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Enhanced Sodium Chloride disturbs the Genetic stability in *Ocimum tenuiflorum* L.

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ABSTRACT

The present investigation was carried out to evaluate the effects of NaCl stress on genetic stability of lines of *Ocimum tenuiflorum* L. using RAPD markers. For genetic characterization of controlled and NaCl stressed plants, the extracted DNAs were subjected to RAPD markers, using PCR amplification. For this purpose a total of seven primers viz, OPH_01_F, OPH_01_R, OPH_01_RC, OPI_05_R, OPI_05_RC, OPG_10 and OPC_06 were used. PCR amplification was followed by gel electrophoresis (1.2% agarose gel) and visualized by UV light, which revealed the presence of monomorphic and polymorphic bands in the amplified products. During this analysis, two primers (OPH_01_F and OPH_01_RC) produced a total of 10 bands. Six of the bands were monomorphic i.e. 02 with the control plant, 02 with the 25mM salt stressed plant and 02 with the 50mM salt stressed plants. While with 75mM and 100mM salt stressed plants polymorphic bands were observed showing genetic variation. Profiles generated by the third primer i.e. OPI_05_R showed 4 monomorphic bands with control and NaCl stressed *O. tenuiflorum* L. While, 100mM salt stressed plant showed total loss of bands. In addition, the expression level of control plant was brighter than the salt stressed plants. The expression level was gradually decreased with an increase in NaCl concentration. These results suggested that increase in salt concentration had a damaging effect on genetic stability of *O. tenuiflorum* L., which in turn affected the morphology and physiology of plant. The present piece of work also highlights the importance of salt stress as biomarker.
Key words: *Ocimum tenuiflorum* L, RAPD, salt stress, Genetic stability

INTRODUCTION

Ocimum tenuiflorum L is one of the most popular herbs grown in the world and is commonly called tulsi. The name tulsi means “matchless one” and is derived from “Sanskrit” (Das and Vasudevan, 2006). It can be found growing wild in tropical and sub-tropical regions of the world and is native to Asia (Pakistan, India, Thailand, Iran, and other countries). *O. tenuiflorum* L is often referred to as the "queen of the herbs" (Bhatnagar *et al*., 1993) and “The mother medicine of nature”. It is also called as “Holy Basil” because it is one of sacred plants in India. In addition to the religious significance, *O. tenuiflorum* L has a great medicinal importance as its leaves contain essential oil contents (Yanpallwar *et al*., 2004). It is a member of family Lamiaceae belonging to genus *Ocimum* that is comprised of herbs and shrubs (Das & Vasudevan, 2006).

Plants are influenced by a wide range of environmental stresses such as drought, high and low temperature, salinity, UV stress, pathogen infection and alkalinity (Breusegem *et al*., 2001). In arid and semi-arid regions, salt stress in water and soil is one of the major stresses that reduce the productivity and plant growth (Allakhverdiev *et al*., 2000; Koca *et al*., 2007). A wide number of responses in plants leading to growth inhibition are caused by saline environment (Jaleel *et al*., 2008). The significant reduction of plant growth under saline conditions is a common occurrence (Ashraf & Harris, 2004). However, different plant organs respond to such reductions in a different way (Jamil *et al*., 2005).

Worldwide, more than 45 million hectares of irrigated land have been damaged by salt and 1.5 million hectares are taken out of production each year as a result of high salinity levels in the soil (Munns & Tester, 2008).

For the identification of basil species the DNA markers have been demonstrated to serve as efficient tools due to their characteristics which include freedom from environmental influence, high polymorphism and abundance (Masi *et al*., 2006; Labra *et al*., 2004; Singh *et al*., 2004). In basil genetic diversity studies, various types of DNA markers can be applied including RAPD (Harisaranraj *et al*., 2008), Inter Simple Sequence

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Repeats (ISSR) (Aghaei *et al*., 2012) and AFLP (Labra *et al*., 2004; Stanko *et al*., 2011).

Random amplified polymorphic DNA (RAPD) is a modification of the PCR in which a single oligonucleotide primer, able to anneal and prime at multiple locations throughout the genome that are characteristics of the template DNA that can produce a spectrum of amplification products. RAPD markers have a wide range of applications in gene identification, population genetics, gene mapping, gene regulation, molecular evolutionary genetics and plant and animal breeding. This technique is very efficient to generate large number of markers in short period compared with previous methods. Therefore, such type of techniques can be performed efficiently in common laboratory due to of its applications (Kumar *et al*., 2011).

The present study was carried out with the objective to analyze the genetic destabilization of NaCl stressed lines of *O. tenuiflorum* L using RAPD markers. That may provide strengthening to crop improvement and breeding programs in saline areas at wider scale in future.

MATERIALS AND METHODS

The present study was conducted in Molecular Genetics and Plant Biotechnology Laboratory of Department of Botany, Lahore College for Women University, Lahore.

Plant Material

Certified seeds of *O. tenuiflorum* L were taken from Punjab Seed Corporation, Lahore and were grown in pots in premises of Lahore College for Women University, Lahore. The experiment was carried out in pots filled with sand and organic matter (animal dung) in 3:1 ratio. Measured pH of sand is 7 which is neutral and after addition of animal dung the pH increases to 7.5 the filled pots are aided with salt concentration. After germination, seedlings were thinned to one seedling per pot. Plants were grown in both controlled and NaCl stress conditions. Salt treatments were given to the plants when they were 20 days old. Plants were subjected to five levels of sodium chloride solutions whose concentrations were: 0 (control), 25, 50, 75 and 100mM NaCl. Salt stress was given to every pot after two days' time interval for 30 days. Leaves of controlled and NaCl stressed plants were taken for further analysis.

DNA Extraction

1gm of leaves of both types was weighed and grinded to a very fine powder using Liquid Nitrogen in pestle and mortar separately. DNA extraction was carried out by using the CTAB method (Doyle & Doyle. 1990). Grinded material of both control and NaCl stressed *O. tenuiflorum* L were transferred to 1500µL eppendorf tubes. 2% CTAB buffer was pre-warmed at 60^{o}C and 600µL of it was added to the eppendorf tubes and placed in a water bath or in an incubator at 60^{o}C. Equal volume of chloroform and iso-amyl alcohol in a ratio of 24:1 were added to the falcon tubes after 30 minutes and were centrifuged at 5000 rpm for 15 minutes at temperature 15^{o}C.

After centrifuge, three layers appeared in eppendorf tubes, upper aqueous layer represents DNA which was taken with the help of micropipette and was transferred to another eppendorf tubes. Other layers showed the presence of proteins and RNA. Cold iso-propanol was added to the falcon tubes in a 2/3 V of total liquid. Falcon tubes were shaken gently and were kept in a freezer overnight and were centrifuged next day at 5000 rpm for 15 minutes and at temperature 4^{o}C. DNA pellet appeared in both eppendorf after centrifugation.

500µL wash buffer was added to the eppendorf tubes and centrifuged at 12000 rpm for 5 minutes at 4^{o}C. Supernatant was discarded and DNA pellet was allowed to dry. DNA pellets were allowed to dissolve for a week by addition of 200µL TE buffer into the eppendorf tubes containing DNA pellets.

RAPD Analysis

Seven RAPD primers i.e. OPH_01_F, OPH_01_R, OPH_01_RC, OPI_05_R, OPI_05_RC, OPG_10 and OPC_06 were used for PCR amplifications. Amplification was carried out in a final volume of 25µL containing 10ul of PCR water, 12.5µL of PCR master mix, 0.5µL of primer OPH_01_F, 0.5 µL of primer OPH_01_R, 0.5µL of primer OPH_01_RC, 05µL of primer OPI_05_R, 0.5µL of primer OPI_05_F, 0.5µL of primer OPI_05_RC, 0.5µL of primer OPG_10, and 0.5µL of primer OPC_06 and 2µL of DNA sample. The purity of each DNA sample was determined spectrophotometrically at 260 and 280nm by using the Nano Drop (ND 1000 Spectro-photometer, Gene Ray Biotechnology, Shanghai, China). All the samples were diluted to a concentration of 40 ng/µl with ddH_2O for PCR analysis and all the DNA samples were good quality. One PCR reaction was prepared as negative control which was not containing the DNA. DNA amplification was obtained through 35 cycles in a DNA thermal cycler. The temperature profile was as follow: Initial denaturation step at temperature 94°C for 5 min (94°C for 30 sec., annealing temperature 45°C for 1 min. and extension temperature 72°C for 1 min) then final extension at 72°C for 5 min.

RESULTS

Effect of NaCl Stress on Morphology of *O. tenuiflorum* L.

O. tenuiflorum L. plants subjected to different NaCl concentrations showed many morphological changes i.e. change in growth of plants and leaf color. It was observed that plants subjected to low concentration of NaCl i.e. 25mM showed normal growth similar to the control. Whereas at higher concentrations of NaCl i.e. 50mM, 75mM and100mM there was a significant gradual reduction in the growth of the plant. Both 50 days old plants (controlled and salt stressed) can be seen in the Fig.1. The salt stressed plants showed stunted growth at higher concentrations of NaCl. Similarly gradually increasing leaf color damage was observed at higher levels of NaCl stress, reaching maximum damage at the highest NaCl concentration of 100 mM which was due to decrease in chlorophyll content that may be due to an increase of chlorophyll degradation or due to decrease of chlorophyll biosynthesis. (Fig.1)

PCR Amplification with RAPD Markers

For the present study, a total of seven primers OPH_01_F, OPH_01_R, OPH_01_RC, OPI_05_R, OPI_05_RC, OPG_10 and OPC_06 were used. Three of all these primers showed amplification with Control and NaCl stressed *O. tenuiflorum* L. and no bands were shown by remaining 4 primers. (Table.1)

Comparison of RAPD profiles of controlled and NaCl stressed *O. tenuiflorum* L. Plants

The final goal of the present research was to compare DNA profiles of controlled and NaCl stressed *O. tenuiflorum* L. plants to evaluate the effect of NaCl stress on their genetic stability (loss of DNA with high concentration of salt and break down of DNA in to small sizes/pieces).The polymerase chain reaction used for genetic analysis requires good quality DNA to ensure successful amplification with reproducible results (Amani *et al*., 2011). Therefore, DNA extracted by CTAB method was used for further RAPD-PCR analysis. RAPD amplification products generated by primers were separated by 1.2 % Agarose Gel Electrophoresis (Rout *et al*., 2009). Out of the seven primers tested three primers gave results .

The obtained product was run on 1.2% agarose gel and observed under UV. The given gel showed the results of RAPD amplification products generated by primers OPH_01_F and OPH_01_RC as shown in Fig 2. With the primer OPH_01_F, 3 monomorphic bands were shown, 01 with the control plant, 01 with the 25mM salt stressed plant and 01 with the 50mM salt stressed plants. While with 75mM and 100mM salt stressed plants polymorphic bands were observed showing genetic variation. The size of the bands ranging between 9000-8000 bps. OPH_01_RC showed the same results with controlled and NaCl stressed tulsi. like primer OPH_01_F (Fig. 2).

Profiles generated by the third primer i.e. OPI_05_R showed 04 monomorphic bands. 01 band with control plant (without NaCl stress), 01 with 25mM salt stressed plant, 01 with 50mM salt stressed plant and 01 with 75mM salt stressed plant. While 100mM salt stressed plant showed total loss of bands. In addition, the expression level of control plant was brighter than that of salt stressed plants which showed that the expression level was gradually decreased with an increase in NaCl concentration. The size of all bands was almost 8000 bps (Fig. 3).

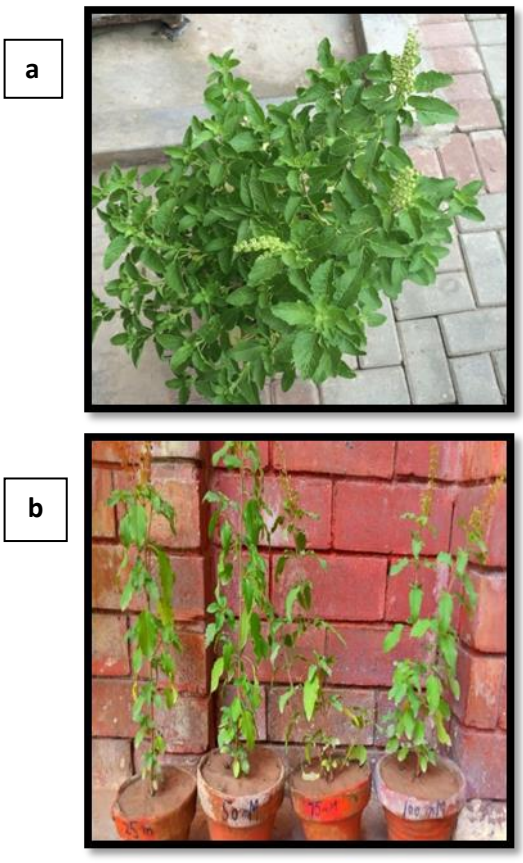

Fig. 1: (a) Control *Ocimum tenuiflorum* L. plant (b) NaCl stressed *Ocimum tenuiflorum* L. plants

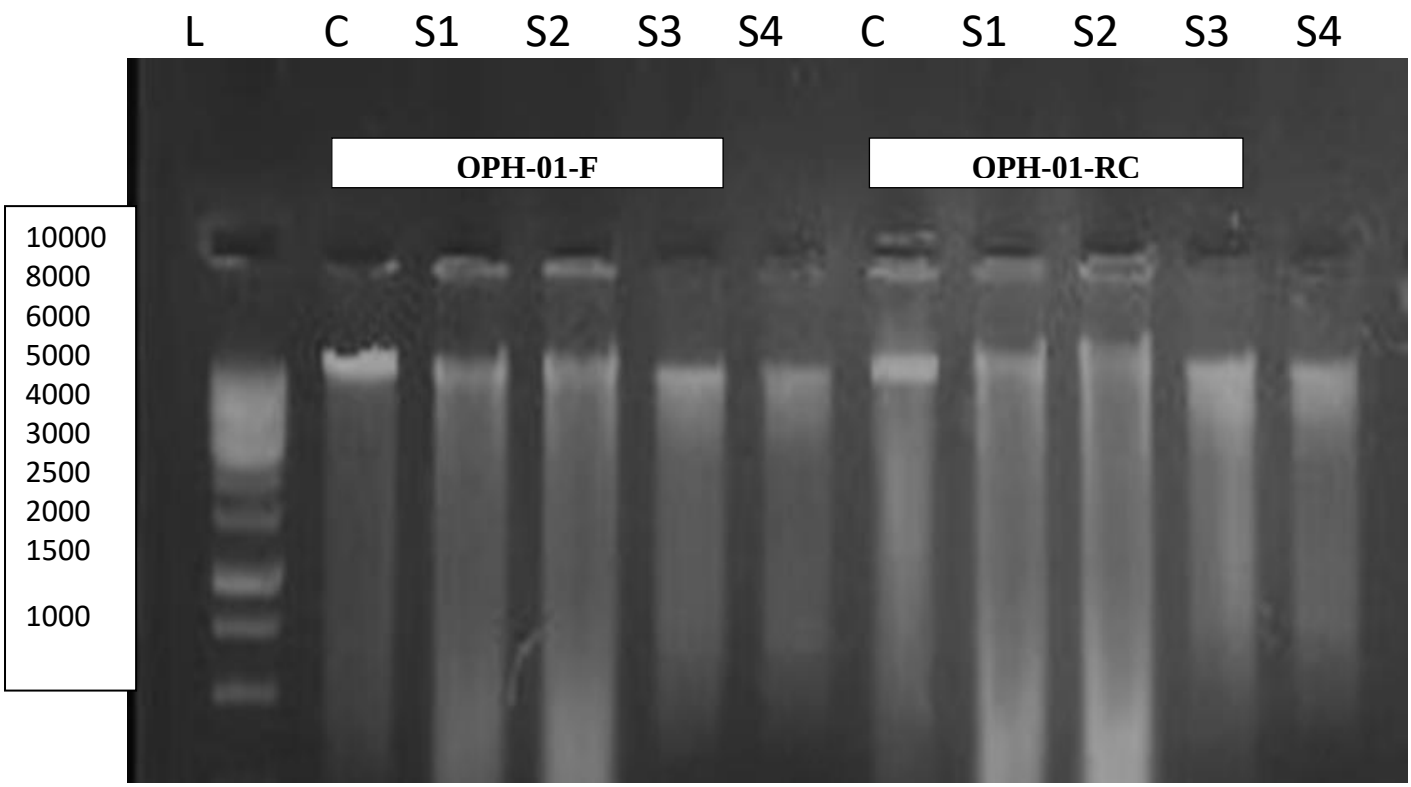

Fig. 2: Amplified DNA Band patterns of control *Ocimum tenuiflorum* L. (C) and NaCl stressed DNA of *Ocimum tenuiflorum* L. (S1= 25mM salt stressed plant, S2= 50mM salt stressed plant, S3=75mM salt stressed plant and S4= 100mM salt stressed plant) using Primer OPH-01-F and OPH-01-RC.

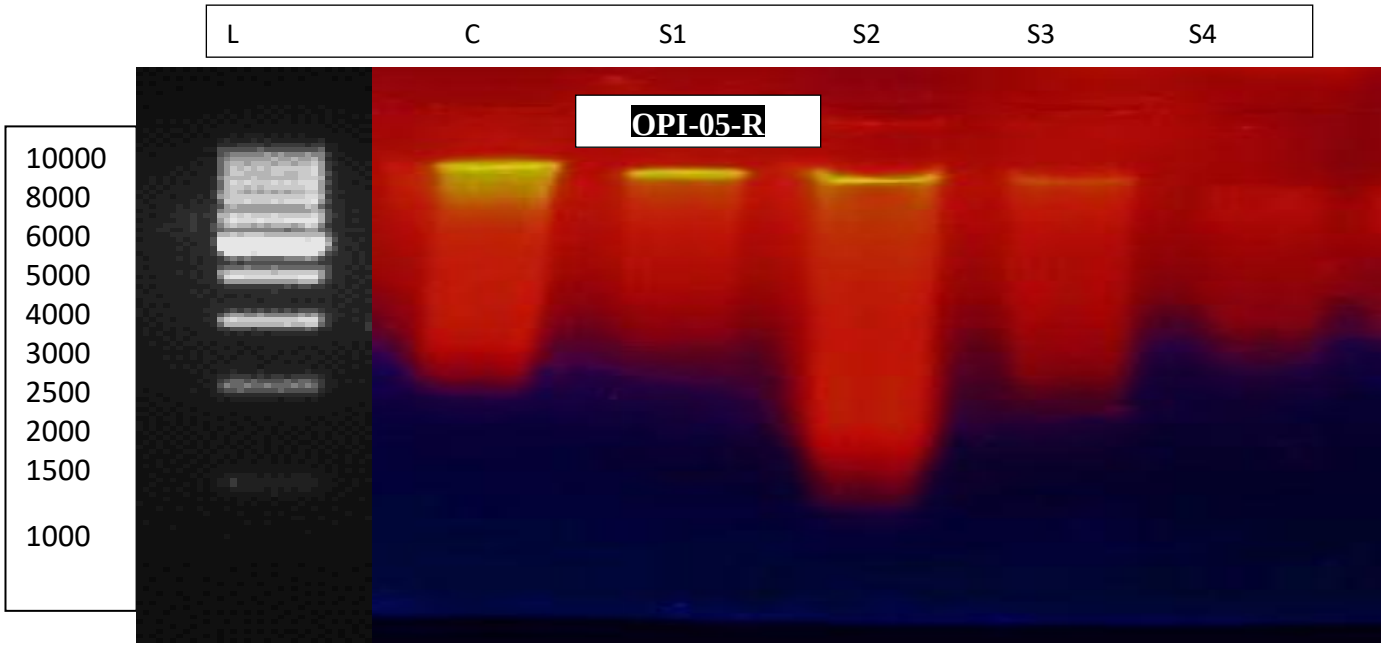

Fig. 3: Amplified DNA Band patterns of control *Ocimum tenuiflorum* L. (C) and NaCl stressed DNA of *Ocimum tenuiflorum* L. (S1= 25mM salt stressed plant, S2= 50mM salt stressed plant, S3=75mM salt stressed plant and S4= 100mM salt stressed plant) using Primer OPI-05-R.

Table 1: Total number of monomorphic and polymorphic bands produced by Primers OPH_01_F, OPH_01_RC and OPI-05-R in control and NaCl stressed DNA samples of *Ocimum tenuiflorum* L. The data represents binary matrix (1 for band presence and 0 for absence) for both samples.

Sample	Total number of bands produced	No. of bands produced with primers		Total Monomorphic bands produced	Total Polymorphic bands produced	Total Loss of Bands
Control Sample	14	OPH_01_F OPH_01_RC OPI-05-R	1 1 1	3	0	1
25mM Salt stressed sample		OPH_01_F OPH_01_RC OPI-05-R	1 1 1	3	0	
50mM Salt stressed sample		OPH_01_F OPH_01_RC OPI-05-R	1 1 1	3	0	
75mM Salt stressed sample		OPH_01_F OPH_01_RC OPI-05-R	1 1 1	1	2	
100mM Salt stressed sample		OPH_01_F OPH_01_RC OPI-05-R	1 1 0	0	2	

DISCUSSION

The present study was carried out with the objective to analyze the genetic destabilization of NaCl stressed lines of *O. tenuiflorum* L. using RAPD markers. During the present piece of work, *O. tenuiflorum* L. plants subjected to different NaCl stresses showed many morphological changes i.e. change in growth of plants, chlorophyll content and leaf color. It was observed that plants subjected to low concentration of NaCl i.e. 25mM showed normal growth just like the control plant whereas at higher concentrations of NaCl i.e. 50mM, 75mM and100mM there was a significant reduction in the growth of the plant. Similarly leaf color damage was observed to increase, progressively at each level of NaCl stress, reaching maximum damage at the highest NaCl concentration of 100 mM.

These results are in accordance with the results of Dhanapackiam & Muhammad (2010) who found that the levels of salinization (40 and 50 mM NaCl) induced a significant decrease in the contents of pigment fractions (chlorophyll a and b) and consequently of the total photosynthetic pigment content as compared with control plants of *O. tenuiflorum* L.

During the present effort, two primers (OPH_01_F and OPH_01_RC) showed a total of 6 monomorphic bands at a locus with control, 25mM and 50mM salt stressed plants indicating that these concentrations did not affect the genetic stability as well as morphology and physiology of *O. tenuiflorum* L. While 4 poly-morphic bands were observed with 75mM and

100mM salt stressed plants showing that these higher concentrations of NaCl started causing genetic damage to the plants affecting their morphological and physiological characters. The influence of genetic background on salt tolerance in soybean was investigated by Khan *et al*., (2013). Ten soybean genotypes were selected and grown under salt stress. Plant growth and random amplified polymorphic DNA (RAPD) analysis were studied. The plant growth in all genotypes was decreased by salt stress in comparison to the control plants. Twenty RAPD primers revealed high polymorphism and genetic variation among ten soybean genotypes studied. The results showed that the closer varieties in the cluster behaved similarly in their response to salinity tolerance.

During the present investigations,, profiles generated by the third primer i.e. OPI_05_R primer showed 4 monomorphic bands with control and NaCl stressed *Ocimum tenuiflorum* L. which included 01 band with control plant, 01 with 25mM salt stressed plant, 01 with 50mM salt stressed plant and 01 with 75mM salt stressed plant. Hundred mM salt stressed plant showed total loss of bands. In addition, the expression level of control plant was brighter than the salt stressed plants, which diminished with the increase in salt stress.

This was supported by the work of Abbas *et al*., (2014) who used eight genotypes of maize to analyze their responses to salt stress (150mM NaCl) at their seedling stage. Eight primers were used for PCR amplification that gave a total of 111 RAPD fragments. 77.7% of these bands were polymorphic. Results of this analysis showed that high molecular weight bands were disappeared under salt stress conditions.

The expression level was gradually decreased with an increase of NaCl concentration. These results suggested that increase in salt concentration had a damaging effect on genetic stability of *Ocimum tenuiflorum* L. and the effect of NaCl induced DNA damage was dose-dependent. This genetic destability could be seen prominently damaging morphological and physiological characteristics of *O. tenuiflorum* L. which most probably resulted from denatured enzymes and subsequently disorganized proteins, due to salt genotoxicity.

Dubey & Ranu (1989) suggested that the decreased number of bands in salt stressed genotype compared with controlled genotypes was associated with denaturing of the enzymes, that were involved in amino acids and protein synthesis under abiotic stress.

High salt concentrations inhibited enzymes by probably disturbing the balance of forces controlling the protein structure. It may be predicted that plants under stress had degraded environmentally-regulated proteins (Abdel *et al*., 2003).

CONCLUSION

From the results of the present study, it is concluded that NaCl acted as a source of genetic variation and changed the DNA patterns. This variation could be used as a useful tool for selection of desirable traits such as salt stress tolerance. The use of molecular markers proved to be a good alternative to the agronomic selection. It may allow a quick selection and provide the breeder with the genetic marker for salt stress on a wider scale, in future.

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