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# RELATIONSHIP BETWEEN SALT TOLERANCE RELATED PHYSIOLOGICAL TRAITS AND PROTEIN MARKERS IN SOYBEAN CULTIVARS (*GLYCINE MAX* L.)

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**ABSTRACT.** This study was conducted to evaluate the salinity tolerance in seedling stage of soybean (*Glycine max* L.). Factorial experiment was done based on randomized complete block design with three replicates. 17 soybean genotypes were used in three salinity stress levels (consisting of control, 75 mM and 150 mM NaCl stress). The experiment was carried out in a greenhouse condition and proline, sodium, potassium, and chlorophyll a, chlorophyll b, chlorophyll a/b and total chlorophyll content were examined. To create salinity stress, NaCl was used in the experiment. The results revealed that different salinity stress had significant effects on all traits except for chlorophyll b and chlorophyll a/b. The cluster analysis in the control and at 75 and 150 mM salinity levels classified genotypes into two, two and three groups respectively. In each condition, the dpx and clean genotypes were placed in a group which the average traits were higher than the other genotypes. This can be generalized to the conditions of control as well as 75 and 150 mM salinity stress. Regression analysis showed possible informative loci encoding protein markers that was probable potential for selection strategies for salt weather proved by complementary tests.

**Key words**: Protein marker; Salinity; Soybean.

## INTRODUCTION

After drought, the salinity is the main and the most common environmental stress in the world, especially Iran (Akhani and Ghorbanli, 1993; Choukr-Allah, 1996). Plant growth through salt stress might be reduced and decreased by osmotic stress and water potential in root growth environment, respectively, or by specific effects of ions in metabolic processes (Greenway and Munns, 1980). Soybean plays an

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important role in world agriculture because of its oil content and protein. It contain about 20% oil and 40% protein, it was devoted the highest cultivated lands in the world for soybean. Genetic variation for parental selection in breeding programs and protein polymorphisms are of great importance and concern in plant breeding. Majority of protein bands of soybean including (11S) glycine and beta-conglycinin (7S), which itself consist of several subunits (Brooks and Morr, 1985). Therefore, this study was performed to evaluate cultivated soybean genotypes in Iran for salinity tolerance. Also, in this paper classification of genotypes through protein markers and physiological traits and their possible association with salt tolerance were considered.

## MATERIALS AND METHODS

**Proline measurement in leaves**

Following Bates *et al.* (1973), proline extraction was performed from the youngest leaves. For this to happen, 100 mg leaf tissue was homogenized in 10 ml of sulfur salicylic acid 3.3%, then the filtered liquid was centrifuged in speed of 4000 rpm and at temperature of 4°C for 10 min, and was added to 2 ml of this extract, 2 ml reagent nynhydrine and 2 ml acetic acid glacial in another separate pipe. The pipes were placed in the water bath for an hour after adding 4 ml of toluene to each tube, were vortexed for 15 to 20 seconds. After forming two separate phases, colored upper phase was separated accurately and was measured in spectrophotometer with a wave length of 520 nm.

**Color measurement of tiny photosynthetic**

Fresh leaf tissue was used to measure chlorophyll 0.2 g leaf tissue with acetone 80% was pulverized gradually to enter the chlorophyll into the acetone solution and finally the solution volume with 80% acetone is brought to 20 ml. The resulting solution was centrifuged for 10 min at 400 rpm and then the absorbance of supernatants was read at wavelengths of 470, 645, 663 nm by a spectrophotometer. Using the following equation, the amount of chlorophyll and carotenoids was obtained: V and W stand for acetone final volume and sample weight in grams, respectively (Arnon, 1967).

$$\text{Chlorophyll a} = (19.3 * A_{663} - 0.86 * A_{645})\ V/100\ W$$

$$\text{Chlorophyll b} = (19.3 * A_{645} - 3.6 * A_{663})\ V/100\ W$$

$$\text{Carotenoides} = 100\ (A_{470}) + 3.27\ (\text{mg chl a}) - 104\ (\text{mg chl b})/22$$

**Measurement of sodium and potassium**

To study the effect of salinity on concentration of sodium, potassium and their distribution in leaves, two weeks after salt stress the samples were selected and put in Avon for 48 hours at 72°C. Dried tissues after being powder in porcelain mortar, for preparation of ashes were put in oven at 550 degrees. Then on each sample was added 10 cc normal hydrochloric acid (HCl), was added and after boiling and absorbing first bubble, the solution made clear with using of filter paper and poured in balloon. Finally,

the volume of balloon by distilled water brought to 100 cc. After calibration of photometer machine with standard solutions the amount of sodium and potassium was measured. Analysis of seed proteins for extraction of proteins with low molecular weight was performed by Payne and Lawrence method (1983). For protein electrophoresis was used of SDS-PAGE method (polyacrylamide gel electrophoresis in presence of sodium sulfate Dsyl). SDS-PAGE was performed with 10% separating gel and 5% stacking gel, according to Laemmli method (1970).

## RESULTS AND DISCUSSION

The analysis of variance for physiologic traits showed that there significant differences among stress levels in all of the studied traits and studied genotypes in all of the traits except chlorophyll b and chlorophyll ab (*Tabel 1*).

Comparison of different levels of salinity on physiological traits showed that with increasing of salinity, the amount of potassium, sodium, chlorophyll a and chlorophyll b was reduced. The most of these traits were in control level and the lowest of them observed in salinity (*Tabs. 2 and 3*).

Proline amount increased with increasing of salinity levels. Also, Kumar *et al*. (2003) reported that in more tolerant conditions, chlorophyll degradation is less. Salinity reduces the amount of leaf chlorophyll (Chiej, 1984; Cramer and Bowman, 1991; Kaya, 2001). With increasing of salinity, the amount of a and b chlorophylls reduced that is appearing of deficiency in their synthesis (Prasad, 1997).

In control level, M7 genotype had highest mean but with increasing of stress levels, the amount of proline increased. So that, in salinity level of 75 mili-molar, 033 genotype had high amount of proline and at 150 mili-molar, JK and Hamilton had highest amount of proline. Main role of proline isn't reducing osmotic potential, but its role is protection of enzymes against dehydration and accumulation of salt (Thomas, 1990). Significant effect of proline on osmotic adjustment is protection of cellular structures and its action on free and sensitivity to salinity has been reported in large number of plants (Koca *et al.*, 2007; Turan *et al*., 2009 ; Desingh and Kanagaraj, 2007; Ashraf and Harris, 2004). Potassium served as a high consumption element in higher plants had important role in enzyme homeostasis. Regulation of cell swelling, electrical load cell, leaf movements and constructing of protein (Yeo and Flowers, 1984; Akinamed *et al.,*2010). Potassium an osmotic regulator weather it was available in soil, will be observed by roots and cause decrease in osmotic potential inside the cell environment. As a result, water losses from plant reduce. One of the effects of salinity can be loss of potassium function in leaves of plant. Increasing the amount of potassium with increasing of salinity stress and toxicity of sodium ions with disorders in No/N can be one of the reasons for decreasing of growth (Flowers *et al*., 1977; Shiro *et al*., 2002).

**Tabel 1 - Analysis of variance table for studding traits between soybean genotypes**

| S.O.V | df | MS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Chl a ($mgr/grf_w$) | Chl b ($mgr/grf_w$) | a/b ($mgr/grf_w$) | a+b ($mgr/grf_w$) | Sodium (ppm) | Potassium (ppm) | Potassium/ sodium (ppm) | Prolin ($\mu mol\ g^{-1}f_w$) |
| Rep | 2 | $0.001^{n.s}$ | $0.001^{n.s}$ | $0.107^{n.s}$ | $0.004^{n.s}$ | $0.073^{n.s}$ | $3.173^{**}$ | $0.679^{**}$ | $0.070^{**}$ |
| Salinity | 2 | $0.131^{**}$ | $0.030^{**}$ | $1.580^{**}$ | $0.189^{**}$ | $2.127^{**}$ | $35.508^{**}$ | $8.689^{**}$ | $3.3556^{**}$ |
| Genotype | 16 | $0.008^{*}$ | $0.001^{n.s}$ | $0.104^{n.s}$ | $0.008^{**}$ | $0.267^{**}$ | $1.205^{**}$ | $0.194^{**}$ | $0.072^{**}$ |
| Salinity×Genotype | 32 | $0.006^{n.s}$ | $0.0001^{n.s}$ | $0.104^{n.s}$ | $0.004^{n.s}$ | $0.069^{n.s}$ | $0.292^{n.s}$ | $0.053^{n.s}$ | $0.040^{**}$ |
| Error | 100 | 0.004 | 0.0001 | 0.102 | 0.003 | 0.085 | 0.512 | 0.078 | 0.016 |
| C.V | | 17.71 | 28.78 | 22.72 | 26.62 | 11.03 | 19.53 | 20.85 | 17.64 |

ns and**: not significant and significant differences in p≤0.01



**Tabel 2 - Comparisons of mean in stress levels for studding traits between soybean genotypes**

| Salinity | Chl a ($mgr/grf_w$) | Chl b ($mgr/grf_w$) | a/b ($mgr/grf_w$) | a+b ($mgr/grf_w$) | Sodium (ppm) | Potassium (ppm) | Potassium/ sodium (ppm) |
|---|---|---|---|---|---|---|---|
| Control | $1.88686^{a}$ | $0.101144^{a}$ | $1.982292^{b}$ | $0.289830^{a}$ | $2.462029^{c}$ | $4.551108^{a}$ | $1.853550^{a}$ |
| Salinity (75 mM) | $0.31152^{b}$ | $0.074080^{b}$ | $1.632643^{c}$ | $0.184722^{b}$ | $3.600711^{b}$ | $3.545034^{b}$ | $1.36680^{b}$ |
| Salinity (150 mM) | $0.110642^{b}$ | $0.052634^{b}$ | $0.697718^{a}$ | $0.183786^{b}$ | $2.864091^{a}$ | $2.894995^{c}$ | $1.032764^{b}$ |

**Tabel 3 - Comparisons of mean in salt stress for studding traits between soybean genotypes**

| Genotype | Chl a ($mgr/grf_w$) | (a+b) ($mgr/grf_w$) | Sodium (ppm) | Potassium (ppm) | Potassium/ sodium (ppm) |
|---|---|---|---|---|---|
| Jk | $0.1090^{c}$ | $0.1898^{cd}$ | $2.386^{d}$ | $3.7039^{abcd}$ | $1.5540^{ab}$ |
| Hamilton | $0.1550^{abc}$ | $0.2295^{abcd}$ | $2.453^{cd}$ | $3.7429^{abcd}$ | $1.5378^{ab}$ |
| Interprize | $0.1800^{ab}$ | $0.2574^{ab}$ | $2.4239^{cd}$ | $3.4017^{cd}$ | $1.4077^{abc}$ |
| Lavina | $0.1184^{bc}$ | $0.1835^{cd}$ | $2.8508^{ab}$ | $3.1992^{d}$ | $1.1750^{c}$ |
| Linford | $0.1271^{bc}$ | $0.1934^{bcd}$ | $2.5264^{bcd}$ | $3.4757^{cd}$ | $1.3964^{abc}$ |
| Liana | $0.1078^{c}$ | $0.1690^{d}$ | $2.8406^{ab}$ | $3.6967^{abcd}$ | $1.3199^{bc}$ |
| t.m.s | $0.1193b^{c}$ | $0.1955^{cd}$ | $3.0252^{a}$ | $3.5374^{cd}$ | $1.2449^{bc}$ |
| Stressland | $0.1230b^{c}$ | $0.2067^{cd}$ | $2.531123^{bcd}$ | $3.7275^{abcd}$ | $1.4927^{abc}$ |
| Safyabad | $0.1378b^{c}$ | $0.2092^{cd}$ | $2.6420^{bcd}$ | $3.2660^{d}$ | $1.2583^{bc}$ |
| O33 | $0.1537^{abc}$ | $0.2283^{cd}$ | $2.5646^{bcd}$ | $3.5990^{bcd}$ | $1.4105^{abc}$ |
| Sahar | $0.1520^{abc}$ | $0.2272^{cd}$ | $2.6821^{abcd}$ | $4.3709^{ab}$ | $1.6851^{a}$ |
| M7 | $0.1666^{abc}$ | $0.2585^{ab}$ | $2.5255^{bcd}$ | $3.2218^{d}$ | $1.2997^{bc}$ |
| b.p | $0.143975^{abc}$ | $0.217802^{bcd}$ | $2.5469^{bcd}$ | $3.5302^{cd}$ | $1.3951^{bc}$ |
| Clean | $0.1664^{abc}$ | $0.2448^{abc}$ | $2.7362^{abcd}$ | $4.4603^{a}$ | $1.6839^{a}$ |
| Gorga | $0.1335^{bc}$ | $0.2125^{abcd}$ | $2.6774^{abcd}$ | $3.6905^{abcd}$ | $1.3994^{abc}$ |
| Calark | $0.1508^{abc}$ | $0.2237^{abcd}$ | $2.7380^{abcd}$ | $3.4911^{cd}$ | $1.3088^{bc}$ |
| dpx | $0.1942^{a}$ | $0.2832^{a}$ | $2.7753^{abc}$ | $4.1674^{abc}$ | $1.5310^{ab}$ |

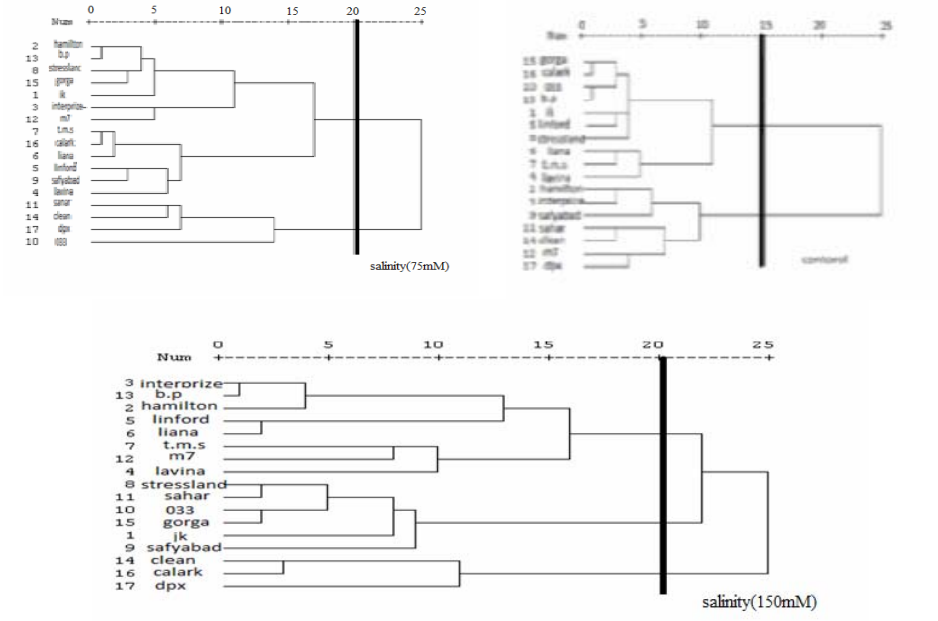


**Figure 1 - Cluster analysis in control and stress condition in studding genotypes**

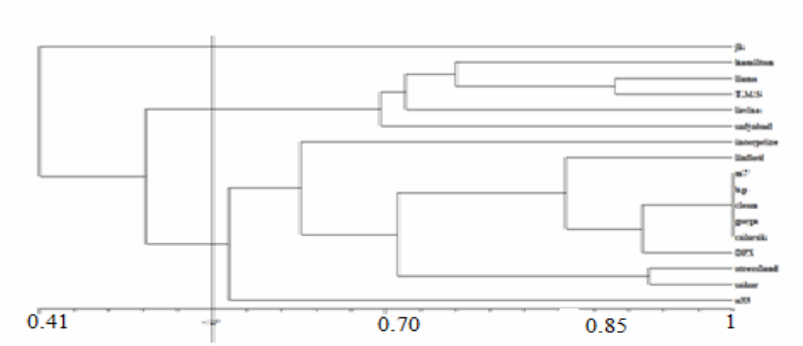


**Figure 2 - Cluster analysis based on seed storage protein electrophoresis**

**Cluster analysis of studied varieties based on physiological traits**

For grouping studding genotypes cluster analysis by method of ward minimum variance using standard squared euclidean distance was done, based on mean physiological traits in control and salinity levels of 75 and 150 mm by cutting tree at distance of 15 units in control level and at distance of 20 units for stress of 75 and 150 mm NaCl and genotypes were divided into two and three groups, respectively (*Fig. 1*).

In control level genotypes 2,3,9,11,12,17 and 14 was placed in second cluster and in first stress level, varieties 10,11,14 and 17 was placed in second cluster and second stress, the third as best cluster involved 14,16 and 17 that in point of studied traits have higher average than whole average. According to cluster analysis 14 and 17 are advisable to three stress conditions (*Fig. 2*).

**Protein markers**

Results of storage protein subunits had shown that there was abundant variation in these subunits in soybean cultivars. So that, the mean of genetic diversity of populations was 0.43 and Shannon index was 0.41. Totally for 17 studied genotypes, we observed 19 protein bands. According to data obtained from table of gene diversity (*Tabel 4*), 15 possible gene loci were polymorphic and percentage of total polymorphic is equal to 78.95 percent. Most gene variation was related to markers 8, 18, 19 and rate was 48%.

Cluster analysis was done based on jacquard similarity coefficient and finally drawing dendrogram was done based on UPGMN method (*Figs. 1-3*). By cutting dendrogram in 0.56 units, three groups were obtained. The first contained JK variety, the second group included Hamilton, Liana, Safyabad, TMS varieties and third group included Sahar, M7, interprize, Laviana, 033, Streesland, BP clean, Calark, Gorgan and OPX. Our results showed that maximum similarity or minimum genetic distance was related to varieties BP and MQ. Additionally maximum genetic distances between

varieties JK and laving, M7 and JK with similarity coefficient of 0.3684 was observed. Convenience of cluster analysis was determined according to significant of cophenetic correlation coefficient (0.8). Patterns of soybean seed storage proteins were shown in *Fig. 3*.

**Association among the studied traits with protein markers**

According to regression analysis, for all studied traits except for potassium in all conditions there was significant relation with some of the studied protein markers and for all of the traits except potassium and sodium occurred. Positive markers recognized: some markers were associated with chlorophyll a, chlorophyll b, chlorophyll a/b and whole chlorophyll. Also, in the case of sodium and potassium/sodium and proline there was some associations occurred (*Tabel 5*).

**Tabel 4 - Genetic diversity based on Shannon and Nei indexes in LMW subunits**

| Locus | Nei's gene diversity | Shannon's information Index | locus | Nei's gene diversity | Shannon's information Index |
|---|---|---|---|---|---|
| 1 | 0.45 | 0.64 | 11 | 0.11 | 0.22 |
| 2 | 0.45 | 0.64 | 12 | 0.11 | 0.22 |
| 3 | 0.41 | 0.60 | 13 | 0.29 | 0.46 |
| 4 | 0.45 | 0.64 | 14 | 0.41 | 0.60 |
| 5 | 0 | 0 | 15 | 0 | 0 |
| 6 | 0 | 0 | 16 | 0.29 | 0.46 |
| 7 | 0 | 0 | 17 | 0.29 | 0.46 |
| 8 | 0.48 | 0.67 | 18 | 0.48 | 0.67 |
| 9 | 0.41 | 0.60 | 19 | 0.48 | 0.67 |
| 10 | 0.11 | 0.22 | | | |

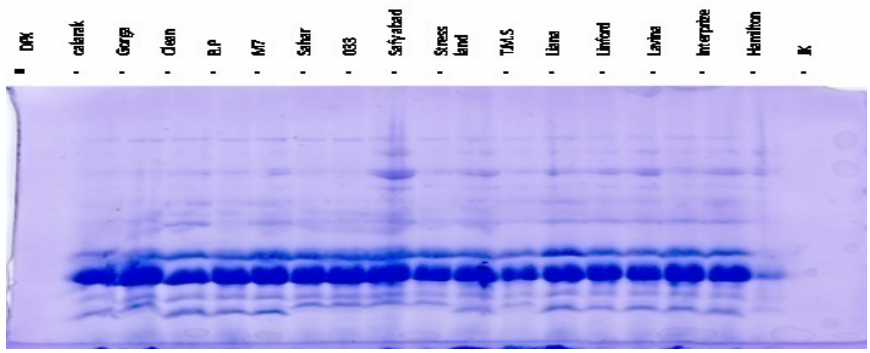


**Figure 3 - Sample gel electerophoresis using seed storage protein in studding cultivars**

**Tabel 5 - Regression coefficient between morpho-physiologic and protein markers**

| Control | Chl a | | | Chl b | | | a/b | | | a+b | | | Sodium | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Salinity (75 mM) | Salinity (150 mM) | Control | Salinity (75 mM) | Salinity (150 mM) | Control | Salinity (75 mM) | Salinity (150 mM) | Control | Salinity (75 mM) | Salinity (150 mM) | Control | Salinity (75 mM) | Salinity (150 mM) |
| Intercep constany | 0.083 | 0.553 | 0.90 | 0.067 | | | 1.202 | 1.465 | | | 0.158 | | 2.417 | 2.835 |
| Pm1 | | | 0.696** | 0.565* | | | -.296* | 0.424* | | | 0.504* | | | |
| Pm3 | | | | | | | | | | | | | | 0.493** |
| Pm4 | | | | | | | | | | | | | | |
| Pm5 | 0.551* | | | | | | 0.737* | | | | | | | |
| Pm9 | | 0.429* | | | | | | | | | | | | |
| Pm10 | -0.617** | | | | | | -0.631** | | | | | | | |
| Pm11 | | | | | | | | | | | | | | |
| Pm12 | | | | | | | | | | | | | | |
| Pm14 | | | | | | | | | | | | | | |
| Pm15 | | -.583** | | | | | | 0.558** | | | | | | |
| Pm17 | | | | | | | | | | | | | | -0.584** |
| Pm18 | | | | | | | 0.374** | | | | | | 0.609* | |
| Pm19 | | | | | | | | | | | | | | |
| $R^2$ | 0.547 | 0.067 | 0.451 | 0.274 | | | 0.868 | 0.453 | | | 0.204 | | 0.328 | 0.324 |

ns and**: not significant and significant differences in p≤0.01

## CONCLUSION

Our results showed excess genetic variance or polymorphism in the case of physiologic traits and protein markers. These findings together with some possible and potential associations with protein markers confirmed evidences of effectuality of protein markers in any salt tolerance selection approaches and proved that this type of biochemical markers became useful tools. It was clear that after some complementary statistical tests (false discovery rate) in order to reveal fidelity of marker associations we can use positive markers in the marker assisted programs.

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