Molecular studies on some barley genotypes in relation to salt stress tolerance

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Abstract: Environmental stress, especially saline soils and saline water, are one of the most important limiting factors for agricultural crops in particular all around the world. Thus, yield enhancement in agricultural crops such as barley under saline conditions is a major goal of plant breeding. Leaf Sample from five barley genotypes and their F_1 offspring were collected at 30 days old seedlings growth under three treatments (control, 7000 and 14000 ppm) of NaCl to develop initial material for salinity tolerance breeding program using biochemical and molecular tools. These genotypes differ genetically in their salt tolerance potentiality and classified to salinity stress tolerant (Arar, Giza 123 and Giza 124), moderate (Mari) and susceptible (Beecher). Based on SDS-PAGE of water soluble protein for all genotypes under study , newly synthesized protein bands of salt treated parents observed at molecular weight (102, 96, 67 and 23) KDa and (28 and 87 KDa) for treated parents and hybrids, respectively . Also Native PAGE was carried out in this experiment to study six isozymes (EST, SKD, FDH, GDH. MDH and PER) patterns. In general theses isozymes patterns were reliable system for discriminating between tolerant and sensitive salinity genotypes under salt stress. Using RAPD-PCR with 5 primer arbitrary oligonucleotide (P18, P86, P24, P92 and P93), the results showed that all barley genotypes are not always identical in their DNA ability to be amplified and the total of amplified bands is 352 PCR bands. On the contrary, primers P18, P86 and P24 were able to generate positive marker, P92 was able to generate negative marker and 93 was able to generate positive and negative marker for salt tolerance. The phylogentic tree succeeded in clustering together the three tolerant parents and moderate parent while sensitive parents in another cluster. These results indicated that protein, isozyme and RAPD analysis are useful molecular tools to indicate genetic polymorphism between the barley genotypes under salt stress.
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Abbreviation: NaCl-sodium chloride; RAPD- random amplified polymorphism DNA; PCR- polymerase chain reaction; EST-esterase; PER-peroxidase; MDH-malate dehydrogenase; GDH-glutamate dehydrogenase; FDH-formate dehydrogenase; SKD- shikimate dehydrogenase.

1. Introduction:

Barely , *Hordeum vulgare L.*, is recognized as one of the most economic and important cereals in the world. By area and production barley is the fourth most important cultivated crop, following, wheat, rice and maize. It can be grown in a wide range of environmental conditions and give satisfactory yields in areas that are not suitable for growing most of the others cereals crops due to problems of abiotic and biotic stress [1-2].

Abiotic stress in fact is the principal cause failure worldwide, dipping average yield for most major crops by more than 50% [3]. Abiotic stress causes losses worth hundreds of million dollars each year due to reduction in crop productivity and crop failure [4]. Among Abiotic stresses, salinity in soil and in irrigation water is very harmful and adversely affects plant growth, development and restrict yield on 40 million hectare of irrigated land in the world [5-6]. Increased Stalinization of arable is expected to have devastating global effects resulting in 30% land loss within next 25 years and up to 50% by the middle of 21st century [7].

When salinity exceeds to optimum tolerance of a plant, the result is stress to the plant, which in turn influences its developmental, structural, physiological and biochemical processes [8], also can cause damages to sensitive plant Species by altering patterns of gene expression including change in cellular structures and impairing membrane function [3-9].

For many years breeding for salt tolerance has been an important task to increase crop productivity under salt stress and choice of parents for crossing is considered an important step in any plant breeding program aimed to an increase in the salinity tolerance of barley which could improve the profitability of some of the more than one billion salt affected hectares present in the world [10]. Using non-conventional approaches such as molecular marker as a strategy to obtain plants with higher performance under salt stress conditions by identify the genes and banding patterns that take place when the plant become growing under salt stress may further accelerate the progress of such breeding programs [11]. Molecular markers developed by

analysis of proteins, isozymes and randomly amplified polymorphism DNA (RAPD) has shown excellent potential to assist selection of quantitative traits [12].

Sodium dodecyl sulphate polyacrylamid gel electrophoresis (SDS-PAGE) is most economical simple and extensively used biochemical technique for analysis of genetic structure of germplasm. Gel electrophoresis can directly equate variation in protein banding patterns to gene coding various proteins and proved to be useful in revealing polymorphic loci that encode isozyme or proteins [13]. The important of protein profiling has long been acknowledged in plant abiotic stress studies and previous study have provided useful information on individual enzyme or transporters, measuring their stress-dependent change in quantity, activity, as well as modifications of structure protein, protein interaction , stress dependent protein movement [14]. Salinity has been reported to cause either decrease or increased in the level of soluble proteins, a complete loss of present protein and the synthesis of new protein in barely [15]. **Karimazadeh *et al.***, [16] observed changes in the electrophoresis pattern of water-soluble proteins from barley cultivars and pointed out accumulation of stress proteins in leaves on exposure to salinity. **Ali *et al.*,** [17] showed 12 polymorphic bands with different expression in the six barley genotypes. Salt tolerance genotypes under salt treatment were characterized by specific band no. 10 with approximate molecular weight of 17.54 KDa, this specific bands of water soluble protein profiles may used as marker for identification of genotypes under salt stress.

Isozymes have proven to reliable genetic markers in breeding and genetic studies on plant species [18-19]. **Wier** [20] indicated that the ability to observe allelic variation and designated allozyme polymorphism at isozyme loci is useful tools to examine genetic processes for different genotypes under stress conditions. In order to take advantage of isozyme markers potential for genetic studies and plant breeding programmes, the knowledge of their inheritance is a prerequisite. Once the genetic control of enzyme systems is known, allozyme or isozymes can be designated more accurately. Within the genus Hordeum, isozymes have been used to study genetic variation under abiotic stress. Salinity like other abiotic stresses alters general metabolic processes and enzymatic activities, causing increased production of reactive oxygen species (ROS) and lead to oxidative [21]. ROS-mediated membrane damage has been demonstrated to be a major cause of cellular toxicity by salinity in crops [22]. To minimize the effect of oxidative, plant cell have evolved a complex antioxidant system, which is composed of low molecular mass antioxidants (Glutathione and Malate) as well as ROS scavenging enzymes, such as Superoxide dismutase (SOD), Esterase (EST), Malate dehydrogenase (MDH) and Glutamate dehydrogenase (GDH) [23].

Crop plants depend on the broad genetic base of variation for resilience and adaptability for ever changing environments and pathogens. In recent years, attention has increasingly focused on the DNA molecule as a source informative polymorphisms, because each individual's DNA sequence is unique. DNA polymorphisms as DNA fingerprinting is becoming the technique of choice for laboratory assessment of cultivar identify. Characterization of genotypes using DNA fingerprinting techniques provides quantitative estimates of genetic structure and the information required for a rational utilization of germplasm in breeding programme [24]. The random amplified polymorphic DNA (RAPD) assay, which detects nucleotide sequence polymorphism by means of the polymerase chain reaction (PCR) and a single primer of arbitrary nucleotide sequence, have been developed and used in genetic and breeding studies in barley [24-25]. It is useful method for provides virtually unlimited number of markers to compare individual genotypes under normal and stress conditions , considering easy handling, cheaper cost assay and it is possible to carry out large scale screening of breeding populations and genetic resources [26-27-28]. Also, **Albayrak and Gözök** [29] indicated that, RAPD-PCR can be used as a tool in the selection of commercially important traits such as resistance against diseases, drought and salinity present in wild barley lines.

Therefore in this view, using five barley genotypes and their F_1 offspring, the objective of present study was to attempts biochemical (protein and isozyme) and molecular (RAPD-PCR) markers associated with salt tolerance in barley genotypes and to assess the level of genetic diversity relationship among them using RAPD molecular marker procedure. This relationship could be used by breeder in establishing strategies for selecting early generation materials in variety developmental programs.

2. Material and Methods:

The present study was carried out during period 2007/2008 and 2008/2009 barley growing seasons, at the experimental farm of the Faculty of Agriculture, El-Jabal El-Gharbei-Zaweia University, El-Zaweia, Libya. Five barley (*Hordeum vulgare* L.) genotypes differing in tolerance to salinity were obtained from Barley Department, Agriculture Research Center, Giza, Egypt, to including in this study. The origin and pedigree of these genotypes are presented in Table 1.

Using diallel mating system, in 2007-2008 season the five parents were crossed in all possible combination to obtain a total of 10 F_1 hybrids.

Table 1. The entry name, pedigree and degree of salt tolerance of the studies barley genotypes.

Genotypes	Pedigree	Origin	Degree Of Salt Tolerance
Giza 123	Giza 117/FAO 86 (Giza 117 = Baladi 16/Palestine 10)	Egypt	High Tolerant
Giza 124	Giza 117/Bahteem 52// Giza 118/FAO 86	Egypt	Tolerant
Beecher	Atlas/Vaughn	Syria	Sensitive
Arar	Perga/Sekitorisai	Syria	Tolerant
Mari	Bouns X Ray-mutant	Syria	Moderate

In 2008/2009 seeds of the five parents with their ten hybrids (15 entries) were sown, in plastic pots (300 mm) filled with 2 Kg of soil mixture containing clay soil, sand and petmous at 1:1:1 ratio, in the green house. 10 seeds of each of the 15 entries were sown in each pot per entry with three replications and all pots were watered with tap water (300 ppm salt) up to 14 days after sowing. On day 15 salt treatments of 7000 ppm, 14000 ppm NaCl with unsalted treatments as control were applied. All treatments were designed in split-plots design with three replicates, where the three salinity treatments arranged randomized within the main plots.

Biochemical and molecular genetic analysis:

Leaf samples from each entry were collected at 30 days old seedlings grown under control and saline conditions and placed directly in deep freezer at -80Cº until they were used for biochemical and molecular analysis.

1- Soluble protein analysis using SDS-PAGE:

SDS-PAGE was used to compare among the 15 entries under different salt treatments by their protein finger prints such as water soluble protein as follow:

Sample extraction: 0.5 g of each leaf sample was manually ground in cold pestle mortar to a fine powder under liquid nitrogen and mixed with 2 ml water-soluble extraction buffer containing 1M Tris HCl, pH 8.8, 0.25 M EDTA. Samples were transferred to eppendorf tubes and left in refrigerator overnight, then vortexed for 15 seconds and centrifuged at 12000 rpm at 4Cº for 20 minutes. The supernatants were collected and considered as the soluble leaf protein extract. Protein concentration was estimated using Bradford's method – **Bradford** [30] - by measuring absorbance at 595 nm using spectrophotometer and expressed as µg/g fresh weight. A standard curve was prepared with bovine serum albumin.

Application of samples:

A volume of 50µl of protein fraction was added to the same volume of LAN's buffer (10 % SDS, Glycerol, 1 M Tris HCL, pH 8.8, 0.25 M EDTA) in eppendorf tube, and 10 µl 2-Mercaptoethanol was added to the each tube and boiled in water bath for 10 min, then 10 µl Bromophenol blue was added to each tube before sample loading. A volume of 15-20 µl, depends on the concentration of protein in the sample were applied to each well by micropipette and control wells were loaded with protein standards. SDS-PAGE was performed by the methods described previously by **Laemmili** [31]. Gels were stained using silver staining as described by **Blum *et al.*,** [32] and after bands becomes clear the gels were photographed and electrophoregrams for each entry under different treatments were scored depends on the presence (1) and absence (0) of bands.

2- Isozyme analysis using Native PAGE:

Enzyme Extraction

0.5 gm from young leaves of each homogenised in 2 ml of cold extraction buffer containing 0.1 M Tris-HCl, 1, pH 8. 1 mM EDTA, o.5 PVP-10, 2mMDTT, 10 mM Mercaptoethanol and 2% PVP were added. Each sample was vortexes for 14 second by electric vortex and centrifuged at 20000rpm for 15 min at 4Cº. The amount of total protein in the supernatant was assayed as described previously in protein analysis, about 15µg of the total protein was applied to native polyacrylamide gel according to Apavatjrut et al., [33].

Enzyme staining

Six enzymatic systems were examined in this study. The gel was stained after electrophoresis according to its protocol and incubated at 37C° in the dark for complete staining adding the appropriate substrate and staining solution. The staining protocol for Esterase's (EST),Glutamate dehydrogenase (GDH), Formate dehydrogenase (FDH) and Shikimate dehydrogenase (SKD) was used according to Jonathan and Wendel [34], While Malate dehydrogenase (MDH) from Falk et al., [35] and peroxidase (PER) by Guikema and Sherman [36].

Gel Fixation

After the appearance of the isozyme bands, the reaction was stopped by washing the gel two or three times with tap water, this was followed by adding the

fixing solution (10 % glacial acetic acid, 20% ethanol and 70% distilled water) Falk et al., [37]. The gel was kept in the fixing solution for 24 hours and rinsed with tap water two times then was photographed and analyzed electrophortically as in Stegemen et al.,[38-39] and developed as reported by Scadalios [40].

3- PCR-RAPD analysis:

DNA isolation: DNA was extracted from leaf tissue from each using a hexadecyltrimethylsmmonium bromide (CTAB) method according to **Maniatis *et al.*,** [41]. 2 grams of frozen barley leaves from each entry were ground in cold pestle mortar with 10 ml buffer (100 mM Tris-HCl pH 8.0, 20mM EDTA, 1.4 M NaCl, 0.2% PVP40 (w/v), 0.2% (v/v) 2-mercaptoethanol) , mixed ,transferred to 50 ml eppendorf tube and incubate at 65 ºC for one hours. After incubation the mixture was centrifuge for 20 minutes with 4000 rpm at room temperature degree. Supernatant was taken and RNAse 1:1000 dilution of RNAse (100 mg/ml) was added and keep it at 37 ºC at 30 minute, then mixed with the same volume of chlorophorm-isomylalcohol (24:1) and centrifuged at 4000 rpm for 30 minutes . DNA was precipitated by the addition of 2/3 volume of cold isopropanol for overnight in 4ºC. the supernatant was removed from the tube and the pellet was washed with wash buffer (70 % ethanol), centrifuged again for 10 minutes with 1000 rpm at 20ºC and the pellet was dried under vacuum. The DNA pellet was resuspended in 100 µl of deionized H_2O and incubated at 50ºC for 15 min, centrifuged for 5 min with 1000 rpm at 20ºC then the solution was transferred to a new microfuge tube.

Polymerase Chain Reaction (PCR): After checking the concentration of genomic DNA by agarose gel electrophoresis for all 15 entries which will used to detect a marker related to salt tolerance, PCR reaction was conducted using arbitrary 10-mer primers (Sigma Company) as shown in table 2.

Table 2. List of five arbitrary primers and their nucleotide sequences used to generate RAPD markers in barley

Primer code	Sequence
P18	5'-GGGCCCTTTA-3'
P24	5'-ACAGGGGTGA-3'
P86	5'-GAGCTCGCGA-3'
P92	5'-CCTGGGCTTT-3'
P93	5'-GGGGGGAAAG-3'

Each PCR mixture was 25 µl containing 12.5 µl of master mix (Fermentas), 0.1 µl of each primer, 0.1 µl of plant genomic DNA and the volume was completed by deionized autoclaved water. The reaction were performed in a thermal cycle (Perkin Elmer) with the following temperature conditions: 94 ºC for 4 min, followed by 45 cycles of 94 ºC for 30 sec, 36 ºC for 30 sec, 72 ºC for 2 min and ending with 72 ºC for 8 min.

PCR products were analyzed using 1.4% agarose gel electrophoresis and visualized with ethidium bromide staining. The size of the fragments were estimated using Qx174RFDNA / HaeIII fragments as a standard DNA, which consisted of 5 double stranded DNA fragments with size of (1353, 1078, 872, 603, 310 and 271 bp). RAPD data were scored for presence (1), absence (0).

Hierchical cluster procedure:

An assessment of genetic divergence and cluster analysis between barley genotypes was analyzed through clustering analysis based on data from RAPD-PCR analysis with 5 primers on the basis of genetic distances according to **Johnson and Wichern** [42], the multivariate analysis was done by SPSS program.

3. Results and Discussion:

Electrophoresis technique for protein and isozyme polymorphism have been used as identification and quantitation methods, which provide association between the altered expression of specific genes and changes in the environmental stress. These changes in expression of genes would be involved in adaptation and could be used as molecular markers for salt stress [17].

3.1 Soluble protein analysis

Electrophoresis analysis was carried out on water-soluble SDS-protein fraction for 5 parental barely genotypes and their hybrids under control and two salt treatment. Densitometer analysis of W.S.P S.D.S-PAGE representing Protein bands with different molecular weight ranged from 18 KDa. to 130 KDa (data not show). All the bands did not exhibit a specific trend to salt tolerance. Total number of bands ranged from 24 to 33 under control, 25 to 34 under 7000 ppm and 28 to 35 under 14000 ppm as shown in Figure 1. More bands (33, 31, 33) under 14000 ppm were exhibited in the tolerant parents (Arar, G.123 and G.124), respectively followed by Mari as moderate genotypes (30 bands), while the sensitive ones (Beecher) showed relative low number of bands (28), this results agree with **Rashed *et al.*,** [43]. Various investigator suggested that the decrease number of bands in sensitive genotype compared with tolerance genotypes is associated with denaturing of the enzymes involved in amino acids and protein synthesis under abiotic stress [44]. **Katja *et al.*,** [2] concluded that more protein are affected by stress-specific regulation in

the less tolerant barley genotype.

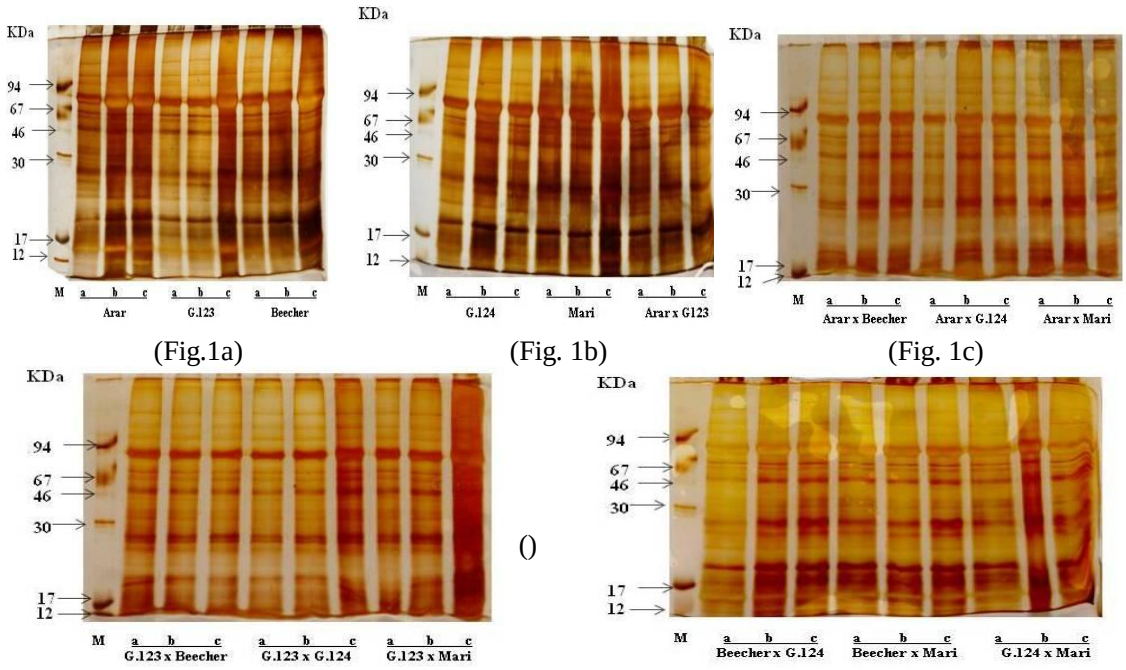
Based on SDS-PAGE of water soluble protein for all genotypes under study, there are nine bands finding in all genotypes (Fig. 1). The newly synthesized protein bands of salt treated parents observed at molecular weight (102, 96, 67 and 23 KDa.). The tolerant genotypes G123, G124 and Arar exhibited also higher intensity in the appearance of bands under salt stress than the sensitive and moderate genotypes Beecher and Mari, respectively.

The band number 11 at molecular weight (96 KDa) which presents in G123 under 7000 and 14000 ppm, all the treatments in G124, and also the band number 16 at molecular weight (67 KDa) which presents in Arar and G124 under 14000 ppm, G123 under 7000 and 14000 ppm might be used as a molecular marker for salt tolerance in barley, as they present in tolerant parent under treatment only while the sensitive parents did not exhibit these bands (Figs. 1 a & b). The newly synthesized protein band in treated hybrids is observed at 87 and 28 KDa. in most of the hybrids (Figs.1 b, c, d & e). These expression of this polypeptide might have been due to the plant adaption to NaCl via expression of a stress resistance gene. This results supports the previous results of **Ali *et al.,*** [17] and ***Vahid et al.,*** [45], since they indicated that the 17.54 KDa and 50 KDa protein was salt enhanced in salt barley and sorghum genotypes, respectively. On the other hand, no negative molecular marker associated with salt tolerance in barley genotypes was detected in this experiment. Other results indicate that the 32 KD protein was salt enhanced in sensitive barley genotypes (**Bendary**) [46].

There are quantitative (band intensity) differences for water soluble proteins under salinity stress compare to control. All Genotypes exhibited higher intensity in the appearance of bands under salt stress whereas were faint in control treatment. These fluctuated effects of the salt stress on the number and intensity of protein bands were detected in previous study by **Hurkman and Tanka** [47-48], and **Diana *et al.,*** [49], who considering that the band intensity is directly related to protein concentration. Higher plants exposed to abiotic stress such as drought condition exhibit a characteristic set of cellular and metabolic response, including a decrease or increase in the synthesis of protein , **Bayoumi *et al.,*** [50].

3.2. Isozymes analysis

Barley, *Hordeum vulgare L.*, was among the first plants studies with isozyme technique, in the current study six isozyme patterns were studies as follow.



(Fig.1a) (Fig. 1b) (Fig. 1c)

(Fig. 1d) (Fig. 1e)

Figure 1 a, b, c, d & e. Protein profile on SDS-PAGE of 15 barley Genotypes under salt stress. Lane M) Protein Marker; lane a) Control; Lane b) 7000 ppm and Lane c) 14000 ppm.

3.2.1. Esterase polymorphism

Esterase isozyme showed high level of variation (81%) with nine polymorphic enzymatic bands out of total eleven enzymatic bands, these bands showed clear different between sensitive and tolerant parental genotypes (Table 3). Bands number six appeared in tolerant parents (Arar and G.123) under 7000 and 14000 ppm and also G.124 and Mari under 7000 ppm. While it is disappeared in sensitive parent (Beecher), this band might be induced as results of response to salt stress. on the other hands, bands number eight showed constitutive occurrence in the sensitive parents under salt treatment while it was absence in both control and salt stress in other genotypes, also band number two was appeared in all genotypes under control while it was appeared in sensitive and moderate genotypes under control and 7000 ppm, these bands could be used as a negative marker for salt stress in barley. These results indicated that esterase isozyme gave reliable to obtain molecular markers linked with salt tolerance in barley.

3.2.2. Shikimate dehydrogenase polymorphism

Shikimate dehydrogenase isozyme showed high level of variation (100 %) with six polymorphic enzymatic bands which were not necessarily present in all genotypes. Electrophoresis patterns of SKD are presented in ideogram (Table 4). All parental genotypes and G.124 x Mari shared bund number one under control and 7000 ppm while it was appeared in all hybrids under control, also band number three were appeared in all genotypes under control. This was interpreted as due to the effect of salt stress which may cause some shift in gene expression and stop gene expression. General variation between control and salt treatments represented in two bands number two which were presented only under salt treatment while absence under control in all genotypes and band number four which was appeared only under 7000 ppm in Arar, G.123 and Mari and all salt treatment in G.124. The hybrids showed also one adaptive band, number four, for salt treatment which appeared under 7000 and 14000 ppm in some hybrids. These result clearly suggested the presence of an association between salt tolerance and the presence of this band. The SKD isozyme system was reliable system for discriminating parents and their hybrids under saline condition.

3.2.3. Formate dehydrogenase polymorphism

Data of FDH isozyme for barely parental genotypes and their hybrids are shown as ideogram (Table 5). Concerning the presence of a given band, it can be concluded that only two bands were polymorphic for all genotypes in the present study. The band number one appeared in the tolerant parents, moderate and all hybrids except Arar x G.124, G.123 x Beecher, G.124 x Beecher, Beecher x Mari and G.124 x Mari under 7000 and 14000 ppm and in sensitive parent and previous hybrids under 14000 ppm only, this band considered as adaptive band for salt stress. In the mean time, all parental genotypes and hybrids except Beecher, Arar x G.124, G.123 x Beecher, G.124 x Beecher, Beecher x Mari and G.124 x Mari exhibited the occurrence of band number two with high intensity under control while this band was uniquely and constitutively exhibited in the other genotypes under 7000 ppm, this may due to the different in gene expression under stress. This band might be considered as a marker negatively associated with salt stress.

3.2.4. Malate dehydrogenase polymorphism

The Malate dehydrogenase isozyme extracted from leaves of salt sensitive and tolerant barley parents and their hybrids exhibited a total of five bands as shown in Table 6. One band number two was exhibited among the profiles of all genotypes which considered as common band. The tolerant parent Arar and hybrids G.123 x Beecher, G.123 x G.124, Beecher x G.124, Beecher x Mari and G.124 x Mari exhibited the same MDH pattern in both control and salt treated plants. In addition to the tolerant parent G.124 and Moderate parent Mari exhibited also the same MDH pattern in control and salt treatment except that control plant showed absence band number one under control. The sensitive parent Beecher manifested only four band (no. 1, 2, 3 and 5) and three band (no 1, 2 and 5) under 7000 and 14000 ppm, respectively. While the control plants revealed three band number 2, 3 and 5. Only band number four was uniquely and constitutively exhibited in the tolerant and moderate parents with complete absence in sensitive parents and also band number three which appeared in all parental genotypes except Beecher under 14000 ppm. In their hybrids there not clearly different between hybrids under control and salt stress.

3.2.5. Glutamate dehydrogenase polymorphism

Electrophoresis patterns of GDH are presented in Table 7. The five parents and their hybrids exhibited one band number three in both control and salt treatment except Arar, G.123 under 14000 ppm. The parents genotypes under control showed presence of band number two while this band was absence under salt stress, also this band was appeared under control in Arar x G.123, Arar x Beecher, Arar x Mari, G.13 x G.124. G.123 x Mari and Beecher x G.124 while in the rest of hybrids it was appeared only under control and 7000 ppm. This interpreted due to the effect of salt stress which might cause some shift in gene expression in genotypes. In the mean time, new band number one present to appear in the hybrids Arar x G.123 , Arar x Beecher, Arar x Mari, G.13 x G.124 and G.124 x

Beecher under 7000 and 14000 ppm, while appeared in another hybrids under 14000 ppm only. This result clearly suggests that the band number one in the hybrids genotypes could be considered as salt shock protein bands which appeared after saline treatments.

3.2.6. Peroxidase polymorphism

As in ideogram (Table 8), a total of three bands number two, three and four were characterized in the parental genotypes and their hybrids which were present in some genotypes and absent in the others except band number one which was present in all genotypes, this band could be considered as a common band for all the study genotypes.

The tolerant parental genotypes, Arar x G.124 and G.124 x Mari revealed the band number two under control and salt treatment while this band was absence in Beecher, Arar x Beecher, Arar x Mari and G.124 x Mari under 7000 and 14000 ppm, which may be due to the different in gene expression under salt stress. In general the electrophoresis banding pattern of peroxidase isozyme revealed that the variation between the sensitive and tolerant genotypes under salt stress, represented in one band number two which was appeared under salt treatment in all tolerant parental genotypes and disappeared in sensitive parent. This result clearly suggested the presence of an association between salt tolerant and the presence of this band.

Sang *et al.*, [51] reported that increases in the expression of activities and isoform of some antioxidant enzymes such as Ascorbate peroxidise were associated with decrease in hydrogen peroxidise in the salt-stressed barley and the quantitative and qualitative aspect of changes are often related to the level of resistance to salinity. **Metwali *et al.*,** [52] revealed that plant grown under salinity showed induction or suppression in the synthesis of few polypeptides. Isozymes of EST, MDH and GDH showed differences under salt stress, these differences might reflect the gene activation for the adaptation of plants to salt conditions and provide good marker for discrimination among salt-tolerant and salt-sensitive accessions.

3.3. Molecular genetic marker

Genomic DNA of the barley genotypes were extracted and were used in performing Randomly Amplified polymorphic DNA (RAPD). 5 arbitrary oligonucleotide primers, base sequence and number of fragment amplified using these different primers showed that: the number of amplified fragments different from one genotype to another indicating that all barley genotypes are not always identical in their DNA ability to be amplified and these primers have amplified 352 PCR bands (Table 9). A maximum of 102 fragments were amplified with primer P93 and minimum of 45 fragments were amplified with primer P86. These results agree with **Adrian *et al.*,** [53], **Baum *et al.*,** [54] and **Noli *et al.*,** [55]. The five primers produced multiple band profiles with a number of amplified DNA fragment ranging from Zero to eleven. Three primers P18, P29 and P39 were reacted and generated PCR product with all genotypes, whereas primer P24 and P86 reacted only with twelve and fourteen genotypes, respectively.

All five used RAPD primers generated polymorphic bands, The P93 primer recorded the highest percentage polymorphism (76%) as it revealed 10 polymorphic bands in 13 amplified fragments, while the P92 primer recorded the least percentage (30%) by revealing 7 polymorphic bands in 13 amplified fragments (Table 10).

Table 3. Ideogram of esterase (EST) isozyme of five parental genotypes and their hybrids under control and salt treatments

bands	A.			G.123			B.			G.124			M.			A. x G.123			A. x B.			A. x G.124			A. x M.			G.123 x B.			G.123 x G.124			G.123 x M.			B. x G.124			B. x M.			G.124 x M.		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	-	+	+	-	+	+	-	-	+	-	+	+	-	-	+	-	+	+	-	+	+	-	-	+	-	+	+	-	-	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+
2	+	-	-	+	-	-	+	+	-	+	-	-	+	+	-	+	-	-	+	+	-	+	-	-	+	-	-	+	+	-	+	-	-	+	-	-	+	+	-	+	+	-	+	+	-
3	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-
4	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	+	-	+	-	-	+	-	-	+	+	+	+	+	-	+	-	-	+	-	-
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	-	+	+	-	+	+	-	-	-	-	+	-	-	+	-	+	+	-	+	-	-	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	-	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	-	-
8	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 4. Ideogram of Shikimate dehydrogenase (SKD) isozyme of five parental genotypes and their hybrids under control and salt treatments

bands	A.			G.123			B.			G.124			M.			A. x G.123			A. x B.			A. x G.124			A. x M.			G.123 x B.			G.123 x G.124			G.123 x M.			B. x G.124			B. x M.			G.124 x M.		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+	-	+	+	-
2	-	+	+	-	+	+	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	+	-	+	+	-	-	+	-	+	+	-	+	+	-	+	+
3	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
4	-	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	-
5	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 5. Ideogram of formate dehydrogenase (FDH) isozyme of five parental genotypes and their hybrids under control and salt treatments

bands	A.			G.123			B.			G.124			M.			A. x G.123			A. x B.			A. x G.124			A. x M.			G.123 x B.			G.123 x G.124			G.123 x M.			B. x G.124			B. x M.			G.124 x M.		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	-	+	+	-	+	+	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	-	+	-	+	+	-	-	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	-	+
2	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-	+	-	-	+	-	-	+	+	-	+	-	-	+	+	-	+	-	-	+	-	-	+	+	-	+	+	-	+	+	-	+	+	-

Table 6. Ideogram of Malate dehydrogenase (MDH) isozyme of five parental genotypes and their hybrids under control and salt treatments

bands	A.			G.123			B.			G.124			M.			A. x G.123			A. x B.			A. x G.124			A. x M.			G.123 x B.			G.123 x G.124			G.123 x M.			B. x G.124			B. x M.			G.124 x M.		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+

Table 7. Ideogram of glutamate dehydrogenase (GDH) isozyme of five parental genotypes and their hybrids under control and salt treatments

bands	A.			G.123			B.			G.124			M.			A. x G.123			A. x B.			A. x G.124			A. x M.			G.123 x B.			G.123 x G.124			G.123 x M.			B. x G.124			B. x M.			G.124 x M.		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	+	-	+	+	-	-	+	+	-	+	-	+	+	-	-	+	-	-	+	+	-	+	-	-	+
2	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+	-	-	+	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 8. Ideogram of peroxidase (PER) isozyme of five parental genotypes and their hybrids under control and salt treatments

bands	A.			G.123			B.			G.124			M.			A. x G.123			A. x B.			A. x G.124			A. x M.			G.123 x B.			G.123 x G.124			G.123 x M.			B. x G.124			B. x M.			G.124 x M.		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

A.= Arar, G.123=Giza 123, B.=Beecher , G.124=Giza 124 , M.=Marei.
- = absent + = present
A= control, b= 7000 ppm, c= 14000 ppm

Table 9. List of primer, their nucleotide sequences and amplification results with 15 barely genotypes.

Primer code	Sequence 5` to 3`	Number of amplified fragment in each genotypes														Total
		Arar	Giza 123	Giza 124	Marei	Beecher	A. x G.123	A. x G.124	A. x M.	A. x B.	G.123 x G.124	G.123 x M.	G.123 x B.	G.124 x M.	G.124 x B.	M. x B.	
P18	GGGCCCTTTA	5	5	5	3	2	5	5	2	4	5	5	3	5	2	3	59
P24	ACAGGGGTGA	6	0	5	3	0	5	5	4	6	4	0	3	5	5	5	56
P92	CCTGGGCTTT	6	4	8	8	8	5	5	4	9	4	5	7	7	5	5	90
P93	GGGGGGAAAG	4	5	9	7	8	7	7	4	8	8	11	5	6	7	6	102
P86	GAGCTCGCCA	3	4	3	4	1	5	5	2	4	4	4	3	3	3	0	45
Total		24	18	30	25	18	27	27	16	31	25	25	21	26	22	19	352

A. = Arar M. = Mari B. = Beecher G.123= Giza123 G.124=Giza124

Table 10: Polymorphism rate for the 15 barley genotypes using P18, P24, P92, P93 and P86 primers.

Primer code	Total amplified band	Polymorphic band	Polymorphism (%)
P18	10	3	30
P24	11	8	72
P92	13	7	53
P93	13	10	76
P86	9	6	66
Total	55	33	

The size of amplified fragment ranged from 310 bp to 1853 bp approximately (Figure 2). When the oligonucleotide P18 was used it produced amplified DNA segments of 945 and 772 bp in three salt tolerant genotypes only (Fig. 2a). This primer also produced an amplified segment of 1148 bp in salt tolerant parents and moderate parent as shown in Figure 8. Also these bands present in the hybrids for these genotypes. These fragment which appeared in the tolerant genotypes but not in sensitive genotypes referred to be positive markers for salt tolerance.

The results of RAPD analysis using Primer P24 are illustrated in Figure 2b. The primer produced zero bands in G.123, Beecher and G.123 x Mari to six bands in Arar and Arar x Beecher .The primer produced three common bands in all genotypes of molecular weight 872, 603 and 310 bp. The other bands were polymorphic as they present in some genotypes and absent in others. The oligonucleotide of P24 produced one amplified DNA segment of 1335 bp in two salt tolerant parents Arar and G.124,

this one positive marker can be used to distinguish the salt tolerant genotypes.

Figure 2c represented the amplified fragment patterns of primer 86. The molecular weights of PCR products generated by this primer ranged from 450 to 1110 bp. Moreover no reaction were detected with hybrid Mari x Beecher, which means that this primer had no complementary sequence with this genotypes. From the RAPD profiles generated by this primer, one common band was produced in all genotypes with molecular weight of 520 bp. Bands with molecular weight 1110 and 450 bp were absent in sensitive genotypes and presence in all genotypes. This fragments which appeared in all genotypes except Beecher referred to be positive marker for salt tolerance.

The results of RAPD analysis using primer 92 are illustrated in Figure 2d. It is interesting to note that the parental genotypes Beecher and Mari as a sensitive and moderate genotype have one bands of Molecular weight 872 bp which are present in these genotypes only and absent in salt tolerant genotypes. Also Beecher has one band of MW 1150 bp which also present only in sensitive parent and absent in other parents, these bands could be used to distinguish these parental genotypes from other and also these two negative DNA markers can then be used to distinguish the salt tolerant genotypes, represented by Arar, G.123 and G.124 from the salt sensitive and moderate ones, represented by Beecher and Mari.

Figure 2e represented the amplified fragment pattern of primer 93. There are two DNA negative marker at 872 and 1450 bp. First negative marker was observed only in salt sensitive genotypes Beecher while second negative marker was observed only in Beecher and Mari, this fragments is referred to negative salt tolerance marker. Also this primer produced an amplified DNA segments of 1353 bp in three most tolerant parental genotypes Arar, G.123 and G.124 which was not observed in salt sensitive parent Beecher and also moderate parent Mari, so this segment might be used as a dependable marker linked to salt tolerance.

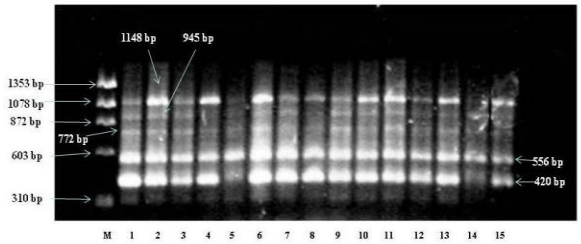


Fig.2 a) RAPD –PCR with primer (P18)

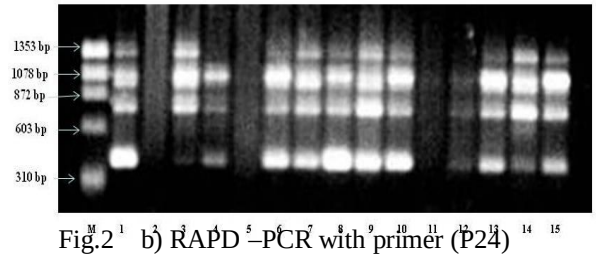


Fig.2 b) RAPD –PCR with primer (P24)

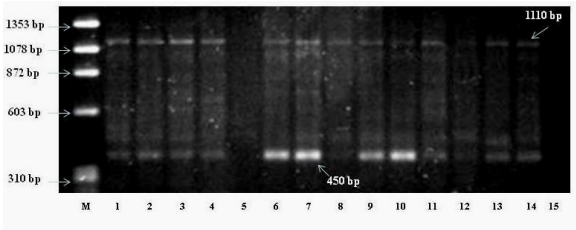


Fig.2 c) RAPD –PCR with primer (P86)

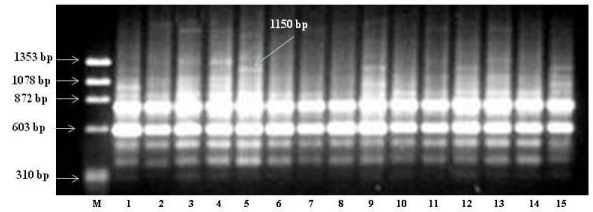


Fig.2 d) RAPD –PCR with primer (P92)

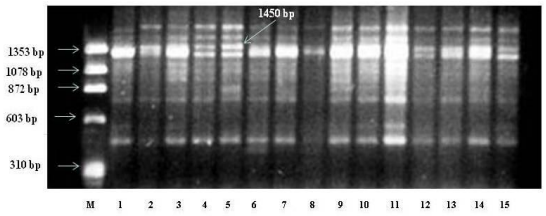


Fig.2 e) RAPD –PCR with primer (P93)

Figure 2 a, b, c, d & e. DNA banding pattern generated by RAPD-PCR with different primers P18, P24,P86, P92 and P93 in 15 barley genotypes. Lane M: DNA marker, lane 1-15:Barley genotypes as follow; (1) Arar, (2) G.123, (3) G.124, (4) Mari, (5) Beecher, (6) Arar x G.123, (7) Arar x G.124, (8) Arar x Mari, (9) Arar x Beecher, (10) G.123 x G.124, (11) G.123 x Mari, (12) G.123 x Beecher, (13) G.1224 x Mari, (14) G.1224 x Beecher and (15) Mari x Beecher.

Table 11. Squared Euclidean distance between barley genotypes on the basis of combined RAPD-PCR products from five primers

	A.	G.123	G.124	M.	B.	A. x G.123	A. x G.124	A. x M.	A. x B.	G.123 x G.124	G.123 x M.	G.123 x B.	G.124 x M	G.124 x B.
G.123	10													
G.124	13	9												
M.	15	14	11											
B.	21	14	19	10										
A. x G.123	11	12	11	10	18									
A. x G.124	8	13	10	11	19	3								
A. x M.	13	12	17	16	16	12	15							
A. x B.	10	15	12	11	17	9	8	17						
G.123 x G.124	10	9	10	11	15	3	6	11	10					
G.123 x M.	16	9	12	13	15	9	10	19	14	8				
G.123 x B.	12	9	16	7	9	13	14	13	12	12	14			
G.124 x M.	10	11	10	7	15	7	10	13	10	6	14	8		
G.124 x B.	10	11	12	9	13	7	10	7	12	6	14	8	6	
M. x B.	14	15	16	11	11	11	14	11	14	10	18	8	8	6

A. =Arar, G.123 = Giza 123, G.124 = Giza 124, M. = Mari & B. = Beecher

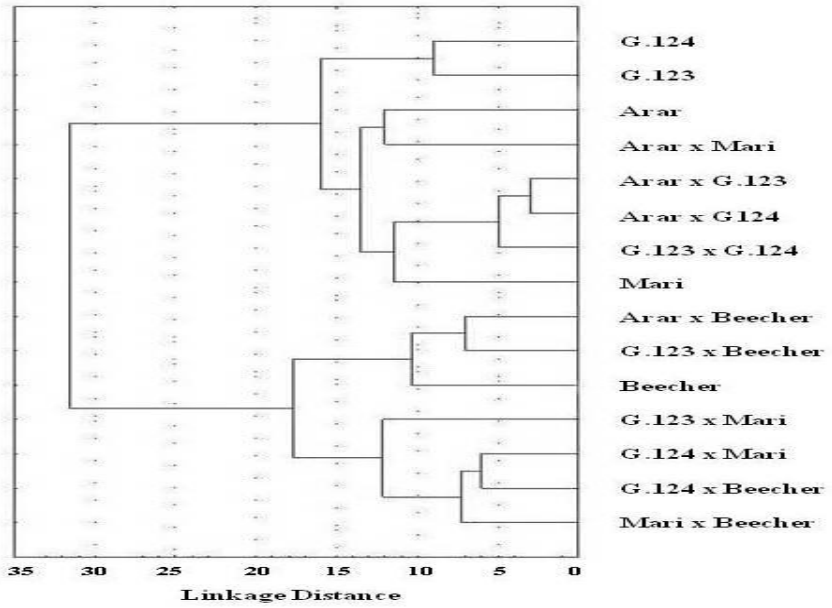


Figure 3. Linkage dendrogram for barley genotypes on the basis of combined RAPD-PCR products from all primer

On the contrary, we concluded that Primers number 18, 86 and 24 were able to generate positive marker for salt tolerance while primer number 92 was able to generate negative marker for salt tolerance and primer number 93 was able to generate positive and negative marker for salt tolerance. **Lucia *et al.*,** [56] used long primer PCR markers specifically targeted to sequence involved in the response to abiotic stress to analyse genetic marker within and among wild barley populations, the results demonstrate the effectiveness of PCR-based molecular markers targeted to environmental regulated genes in detecting useful variation and thus in monitoring the impact exerted by adaptation to environmental on genetic differentiation. Our results are also in parallel with those of **Ali *et al.*,** [17] who identified amplified band with molecular size 2000 and 500 bp in tolerant parents and was not detected in sensitive parents in barley by using primer D20 and Z7, respectively

Interestingly, many bands were shown to appear in tolerant parents but absent in their hybrids. There is no way that we can make linkage between these RAPD marker and salt tolerant gene. In other word, they can be successfully used as RAPD marker for salt tolerant parent but not for salt tolerance gene (s). The result of F_1 against different primers indicated that RAPD marker are dominant, where they were present at F_1 generation. However no explanation can be given to interpret absence of this marker at F_1 generation. As a conclusion the RAPD analysis seems to be one of the powerful tools for detecting polymorphism and could be discriminate between all the five parental genotypes. The results are also

agreement with the finding of **Geiese *et al.,*** [57], and **Adnan and Katsuhiko** [58].

Cluster analysis

The barley genotypes were subjected to hierarchical Euclidean cluster analysis to determine the genetic divergence between parents and their corresponding F1 hybrids. The actual values of genetic distance which based on RAPD using different five oligonucleotide primer are give in table 11. The genetic distance obtained between these 15 genotypes were ranged from 6 to 21. The magnitude of genetic distance measured the extent of genetic diversity between the genotypes. Considering the genetic divergence between parental genotypes, the minimum distance (9) was recorded between Arar and G.123 (tolerant parents), while the maximum genetic distance of (21) was between Arar (Tolerant) and Beecher (sensitive). The distance observed among hybrids were found to be lower in both magnitude and range than those observed between the parental genotypes, indicating that the parental genotypes were widely dispersed from their F_1 hybrids and these 10 F1 hybrids had intermediate genetic background between their corresponding parents, where the least genetic distance (3) was observed between Arar x G.123 and Arar X G.124, Arar x G.123 and G.123 x G.124. The genetic divergence among fifteen genotypes is shown diagrammatically by linkage dendrogram (Figure 3), which resulting from combined data of RAPD-PCR analysis using different five primers (Fig. 2). The fifteen genotypes were grouped into two clusters. Cut off point at 20 dissimilarity point (genetic distance) was fixed as minimum dissimilarity. **Email *et al.,*** [59] indicate that determining true genetic dissimilarity between individuals is an important and decisive point for clustering and analyzing diversity within and among populations.

The barley parental and their hybrids genotypes were distributed in two clusters. Cluster 1 consist only salt and moderate tolerance parental genotypes and most of their hybrids, while cluster number 2 consisted sensitive parent with their hybrids. These data indicated that considerable variation was created by hybridization in some hybrids but not all. It is interesting to note that the two parental genotypes Giza 123 and Giza 124 were grouped in a single sub-cluster, which may due to similarity in their genetic structure and common selection history. **Pahang *et al.,*** [60] found DP555BR and Dp449BR shared cv.DP5690 in their pedigree but they were grouped separately and they concluded that pedigree information or geographic origins of cultivars may not accurately reflect genetic relatedness among genotypes, whereas DNA markers could better reveal the genotypic relationships when there are sufficient markers and they are distributed across all chromosomes.

4. Conclusion:

The protein of 102, 96, 67, 23 KDa mw that are synthesized either specifically or at a higher rate under salt stress play an adaptive role in plant during osmotic adjustment, protecting the key cytoplasmic enzymes and protein synthesizing apparatus against adverse effect of high salt concentrations. Using isozymes such as EST, PER, SKD, GDH, FDH and MDH as genetic marker for genotype identification in barley under salt stress would be useful for displaying the effect of salt stress among the barley genotypes under salt stress. It is evident from this study that the RAPD assay is important since it is relatively easy to obtain valuable data and it can be useful in barley breeding programmes, where breeders can select related or unrelated parental germplasm to maximize variability in barley breeding programme under abiotic stress. During inter-mating accessions with grater genetic distance may provide unique genetic combination and useful variation for breeding. Construction of genetic relatedness tree can done using RAPD molecular marker, the RAPD dendrogram revealed that the closer the geographical locations and salinity tolerance the closer the genetic relationships. The use of molecular markers will be good alternative to the agronomic selection, where it allow a quick selection and provides the breeder with the genetic marker for salt stress.

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