4. Peroxidase (POD) isozyme marker related to salinity stress

The differential electrophoretic profile of POD isozyme extracted from the leaves of six soybean cultivars growing under effect of three concentrations (0, 75 and 150 mM) of NaCl were observed. After the native polyacrylamide gel electrophoresis (PAGE) analysis, we identified up to seven distinct bands of POD isozymes in six soybean cultivars at different RF values varying from 0.24 to 0.67 (**Table 8, Fig. 4**). In Giza 21, a new band was reported at both of 75 and 150 mM NaCl compared with control plants. In Giza 22, two new bands at both of 75 and 150 mM NaCl were detected compared with control plants. In case of Giza 35, two new bands appeared at both of 75 and 150 mM NaCl and one new band were observed at 150 mM NaCl as compared with non-stressed plants. In Giza 82, one new band was observed at both NaCl concentrations and one new band at 150 mM NaCl compared with non-stressed plants. For Giza111 and Crawford, a new band was detected at both of 75 and 150 mM NaCl compared with non-stressed plants. Our results also revealed that the intensities of seven POD isoforms were enhanced under salinity stress. (**Fig. 4**). The increase of band intensity and appearance of new bands may be an indication of an increase in POD activity under salinity conditions.

Enzymes encoded by different alleles of a specific locus or by separate loci commonly show different electrophoretic mobilities due to variations in the amino acids sequence of the enzyme molecules, which in turn dependent on the sequences of nucleotides in DNA (**Micales *et al*., 1992**). The multiple isoforms of enzymes is one of the primary control mechanisms of cellular metabolism in plants and the change in the isozyme profiles plays an important role in the cellular defense against salt stress **(Amal *et al*., 2010)**. The profiles of POD isozyme showed that the number of produced bands between six soybean cultivars was related to NaCl concentrations and the soybean genotype. The preview results indicated that few bands disappeared or newly appeared in the salt stress as compared to control. These results are in parallel with those of several authors, who used some isozymes pattern electrophoresis variations to evaluate some cultivars under different salt stress to be used as molecular markers **(Rashed *et al*., 1994; Rashed *et al*., 2006; Sayed and Gabr, 2013; Moharramnejad and Valizadeh, 2014; Saad-Allah, 2015)**.

Concerning to the positive markers that may be related to salt - tolerance, our results showed that Giza 35 have the highest numbers (3) at both NaCl concentrations compared to others. These results resemble those obtained by **El-Baz *et al*., (2003)** who used peroxidase isozyme and protein pattern as a marker for salt stress tolerance in cucumber plants. They are found that the profile of peroxidase isozyme was modified during salt stress conditions, also a new subset of proteins induced by salt stress compared to control plant was observed. This behavior may be due to its ability to tolerate salt stress or due to the effect of salt stress which may cause some shift in gene expression.

Rashed *et al*., (1994) observed occurrence of different response in the decrease of intensity rather than in the isoforms of peroxidase in favor of salt tolerant genotype under stress. **Hassanein (1999)** detected presence of 9 different esterase isozymes in embryos of seeds germinated in 105 mM NaCl of peanut (*Arachis hypogaea*) plant, whereas only 5 of them were detected in the embryos of untreated seeds. The ability of peanut to grow at high concentrations of NaCl may be due to the alteration in gene expression. **Diego *et al*. (2003)** found that the POD activity significantly increased in salt-tolerant cotton cultivars but was unchanged in salt-sensitive cotton cultivars under salt stress. **Rahnama and Ebrahimzadeh (2006)** reported that POD isozyme profiles at 100 mM NaCl were different from that of the control. These differences were quantitative and were expressed more in terms of increased or decreased isozymes activities. **Gao *et al*. (2008)** recorded that increased POD activities might enable plants to protect themselves against salt stress. In fact, the POD enzyme has been experienced in a numerous cases of stress effects **(Turhan et al., 2008; Németh *et al*., 2009; Saad-Allah, 2015)**. Their susceptibility can be related to their gene transcriptions influenced by abiotic stress factors **(Jaleel *et al*., 2009).** POD and SOD isozymes showed positive correlations for salinity stress of alfalfa plant (**Valizadeh *et al*. 2013**). Also, SOD isozyme was found to be associated to salt tolerance in genotypes of red bean **(Moharramnejad and Valizadeh, 2014).**

Table 8: Effect of two concentrations (75 and 150 mM) of NaCl salt stress on peroxidase isozyme patterns for six soybean cultivars. Soybean cultivars involved (Giza 21, Giza 22, Giza 35, Giza 82, Giza 111 and Crawford. (C) Control plants (treated with 0 mM NaCl), (+) presence of band, (-) absence of band, (RF) relative mobility. RF is the distance traveled by each protein divided by the distance traveled by the tracking dye. Color signs refer to the positive bands.

Band No.	RF	Giza 21			Giza 22			Giza 35			Giza 82			Giza 111			Crawford		
		C	75	150	C	75	150	C	75	150	C	75	150	C	75	150	C	75	150
1	0.24	-	+	+	-	+	+	-	+	+	+	+	+	-	+	+	-	+	+
2	0.35	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-
3	0.45	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	0.54	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	+	+	-
5	0.62	+	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+
6	0.64	-	-	-	+	-	-	+	+	+	-	+	+	-	-	-	-	-	-
7	0.67	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Total		3	4	4	5	6	5	4	6	7	5	4	6	4	4	5	4	5	4
Positive bands			1	1		2	2		2	3		1	2		1	1		1	1

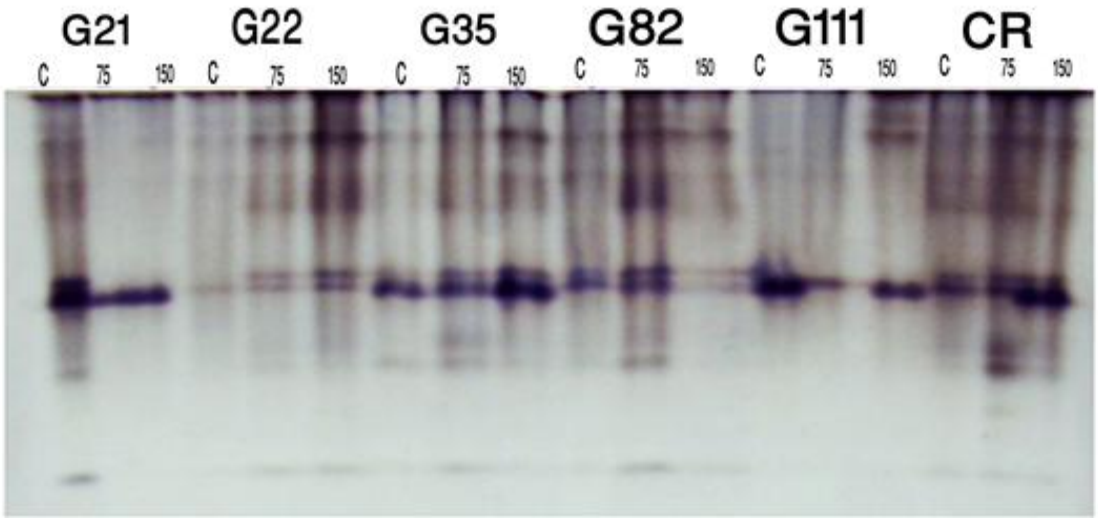


Figure 4: Native polyacrylamide gels for the peroxidase isozymes in six soybean cultivars treated with 75 and 150 mM NaCl. Soybean cultivars involved Giza 21 (G21), Giza 22 (G22), Giza 35 (G35), Giza 82 (G82), Giza 111 (G111) and Crawford (CR). C= Control plants (treated with 0 mM NaCl).

The change in the isozyme profile due to salinity stress through the appearing or disappearing the existing bands can be used as potential marker because the isozymes play an important role in the cellular defense against oxidative stress caused by salt stress **(Shah and Nahakpam, 2012)**. Therefore, our results suggested that the qualitative changes in POD isozymes pattern for six soybean cultivars can be used as molecular marker to differentiate salt tolerant soybean cultivars under salinity stress. Identification such markers linked to salinity tolerance will provide plant breeders a new tool for selecting cultivars with improved salt-tolerance without measuring the phenotype, thus reducing time required and extensive field testing.

4. Conclusion

The data in this study demonstrated the molecular markers associated with salinity tolerance for the six soybean cultivars. Our results suggested that Giza 82 and Giza 35 are comparatively superior than other cultivars with respect to their pattern of RAPD, ISSR and SDS-PAGE markers which is well correlating with their adaptive strategy to salt stress. Identification of specific positive markers could introduce a great benefit for breeding programs to predict the most tolerant cultivars. RAPD and ISSR techniques are useful methods to detect specific markers for salinity tolerance, however subsequent experiments need to be achieved to determine the linkage between these RAPD, ISSR, protein and isozyme markers and the gene(s) responsible for salt tolerance in these soybean cultivars.

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