DETECTION OF MOLECULAR MARKERS ASSOCIATED WITH SALT TOLERANCE IN ALFALFA (*Medicago sativa* L.)

F. M. ABDEL-TAWAB[1], EMAN M. FAHMY[1], M. A. EL- NAHRAWY[2], WAFAA M. SHARAWY[2] AND MERVAT R. I. SAYED[2]

1- Genetic Dept., Fac. Agric., Ain Shams Univ., Cairo, Egypt

2- Forage Crops Res. Sec., Field Crop Res. Inst., ARC, Giza, Egypt

Alfalfa (*Medicago sativa* L.) is one of the world's most valuable forage legumes. It is grown for hay, pasture and silage, and is valued highly as a livestock feed. It is characterized by adaptability to wide range of soils and growing conditions, flexibility in being able to grow alone or mixed with grasses and high yields. It is the most cultivated forage legume due to its ability to fix atmospheric nitrogen and its high protein content.

Soil salinization significantly limits crop production and consequently has negative impact on food security. It is one of the major abiotic stresses that affects crop productivity and quality and has been described as one of the most serious threats to agriculture and the natural status of the environment. Increased salinization of arable land is expected to have devastating global effects, resulting in a 30% land loss within the next 25 years and up to 50% by the year 2050 (Lorenzo *et al.*, 2007).

The analysis of the genetic variability within and among populations of cultivated alfalfa can assess future risk of genetic erosion and help in the development of sustainable conservation and genetic improvement strategies. Successful assessment of the genetic diversity of alfalfa has been hampered by the statistical methods available (Stanford, 1951; Flajoulot *et al.,* 2005). Alfalfa species are composed of ecotypes, population complexes adapted to the environment of a given climatic region or to definite habitats within a region (Helmy *et al.,* 2003). Environmental constraints represent the most limiting factors for agricultural productivity and play a major role in the distribution of plant species across different types of environments. Environmental factors, such as drought and salinity are responsible for significant yield reductions. Developing cultivars that tolerate abiotic stresses is one of the major goals of breeding programs of alfalfa. Molecular markers can assist these programs by identifying the important traits, helping in screening the genotypes and selecting them.

Molecular markers can be identified by a range of molecular techniques

such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphism (AFLP), DNA amplification fingerprinting (DAF), sequence characterized amplified regions (SCARs), microsatellites (SSR) etc, (Lin *et al.,* 1996). Also, there are several different DNA analytical procedures that have been used to identify, characterize and determine genetic diversity among cultivars. AFLP is one of the most the recently DNA analysis procedures, which combines assay flexibility with a high degree of sensitivity and reproducibility to yield significantly more information about the plant genome under study than other techniques (Lin *et al.,* 1996). AFLP is a method for genotyping individuals for a large number of loci using a minimal number of PCR reactions. AFLP markers are efficient tools for estimating genetic similarity in plant species and effective management of genetic resources. They are a reliable method of genetic fingerprinting and have been successfully used for characterization and evaluation of genetic relationships in several species (Vos *et al.,* 1995; Neqi *et al.,* 2000).

The objectives of this study were to test the most tolerant and the most sensitive alfalfa (*Medicago sativa* L.) genotypes, their F_1, and F_2 under salt condition for some yield-related traits and obtain molecular genetic markers associated with salt tolerance by bulked segregant's analysis technique using RAPD, ISSR and AFLP analysis.

MATERIALS AND METHODS

This study was carried out in the laboratories and the greenhouse of the Department of Genetics, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, Cairo, Egypt and the Forage Crops Research Department, Field Crops Research Institute, Agriculture Research Center (ARC), Giza, Egypt, during the period from 2007 to 2010. Two landraces; Balat (tolerant parent) and Elkasr (sensitive parent), which were selected from fifteen landraces of alfalfa from a previous study (Sayed, 2004), their F_1 and F_2 seeds were used in this study.

The two contrasting genotypes, their F_1 and F_2 were represented by 200 seeds each, sown in a sand culture for salinity treatment experiment (8000 ppm) compared with the control which was conducted according to Heakel *et al.* (1981). Pots (45 cm height and 50 cm diameter) were filled with fine sand at the rate of 50 kg pot^{-1}. Ten seeds were sown in each pot. Modified-Hogland solution (Johanson *et al.,* 1957) was used as the base nutrient solution every three days until the first cut (pre-treatment). After that, the treatment was started by withholding irrigation for salinity treatment (8000 ppm NaCl) irrigated every 15 days. A split-plot design experiment with three replications was carried out. The two salinity levels (control and 8000 ppm) were in the main plots whereas sub-plot was devoted for the two contrasting parents and their F_1. Samples were taken from each plant to extract DNA for molecular markers techniques.

Measurements were recorded on 10 plants from each pot for the following traits: plant height (cm), number of branches, leaves fresh weight (g), stem fresh weight (g), leaves/stem ratio for fresh weight, total fresh weight (g) and dry forage weight (g). The collected data from the two parents and their F_1 plants were statistically analyzed according to Snedecor and Cochran (1969). The differences among means were compared using Duncan's multiple range test (Duncan, 1955). The F_2 plants were classified into 10 groups depending on their performance under salinity stress to choose the most tolerant and the most sensitive F_2 plants to be used for molecular genetic analysis.

DNA isolation

The DNA isolation protocol was performed as described by Junhans and Metzlatt (1990).

Randomly amplified polymorphic DNA (RAPD)

The PCR reactions were conducted using 13 arbitrary 10-mer primers (Operon Technologies, Inc) (Table 1). The reaction conditions were optimized and mixtures were prepared (25 µl total volumes) consisting of the following: dNTPs (8 mM mix) 2.5 µl, Taq DNA polymerase (5 U/µl) 0.2 µl, 10 X buffer with 15 mM MgCl2 2.5 µl, Primer (10 mM) 1.0 µl, Template DNA (10-50 ng/µl) 1.0 µl, H_2O (dd) 17.8 µl. Amplification was carried out in Stratgene Robo-Cycler Gradient 96 which was programmed for 40 cycles as follows; denaturation 94°C for 4 minutes (one cycle), followed by 40 repeated cycles of 94°C for 1.5 min, 36°C for 1.5 min, 72°C for 2.5 min, and finally one cycle extension at 72°C for 7 min and 4°C (infinitive).

Inter-simple sequence repeats (ISSRs)

ISSR reactions were conducted using five primers, (Table 2). The reaction conditions were optimized and the reaction mixture was consisted of: dNTPs (8 mM mix) 2.5 µl, Taq DNA polymerase (5 U/µl) 0.3 µl, 10 X buffer with 15 mM MgCl2 3.0 µl, primer (10 mM) 2.0 µl, template DNA (50 ng/µl) 2.0 µl, H_2O (dd) 20.2 µl. Amplification was carried out in Stratgene Robocycler Gradient 96 which was programmed for 45 cycles as follows; Denaturation (one cycle) 94°C for 2 min, followed by 30 repeated cycles of 94°C for 30 second, 44°C for 45 sec, 72°C for 1.5 min, and finally one cycle extension at 72°C for 20 minutes and 4°C (infinitive).

Amplified fragment length polymorphism (AFLP)

AFLP procedure was applied according to Vos *et al*. (1995) using the AFLP Analysis System I-invitrogen (cat. no. 10544-013) according to the manufacturer's protocol. Genomic DNA was digested with the restriction enzymes *Eco*R1 and *Mse*1, the adaptors were ligated using T4 DNA Ligase and used in a pre-selective amplification step.

*Mse*1 and *Eco*R1 digestion of genomic DNA and ligation of double-

stranded adaptors were completed in a one-step reaction (37°C, 2 h) using 0.5-1.0 µg of DNA, 2.2 µL of 5 X ligase buffer, 1.1 µL of 0.5 mol/L NaCl, 0.5 µL of 1 mg/mL bovine serum albumin, 1 µL of 50 µmol/L *Mse*I adaptor, 1 µL of 5 µmol/L *Eco*RI adaptor, 0.25 µL *Mse*I, 0.25 µL *Eco*RI, and 0.33 µL of T4 DNA ligase, and then adding water to a total volume of 11 µL. The adaptor ligation reaction was then diluted 10-fold for use in the preselective PCR (4.5 µL DNA solution, 1X PCR buffer, 1.5 mmol/L MgCl2, 1 µmol/L dNTPs, 2.75 µmol/L *Eco*RI preselective primer (5′-GACTGCGTACCAATTCA-3′), 2.75 µmol/L *Mse*I preselective primer (5′-GATGAGTCCTGAGTAAC-3′),

PCRs were conducted in a Stratgene Robocycler Gradient 96 in a total volume of 20 µL using a concentration of 10 pmol for each primer. The preselective PCR included an extension of 72°C for 2 min, which was followed by 29 cycles (each) of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and then a final extension of 10 min at 60°C. The preselective PCR products were diluted 10-fold for use in the selective PCR. An aliquot of the preselective amplification reaction was then used in the selective amplification step with three primers, E-ACC (5′ GACTGCGTACCAATTCAAC 3′); M-CAC, (5′ GATGAGTCCTGAGTAACAC 3′) and M- CTC (5′ GATGAGTCCTGAGTAACTC 3′). Two combinations of *Eco*R1 and *Mse*I primers (E-AAC/M-CAC and E-AAC/M-CTC) were used in a selective amplification. The selective PCR included an initial denaturation of 94°C for 2 min, which was followed by 12 cycles (each) of 94°C for 30s, 65°C for 30s and 72°C for 2 min; then 23 cycles each of 94°C for 20s, 56°C for 30s, and 72°C for 2 min; with a final extension of 10 min at 72°C.

Data analysis

PCR amplification products were scored independently as presence or absence of fragment. Only sharp PCR fragments were scored (not "ghost bands"). Fragments at low intensities were only scored as present when they were reproducible in repeated experiments using Gelworks 1D advanced software (UVP Co., UK). The dominant markers were determined according to Labate (2000) for RAPD, ISSR and AFLP results.

RESULTS AND DISCUSSION

Yield- related traits

The two parents, their F_1 and the six most tolerant and six most sensitive F_2 plants showed marked differences (Tables 3, 4 and Fig. 1) for the following traits; plant height (cm), number of branches, leaves fresh weight (g), stem fresh weight (g), leaves/stem ratio, total fresh weight and dry weight (g) under control and salt treatment (8000 ppm). The data presented in Table (3) showed that the two parents and their F_1 differed significantly for all morphological traits. Data also revealed that the tolerant parent was superior to the sensitive one and F_1. The obtained results in Table (3) revealed that the height of plants for both parents and their hybrid decreased markedly under salinity treat-

ment. The reduction in plant height was recorded to be 35.84% less than the control of the sensitive parent as compared to only 9.95% reduction in the tolerant parent under the same treatment. However, fresh and dry weights of the plants of the two parents and their F_1 were affected by salinity stress. The two traits suffered relatively more in the sensitive parent than the tolerant one. Leaves/stem weight ratio increased under salinity treatment compared with the control. These results are in agreement with Zhou *et al. (*1992) who found that increasing of NaCl concentration led to decrease in the growth of alfalfa. Moreover, Hefny *et al.* (2000) reported that increasing salt concentrations caused reduction in growth parameters and root-stem ratio. Furtherance, Naceur *et al.* (2001) showed also that salinity could reduce plant height and dry weight. Elboutahiri *et al.* (2003) and Helmy *et al.* (2003) reported that NaCl stress resulted in substantial reduction in all the studied parameters.

Recently, Petcu *et al.* (2007) reported that biomass was significantly decreased under salt stress by over 37 % and the effects of salt stress on yield was additive. Shaily *et al.* (2010) reported that salinity effects the growth, development and germination of alfalfa adversely.

Substantial differences between the most tolerant and most sensitive F_2 individual plants were detected for some morphological characters (Table 4 and Fig. 1). Results showed that the tolerant plants were superior to the sensitive ones in all characters except leaves/ stem ratio which increased in the sensitive plants.

Molecular markers related to salinity stress using RAPD

RAPD-PCR technique was used to develop molecular markers for salinity using thirteen 10-mer random primers. All primers successfully amplified DNA fragments for all genotypes and produced different bands number ranging from five to nineteen bands. Five of them showed some molecular markers for salinity tolerance. Primer OP-G05 showed two bands with molecular sizes of 795 and 390 bp which were present in the sensitive parent, F_1 and F_2 sensitive bulk under salinity, while they were absent in the tolerant parent and the F_2 tolerant bulk. So, these bands can be used as negative molecular markers for salinity tolerance in alfalfa plants.

Also, primer OP-L16 showed one band with molecular size of 991 bp which was present in the sensitive parent and F_2 sensitive bulk under salinity, while it was absent in the tolerant parent and F_2 tolerant bulk. So, this band can be used as negative molecular marker for salinity tolerance in alfalfa plants. Three primers (OP-M17, OP-O18 and OP-O20) showed one band each with molecular sizes of 615, 653 and 658 bp, respectively, which were present in the tolerant parent, F_1 and F_2 tolerant bulk under salinity, which were absent in the sensitive parent and F_2 sensitive bulk. So, these bands can be used as positive molecular markers for salinity tolerance in alfalfa plants, (Fig. 2). These

results are in agreement with those of 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.

Echt *et al.* (1992) reported that RAPD markers appeared to be useful for the rapid development of genetic information in alfalfa. Also**,** Dias *et al.* (2004) reported that the RAPD markers were efficient in separating all the accessions analyzed individually. In this respect also, Yang *et al.* (2005) used bulked segregant analysis in combination with RAPD to identify 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. Bortolini *et al.* (2006) used RAPD molecular markers for white clover and their results highlighted the high genetic diversity present between the accessions from different origins and breeding status.

Molecular markers related to salinity stress using ISSR

ISSR primers analysis was used to obtain molecular genetic markers for salinity tolerance from the two parents, their F_1 and the two contrasting bulks of F_2 using five primers (Fig. 3). All ISSR primers succeeded in amplifying DNA fragments. Among the 92 amplified fragments across the five primers, 87 were polymorphic (94.6%). Primer HB-09 showed one band with molecular size of 332 bp which was present in the sensitive parent, F_1 and F_2 sensitive bulk under salinity, while it was absent in the tolerant parent and the F_2 tolerant bulk. So, this band can be used as negative molecular marker for salinity tolerance in alfalfa plants. Also, primer HB-15 gave one band with molecular size of 480 bp which was exclusively present in the tolerant parent and the F_2 tolerant bulk under salinity. So, this band can be used as positive molecular marker for salinity tolerance in alfalfa plants.

These results are in agreement with those of Wei (2004) who reported that DNA fingerprinting can be applied to variety identification and genetic diversity evaluation of *Medicago sativa.* Hassan (2005) reported that ISSR marker is the best choice for the evaluation of diversity and assessing the genetic relationships between *M. oleifera and M. pregrina* genotypes with high accuracy. Also, Said (2005) stated that ISSR markers were useful tools to asses the genetic variations in *Capparis* spp. (caper) and *Solenostemma arghel* (arghel) species which is considered as an important prerequisite for the improvement of these species and for the conservation of their germplasm.

Molecular markers related to salinity stress using AFLP

Two selected combinations were used for amplifications of the digested

DNA fragments for the two contrasting parents, their F_1 and F_2 bulks of alfalfa genotypes (Fig. 4).

Combination of E-AAC/M-CAC primers

Out of a total of 118 fragments, one band showed negative molecular marker with molecular size of 495 bp which was present in the sensitive parent, F_1 and the F_2 sensitive under salinity, while it was absent in the tolerant parent and the F_2 tolerant bulk for salinity. So, this band can be used as a negative molecular marker for salinity tolerance in alfalfa plants.

Combination of E-AAC/M-CTC primers

In this combination out of a total of 128 fragments found, four markers for salinity stress, with molecular size of 700, 750, 540 and 140 bp which were present in the tolerant parent, F_1 and the F_2 tolerant bulk, while they were absent in the sensitive parent and the F_2 sensitive bulk under salinity. So, these bands can be used as positive molecular markers for salinity tolerance in alfalfa plants.

These results are in agreement with those of Powell *et al.* (1996) who suggested that AFLP markers provide high levels of discrimination of complex genetic structures and AFLP markers have the highest effective multiplex ratio. Julier *et al*. (2003) used AFLP to develop markers using specific mapping procedures for autotetraploids. They concluded that, compared to diploid alfalfa genetic maps, their maps cover about 88-100% of the genome and are close to saturation. These maps were valuable tools for alfalfa breeding and were used to locate QTLs. Also, Obert *et al*. (2004) reported that AFLP assay is an efficient method for the identification of molecular markers and is useful in the improvement of numerous crop species.

According to the aforementioned results, alfalfa is a perennial forage crop which remains between 3-5 years in the soil, therefore, elucidation of molecular markers associated with salt tolerance will give an added value to screen for hundreds of landraces and/or elite cultivars in a fast and cost-effective way to aid effective selection for the most promising genotypes to be recommended for growing in salt affected areas of the Egyptian soils.

SUMMARY

Two selected landraces, their F_1 and F_2 plants were tested for salinity tolerance (8000 ppm) in sand culture experiment, during the period from 2007 to 2010. The results indicated that all the morphological traits measurements decreased markedly under salinity treatment compared with the control in the two parents and their F_1. While, leaves/stem weight ratio increased under salinity treatment compared with the control. Molecular genetic studies including randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSRs) and amplified fragment length polymorphism (AFLP) were applied to identify some molecular markers associated with salinity tolerance in the two parents, their F_1 and F_2 bulked plants. The RAPD-PCR using

thirteen random primers showed some molecular markers for salinity tolerance with five of them. Primer OP-G05 showed two bands with molecular sizes of 795 and 329 bp and primer OP-L16 showed only one band with molecular size of 991 bp, these three bands were present in the sensitive parent, F_1 and F_2 sensitive bulk under salinity, and absent in the tolerant parent and F_2 tolerant bulk. Consequently, they can be used as negative molecular markers for salinity tolerance in alfalfa plants. Three primers (OP-M17, OP-O18 and OP-O20) showed one band for each with molecular sizes of 615, 653 and 658 bp, respectively, which were present in the tolerant parent, F_1 and F_2 tolerant bulk under salinity, and absent in the sensitive parent and F_2 sensitive bulk. So, these bands can be used as positive molecular markers for salinity tolerance. The five primers used for ISSR-PCR showed that HB-09 primer exhibited one negative molecular marker while HB-15 primer showed one positive molecular marker. Some AFLP markers were recorded using two primer pairs of E-AAC/M-CAC and E-AAC/M-CTC. A total of 5 AFLP markers (four positive and one negative) out of 246 bands were identified.

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Table (1): Nucleotides sequence of the 13 random (10 mer) primers used for RAPD-PCR technique.

Primer name	Sequence	Primer name	Sequence
OP-A10	5′-GTG ATC GCA G-3′	OP-G05	5′ -CTG AGA CGG A- 3′
OP-A19	5′-CAA TCG CCG T- 3′	OP-I17	5′ -GGT GGT GAT G-3′
OP-C12	5′ -TGT CAT CCC C- 3′	OP-L16	5′ -AGG TTG CAG G-3′
OP-C20	5′- ACT TCG CCA C -3′	OP-M17	5′ -TCA GTC CGG G- 3′
OP-E03	5′- CCA GAT GCA C -3′	OP-O18	5′ -CTC GCT ATC C- 3′
OP-F04	5′ -GGTGATCAGG -3′	OP-O 20	5′ - ACACACGCTG - 3′
OP-F06	5′- GGGAATTCGG- 3′		

Table (2): Nucleotides sequence of the five ISSR specific primers.

Primer	Sequence
HB08	5′ GAGAGAGAGAGACC 3′
HB09	5′ GTGTGTGTGTGTGG 3′
HB12	5′ CACCACCACGC 3′
HB13	5′ GAGGAGGAGGC 3′
HB15	5′ GTGTGTGTGTGTGC 3′

Table (3): Means of some yield-related traits of the two contrasting parents and their F_1 under control and salinity (8000 ppm) treatment.

Treatment	Plant height(cm)	No. of branches	Fresh weight (g plant^{-1})	Dry weight (g plant^{-1})	Leaves/stem ratio
Control	46.42	15.00	12.08	2.37	1.67
8000 ppm	37.29	8.00	5.02	1.09	2.04
t-test	**	**	**	**	**
Tolerant parent (P_1)	60.75	14.00	13.47	2.57	1.99
Sensitive parent (P_2)	22.35	8.00	5.28	1.20	1.70
F_1	42.47	11.00	6.90	1.44	1.88
LSD 0.05	1.20	1.00	0.28	0.15	0.13
P_1 Control	63.93	19.00	18.20	3.32	1.59
8000 ppm	57.57	10.00	8.73	1.81	2.38
P_2 Control	27.23	11.00	7.73	1.70	1.49
8000 ppm	17.47	6.00	2.83	0.70	1.91
F_1 Control	48.10	15.00	10.30	2.10	1.92
8000 ppm	36.83	7.00	3.50	0.77	1.84
LSD 0.05	1.69	1.00	0.40	0.22	0.18

Table (4): F_2 individual plants in the two extreme groups; the most-salt tolerant and the most-salt sensitive according to some yield related traits under salt treatment.

Genotype	Plant No.	Plant height	No. of branches	Leaves fresh weight	Stem fresh weight	Leaves/stem ratio	Plant fresh weight	Plant dry weight
The most- salt tolerant F_2 plants	29	70.26	20	3.50	2.19	1.60	5.69	1.72
	48	60.45	7	3.08	1.94	1.59	5.03	1.29
	79	57.11	3	2.81	2.38	1.18	5.18	1.01
	93	71.24	12	2.91	2.28	1.28	5.17	1.17
	103	61.61	8	2.98	2.19	1.37	5.19	1.14
	109	56.43	5	2.61	2.41	1.09	5.02	1.23
Mean of F_2 Tolerant		62.85	9.16	2.98	2.23	1.35	5.21	1.26
The most- salt sensitive F_2 plants	18	39.20	10	1.13	0.17	6.65	1.29	0.11
	26	48.16	10	1.14	0.23	4.96	1.37	0.13
	31	51.42	8	1.19	0.29	4.10	1.49	0.25
	38	41.16	11	1.21	0.18	6.72	1.15	0.09
	58	64.27	3	1.11	0.27	4.11	1.38	0.13
	59	44.19	4	1.21	0.27	4.48	1.48	0.21
Mean of F_2 Sensitive		48.07	7.18	1.17	0.24	5.17	1.36	0.15

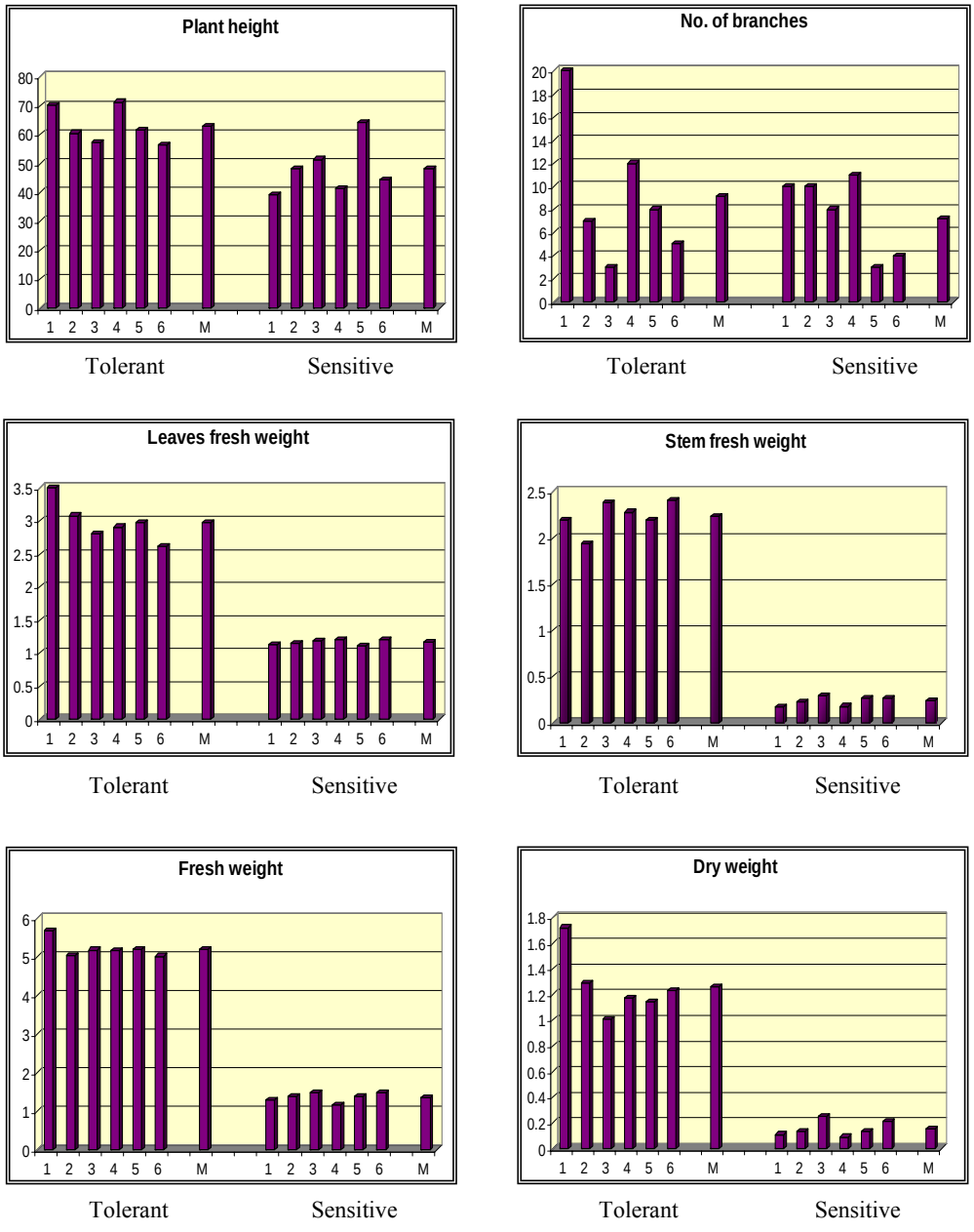

Fig. (1): Histograms of some yield-related traits of the most tolerant and most sensitive F_2 plants under salinity condition by individual six plants each.

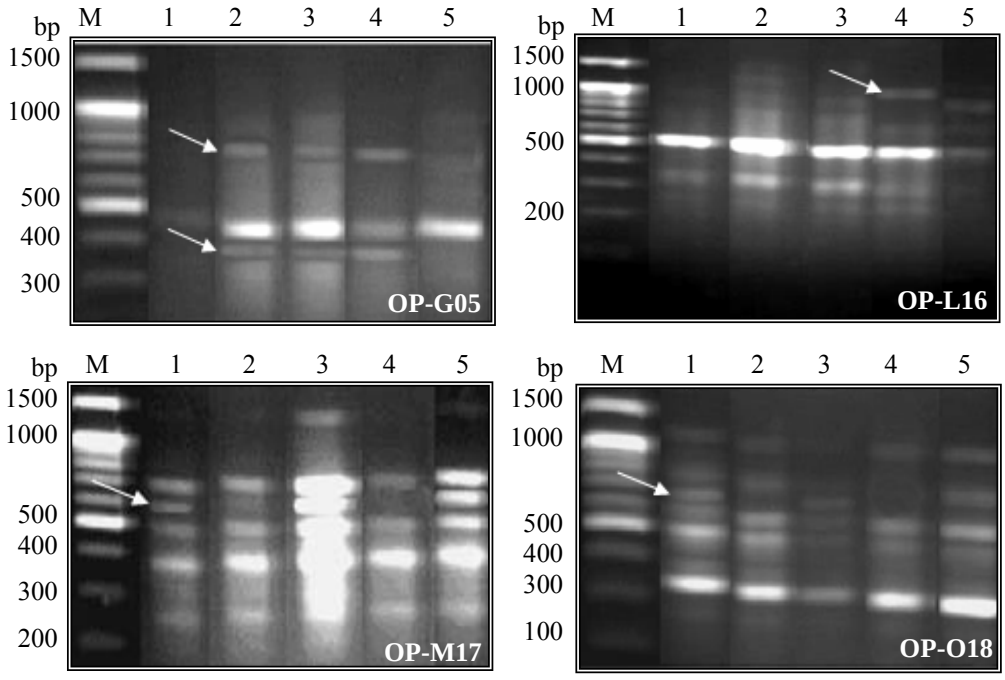

Fig. (2): DNA polymorphism of the alfalfa genotypes amplified with primers, OP-G05, OP-L16, OP-M17, OP-M18 and OP-O20. (M) DNA marker,
(1) P_1 (Tolerant parent)
(2) P_2 (Sensitive parent)
(3) F_1 plants
(4) F_2 (sensitive bulk)
(5) F_2 (tolerant bulk)

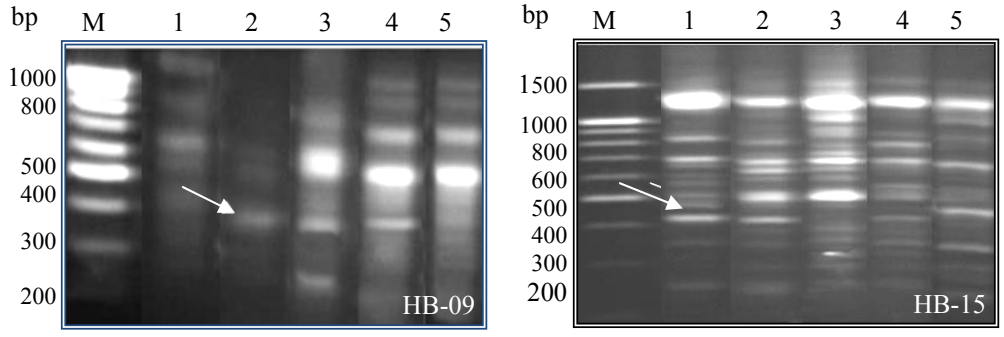

Fig. (3): ISSR profiles of the alfalfa genotypes amplified with primers HB-09 and HB-15.
(M) DNA marker, (1) P_1 (Tolerant parent) (2) P_2 (Sensitive parent)
(3) F_1 plants (4) F_2 (sensitive bulk) (5) F_2 (tolerant bulk)

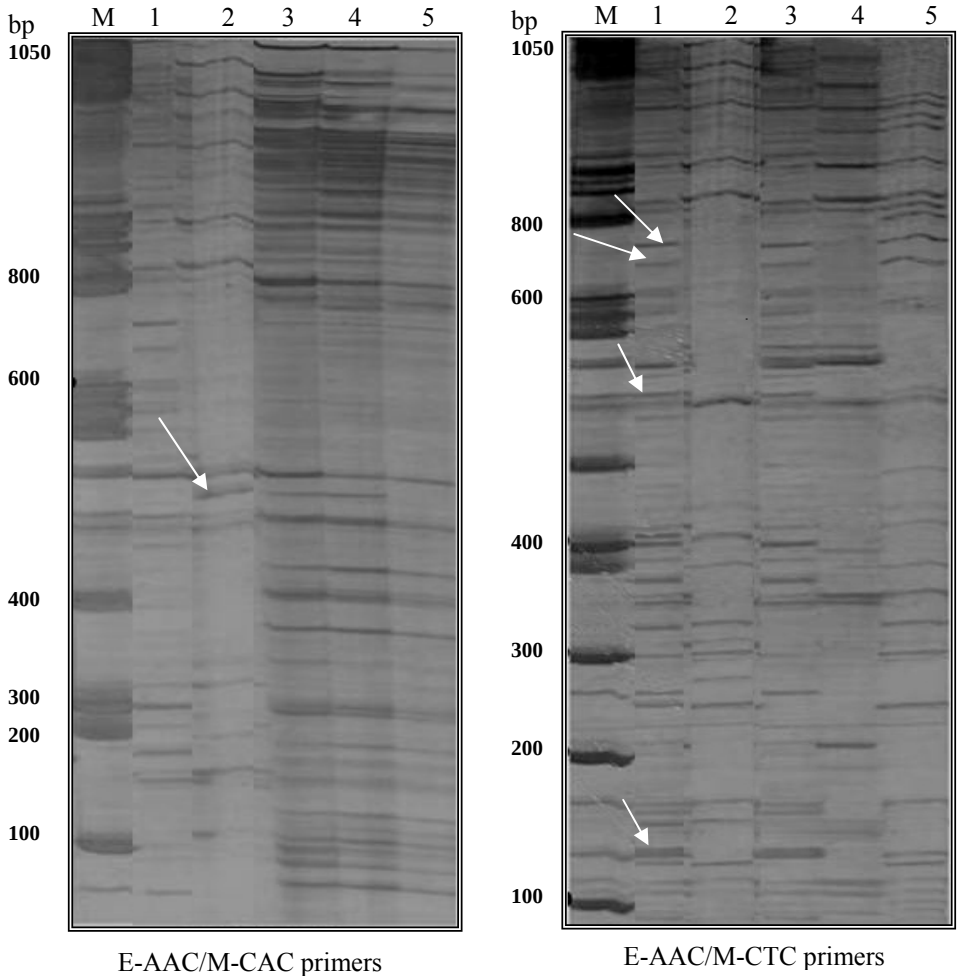

Fig. (4): AFLP profiles of the alfalfa genotypes amplified with two selective primers, E-AAC/M CAC and E-AAC/M-CTC.
(M) DNA marker, (1) P_1 (Tolerant parent) (2) P_2 (Sensitive parent)
(3) F_1 plants (4) F_2 (sensitive bulk) (5) F_2 (tolerant bulk)