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Investigation of the relationship between physiological traits and protein markers in soybean cultivars (*Glysin max* L.)

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Abstract: This study was conducted to evaluate salinity tolerance in the seedling stage of soybean (*Glysin max* L.). A factorial experiment was performed in a randomized complete block design with three replications. Seventeen soybean genotypes were used at 3 salinity stress levels (consisting of control, 75 mM and 150 mM NaCl stresses). The experiment was carried out in greenhouse conditions and proline, sodium, potassium, and chlorophyll a, chlorophyll b, chlorophyll a/b and total chlorophyll content were examined. To create salinity stress, NaCI was used in the experiment. The results revealed that different salinity stresses 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 whose average traits were higher than those of the other genotypes. This can be generalized to the conditions of the control as well as of 75 and 150 mM salinity stress. Regression analysis showed possible informative loci encoding protein markers as a probable potential for selection strategies for salt conditions, as proved by complementary tests.

Key words: Protein marker, Salinity, Soybean.

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Introduction

After drought, salinity is the main and the most common environmental stress in the world, especially in Iran (Akhani 1993; Chuker-Alla 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 important role in world agriculture because of its oil content and protein. It contains about 20% oil and 40% protein. Soybean covers the highest percentage of cultivated land in the world. Genetic variation for parental selection in breeding programs and protein polymorphisms are of great importance and concern in plant breeding. The majority of protein bands of soybean including (S11) glycine and glycine btakan (S7) consist of several subunits (Brooks *et al*. 1985). Therefore, this study was performed to evaluate cultivated soybean genotypes in Iran for salinity tolerance. Also, 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 10ml of 3.3% sulfosalisilic acid, then the filtered liquid was centrifuged at 4000 rpm and at a temperature of 4°C for 10 min, and was added to 2ml of the extract, 2ml reagent Nine dimenhydrinate and 2 ml glacial acetic acid 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, the colored upper phase was separated accurately and was measured in a spectrophotometer with a wave length of 520 nm.

Color measurement of tiny photosynthetic. Fresh leaf tissue was used to measure chlorophyll i.e. 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 was 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 (Ayron 1967).

Chlorophyll a = $(19.3 * A_{663} – 0.86 * A_{645})$ V/100W
Chlorophyll b = $(19.3 * A_{645} – 3.6 * A_{663})$ V/100W
Carotenoids = $100(A_{470})$ + 3.27(mg chl. A) – 104(mg chl. B)/22

Measurement of sodium and potassium. To study the effect of salinity on the concentration of sodium, potassium and their distribution in leaves, two weeks after salt stress the samples were selected and put in an oven for 48 hours at 72°C. After being powdered in porcelain mortar, for preparation of ashes, dried tissues were put in an oven at 550 degrees. Then, 10cc normal hydrochloric acid (HCl) was added on each sample and after boiling and absorbing the first bubble, the solution was clarified using filter paper and poured in a balloon. Finally, the volume of the balloon was made up to 100 cc with distilled water. After calibration of the photometer machine with standard solutions, the amounts of sodium and potassium were measured. Analysis of seed proteins for extraction of low molecular weight proteins was performed by Pine and Lavrs method (1983). For protein electrophoresis, SDS-PAGE method (polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate) was used. SDS-PAGE was performed with 10% separating gel and 5% stacking gel according to Laemmli (1970).

Results and Discussion

The analysis of variance for physiologic traits showed 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* (Tab.1).

Tab. 1. Analysis of variance for studied traits between soybean genotypes

S.O.V.		Rep	Salinity	Genotype	Salinity×Genotype	Error	C.V.
df		2	2	16	32	100	
	Chla (mgr/grf$_w$)	0.001$^{n.s}$	0.131**	0.008*	0.006$^{n.s}$	0.004	17.71
	Chlb (mgr/grf$_w$)	0.00 1$^{n.s}$	0.030**	0.001$^{n.s}$	0.0001$^{n.s}$	0.0001	28.78
MS	a/b (mgr/grf$_w$)	0.107$^{n.s}$	1.580**	0.104$^{n.s}$	0.104$^{n.s}$	0.102	22.72
	a+b (mgr/grf$_w$)	0.004$^{n.s}$	0.189**	0.008**	0.004$^{n.s}$	0.003	26.62
	Sodium (ppm)	0.073$^{n.s}$	2.127**	0.267**	0.069$^{n.s}$	0.085	11.03
	Potassium (ppm)	3.173**	35.508**	1.205**	0.292$^{n.s}$	0.512	19.53
	Potassium/Sodium (ppm)	0.679**	8.689**	0.194**	0.053$^{n.s}$	0.078	20.85
	Proline (μmol g^{-1}fw)	0.070**	3.3556**	0.072**	0.040**	0.016	17.64

ns and **: Non-significant and significant differences at $p \leq 0.01$

Comparison of the effect of different levels of salinity on physiological traits showed that with increasing salinity, the amounts of potassium, sodium, chlorophyll *a* and chlorophyll *b* were reduced. Most of these traits were in the control and the lowest of them was observed in salinity (Tab. 2-3). Proline amount increased with increasing salinity levels. Also, Kumar *et al.* (2003) reported that under more tolerant conditions, chlorophyll degradation is less.

Salinity reduces the amount of leaf chlorophyll (Chyyj 1984; Gramer and Bowman 1991; Kay 2007). With increasing salinity, the amount of chlorophylls *a* and *b* reduced i.e. deficiency in their synthesis occurred (Prasad 1999).

Tab. 2.Comparisons of mean at stress levels for the studied 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]	0.10114 [a]	1.98229[b]	0.28983 [a]	2.46203[c]	4.55111 [a]	1.85355 [a]
Salinity(75mM)	0.31152[b]	0.07408 [b]	1.63264[c]	0.18472 [b]	3.60071[b]	3.54503 [b]	1.36680 [b]
Salinity(150mM)	0.11064 [b]	0.05263 [b]	0.69772[a]	0.18379 [b]	2.86409[a]	2.89499 [c]	1.03276 [b]

Tab. 3.Comparisons of mean under salt stress for studied traits between soybean genotypes

Genotype	Chl a (mgr/grf$_w$)	a+b (mgr/gr)	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]

In the control, M7 genotype had the highest mean but increasing stress levels led to an increase in proline amount. Under salinity level of 75 mili-molar, 033 genotype had a high amount of proline, and at 150 mili-molar, JK and Hamilton had the highest amount of proline. The main role of proline is not to reduce the osmotic potential, but to protect enzymes against dehydration and accumulation of salt (Thomas 1990). The significant effect of proline on osmotic adjustment includes protection of cellular structures and its action on free. Sensitivity to salinity has been reported in a large number of plants (Coca *et al.* 2007; Turan *et al.* 2007; Dsyng and Kanagava 2007; Ashraf and Harris 2004). Potassium serves as a high consumption element in higher plants and plays an important role in enzyme homeostasis, regulation of cell swelling, electrical cell load, leaf movements and protein construction (Yoo *et al.* 1984; Milk *et al.* 2002).

Potassium is an osmotic regulator available in soil, absorbed by roots and causes a decrease in osmotic potential inside the cell environment. As a result, water losses from the plant reduce. One of the effects of salinity can be loss of potassium function in plant leaves. The increase in the amount of potassium with increasing salinity stress and toxicity of sodium ions with disorders in No/N can be one of the reasons for growth reduction (Flowers *et al*. 1977).

Cluster analysis of studied varieties based on physiological traits. In order to group the studied genotypes, cluster analysis by Ward's minimum variance method using standard squared Euclidean distance was conducted, based on mean physiological traits in the control and at salinity levels of 75 and 150 mm by cutting tree at a distance of 15 units in the control and at a distance of 20 units for 75 and 150 mm NaCl treatments, and genotypes were divided into two and three groups respectively. In the control, the genotypes 2, 3, 9, 11, 12, 17 and 14 were placed in the second cluster and at the first stress level, varieties 10, 11, 14 and 17 was placed in the second cluster and under the second stress, and the third as the best cluster involving 14, 16 and 17 that had higher average traits than the whole average. According to the cluster analysis, 14 and 17 are advisable to three stress conditions.

Protein markers. Results of screening of storage protein subunits using electrophoresis show that there was abundant variation in these subunits in soybean cultivars. The mean of the genetic diversity of populations was 0.43 and Shannon index was 0.41. For 17 studied genotypes, a total of 19 protein bands was observed. According to the data obtained from Table on gene diversity (Tab.4), 15 possible gene loci were polymorphic and the percentage of total polymorphism is equal to 78.95 percent. Most gene variations were related to markers 8, 18, 19 and the polymorphism rate was 48%.

Tab. 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			

The cluster analysis was done based on the Jacquard similarity coefficient and finally a tree diagram was designed based on the UPGMN method (Figure 1-3). By cutting the tree diagram in 0.56 unit, three groups were obtained. The first includes varieties JK, the second group includes Hamilton varieties, Liana, Safyabad, T.M.S, the third group included Saharvarietes, M.7.interprize Laviana, 033, streesland, B.P clean, Calark, Gorgan, OPX,, Maximum similarity or minimum genetic distance was related to varieties (B, P, Mq). (Clean, MV), (B.P, Clean), (Gorga, Clean), (Gorga, B.P), (Corga M.7), (Calark, M7), (Calark, B.P), (Calark, Clean), (Gorga, Calark) the similarity coefficient was 1 and minimum similarity and maximum genetic distances between varieties (JK and laving), (M7 and JK) with similarity coefficient of 0.3684. Convenience of cluster analysis was determined according to the significance of the cophenetic correlation coefficient (0.8). Examples of patterns of storage proteins were shown in soybean seed (Figure 3).

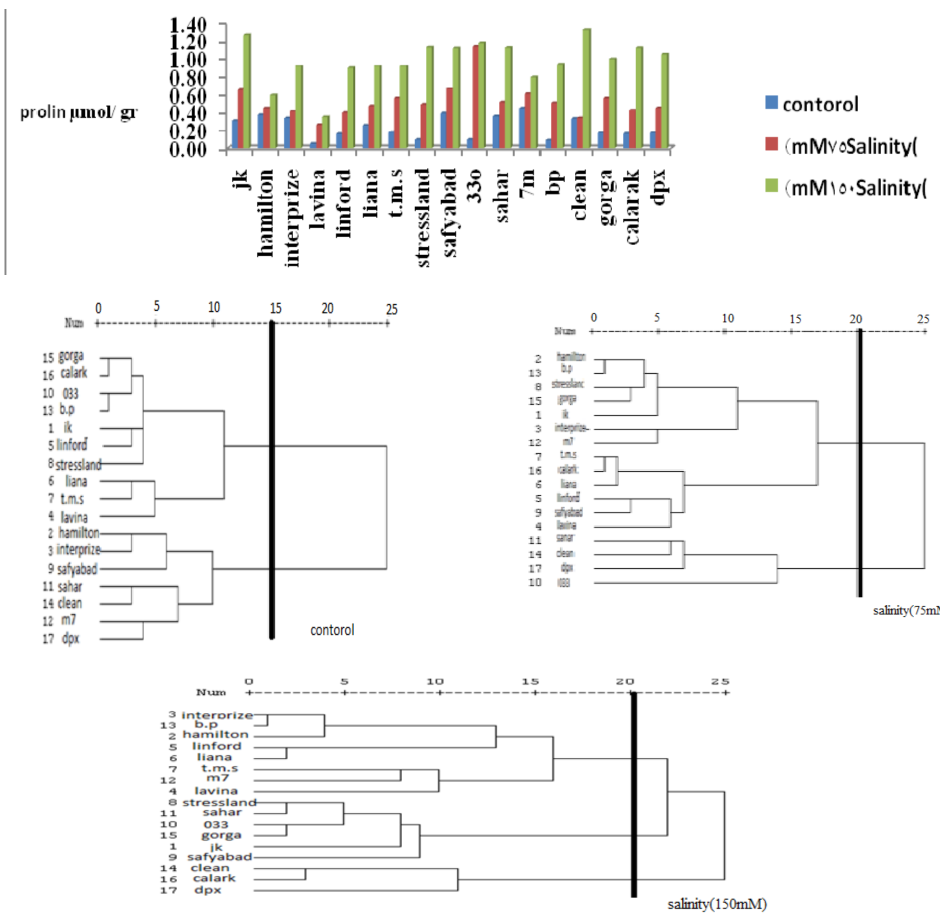

Fig.1. Cluster analysis under control and stress condition in studied genotypes

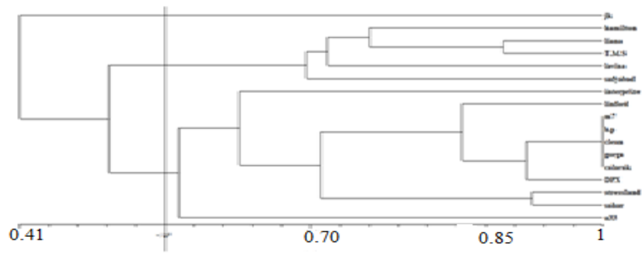

Fig 2. Cluster analysis based on seed storage protein electrophoresis

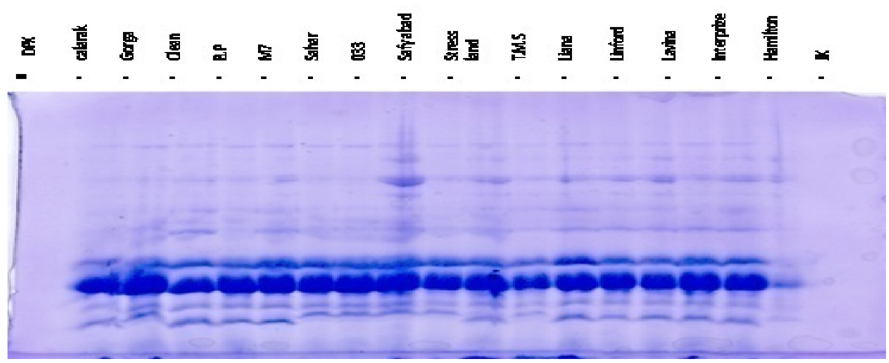

Fig 3. Sample gel electrophoresis using seed storage protein in studied cultivars.

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

Tab. 5. Regression coefficient between morpho-physiologic and protein markers

	Chl a			Chl b			a/b			a+b			sodium		
	control	Salinity (75mM)	Salinity (150mM)	control	Salinity (75mM)	Salinity (150mM)	control	Salinity (75mM)	Salinity (150mM)	control	Salinity (75mM)	Salinity (150mM)	control	Salinity (75mM)	Salinity (150mM)
Intercep. constancy		0.083	0.553	0.90	0.07			1.20	1.47			0.16		2.42	2.84
Pm1				0.69**	0.57*			-.296*	0.42*			0.50*			
Pm3															0.49**
Pm4															
Pm5		0.551*						0.74*							
Pm9			0.43*												
Pm10		-0.617*						-0.63**							
Pm11															
Pm12															
Pm14															
Pm15			-0.58**						0.56**						
Pm17															-0.58**
Pm18								0.37**						0.61*	
Pm19															
R^2		0.547	0.07	0.45	0.27			0.87	0.45			0.20		0.33	0.32

ns and **: Non-significant and significant differences at 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. The resulting associations must be proved by another statistical method or complementary tests before being used as positive markers in any marker assisted programs.

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ISPITIVANJE ODNOSA IZMEĐU FIZIOLOŠKIH OSOBINA I PROTEINSKIH MARKERA KOD SORTI SOJE (*GLYSIN MAX* L.)

-originalni naučni rad-

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Rezime

Ovo istraživanje izvršeno je u cilju ispitivanja tolerancije biljaka na salinitet tokom faze klijanaca kod soje (*Glysin max* L.). Faktorijalni ogled izvršen je po slučajnom blok sistemu u tri ponavljanja. Ukupno 17 genotipova soje korišćeno je pri 3 nivoa stresa na salinitet (kontrola, 75 mM i 150 mM NaCl). Ogled je izvršen u zaštićenim uslovima, uz ispitivanje sadržaja prolina, natrijuma, kalijuma, hlorofila a, hlorofila b, hlorofila a/b i ukupnog hlorofila. Za stvaranje uslova za stres na salinitet primenjen je NaCl. Dobijeni rezultati pokazali su da različiti stres na salinitet ima značajan uticaj na sve osobine, osim na hlorofil b i hlorofil a/b. Na osnovu klaster analize u kontrolnoj varijanti i na varijantama sa primenom 75 i 150 mM NaCl, genotipovi su svrstani u dve, dve odnosno tri grupe. U svakoj varijanti, dpx i čisti genotipovi svrstani su u grupu sa prosečnim vrednostima osobina višim nego kod drugih genotipova, što se može generalizovati i na uslove kontrolne varijante, kao i varijante stresa na salinitet sa 75 i 150 mM Na Cl. Regresiona analiza je pokazala moguće informativne proteinske markere koji kodiraju lokuse kao mogući potencijal za strategije selekcije na slane uslove, a što je dokazano komplementarnim ispitivanjima.

Ključne reči: proteinski marker, salinitet, soja.