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Changes in Activity Profile of Some Antioxidant Enzymes in Alfalfa Half-sib Families under Salt Stress

M. Valizadeh[1*], S. Moharamnejad[1], M. Ahmadi[1], and H. Mohammadzadeh jalaly[1]

ABSTRACT

In order to evaluate salt (NaCl) tolerance in alfalfa (*Medicago sativa* L.) half-sib families, a factorial experiment on the basis of completely randomized design, with 20-25 individuals in pot (replicates) was carried out under field conditions at the Research Station of University of Tabriz. Electrophoretic analyses were performed by using 7.5% slab polyacrylamide gels. Two antioxidant enzymes including superoxide dismutase (SOD) and peroxidase (POX) and one common isozymic system namely esterase (EST) were stained and for each isozymic band the "density × area" scores onto gels were evaluated by MCID software as enzymatic activity. Plant materials consisted of 12 half-sib families that were obtained from a "polycross nursery progeny test" in the same station, a few years ago. The applied salt stress (9±0.2 ds m^{-1}) reduced plant height, dry weight, leaf weight and stem weight about 31.7, 37.5, 33.7 and 34.7 percent, respectively. Significant correlations were observed between plant height and some antioxidant isozyme activities. Salt stress increased activities of some SOD and POX isozymes but it was ineffective on the activity of EST. Among the families, Ranger cultivar and Zaghl-aghaj and Taze-kand landraces which displayed maximum height and other characteristics exhibited the highest increments in respect of isozyme activities, indicating that antioxidant analysis by gel electrophoresis could be a useful tool for salt stress tolerance studies.

Keywords: Alfalfa half-sib families, Isozyme activities, Salt stress.

INTRODUCTION

Exposure of plants to abiotic stresses such as high salinity, drought, severe light and temperature leads to major losses in crop productivity. A study on global land use pattern reveals that 7% of the world's land area, amounting to 1,000 million hectares, has become saline (Tester and Davenport, 2003). The genetic mechanisms of salt tolerance have to be defined in order to efficiently develop more salt tolerant crops. There is no question that salt stress alters gene expression in a tissue-specific and time dependent manner. However, use of molecular techniques in conjunction with physiological and biological analyses to identify potential genetic mechanisms for salt tolerance is a valid approach for the development of salt tolerant plants.

The modifications of gene expression due to different environmental conditions are a common response in the metabolism of plant cells (Ski, 2003). Gene activation due to environmental stimuli plays an extremely important role in the adaptation of plants to unfavorable conditions and promotes the appearance of specific proteins (Sachs and Ho, 1986). One of two major origins of alfalfa (*Medicago sativa* L.) is northwest of Iran, and it is the most important forage species in this country with more than 600 thousand hectares under cultivation (Valizadeh *et al*., 2002).

[1] Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran.
[*]Corresponding author, e-mail: mvalizadeh@tabrizu.ac.ir

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Improving yield and quality traits are important objectives in herbage breeding programs (Heyn, 1984). Smith *et al*. (1997) ranked forage traits in terms of their nutritional value for dairy production. Improved digestibility was the most important criteria and high crude protein and low fiber content was ranked as moderate priority in terms of quality objectives. Salinity research in alfalfa has focused primarily on germination rate (Dobrenz *et al*., 1989), seedling establishment (Mckimmie and Dobrenz, 1987) and plant grown in greenhouse screening system (Monirifar and Barghi, 2009) in the presence of NaCl. Salt stress, like other abiotic stresses can lead to oxidative stress (Ashraf, 2009) through the increase in reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which are highly reactive and may cause cellular damage through oxidation of lipids, proteins and nucleic acids (Ying *et al*., 2006). In order to protect cell membranes and organelles from ROS damaging effects, plants are equipped with an enzymatic and non enzymatic antioxidant system. It has been reported that a significant correlation exists between the activities of antioxidative enzymes and the salt tolerance of plants (Chinta *et al*., 2001; Diego *et al*., 2003; Nader *et al*., 2005). This system consists of low-molecular-weight antioxidants, such as ascorbate and glutathione, as well as several enzymes such as superoxide dismutase (SOD) which convert O^-_2 to H_2O_2 and peroxidase (POX), catalyzing the breakdown of H_2O_2 (Mittler, 2002; Thomas *et al*., 2005; Turkan *et al*., 2005; Gaber, 2010). Wang *et al*. (2009) studied antioxidant responses of three *Medicago* species including alfalfa to 300 mM NaCl during seed germination stage and found a much weaker glutathione reductase (GR) activity in seeds of alfalfa in the controls and salt treatment and different responses were reported for other species. Wang and Hang (2009) reported that increased salt concentrations led to a significant alteration in SOD, POX, APX and CAT activities in alfalfa. Similar increases in the activities of SOD and POX have been reported in *Morus alba* (Chinta *et al*., 2001), cotton (Diego *et al*., 2003), common bean (Nagesh and Devaraj, 2008) and tomato (Dogan, 2012).

Progenies from seed of lines that were subject to outcrossing with other selected lines growing in the same nursery are mainly half-sib families in outbreeding plant species such as alfalfa. These families are used for estimating the heritability of the quantitative character under investigation and developing synthetic varieties (Hill *et al*., 1998). Ewens and Felker (2010) applied half-sib families to study the genetics of insect resistance in *Prosopis alba* and Johnson *et al*. (1992) investigated heritability estimates for germination attributes in alfalfa using half-sib families, under salinity condition.

The present research was carried out in an attempt to study the relationships between agronomical traits and antioxidant isozyme responses under salinity condition in alfalfa half-sib families.

MATERIALS AND METHODS

Plant Materials

Plant materials used in this study consisted of 12 half-sib families (Table 1) that were obtained from a polycross nursery progeny test, at the Research Station of University of Tabriz (Valizadeh *et al*., 2002).

Twelve half-sib families were grown at their second growth season using a pot for each in the Research Station of University of Tabriz with one saline (9±0.2 ds m^{-1}) and one normal irrigation levels. A factorial experiment on the basis of completely randomized designs, with 20-25 individuals (replicates) was carried out under field conditions. Various traits such as plant height, dry weight, leaf weight, stem weight

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Table 1. List of alfalfa half-sib families analyzed in this study.

No.	Families	Locality	Country
1	Leylan-hamid	Arak	Iran
2	Galebani	Marand	Iran
3	Ghara-yonje	Maraghe	Iran
4	Maman-famenin	Hamedan	Iran
5	Amo-zeynetdin	Tabriz	Iran
6	Taze-kand	Nagadeh	Iran
7	Zoghal-aghaj	Tabriz	Iran
8	Selvana	Urmia	Iran
9	Shazand	Arak	Iran
10	Maopa	-	USA
11	Ranger	-	USA
12	Chaleshte	Chahar Mahale Bakhtiyary	Iran

were recorded from 20-25 plants of each half-sib family.

Enzyme Extraction and Electrophoresis

The crude extract of fresh and healthy leaves from adult plants were prepared with separate mortars and pestles in a Tris-HCl extraction buffer pH 7.5 (Tris 50 mM, sucrose 5%, ascorbic acid 50 mM, sodium metabisulfite 20 mM, PEG 2% and 2-Mercaptoethanol 0.1% before use, Valizadeh *et al*., 2011) with a ratio of 0.5 mg μl^{-1} (1W:2V) and centrifuged at 4ºC and 10,000 rpm for 10 minutes using small Eppendorf tubes. Enzyme extracts were immediately absorbed onto 3×5 mm wicks cut from Whatman 3 mm filter paper and loaded onto 7.5% horizontal slab polyacrylamide gels (0.6×15×12 cm) using TBE (Tris-Borate-EDTA) electrode buffer (pH= 8.8). Electrophoresis was carried out at 4°C for 3 hours (constant current of 30mA, and voltage of 180V). For each half-sib family, analysis was repeated three times, each time from bulked material of at least five individuals.

Three enzymatic systems were analyzed in this study. The slab gels were stained after electrophoresis preparing two slices of slab gel. The staining protocol for EST and SOD was performed according to Soltis and Soltis (1990) and POX according to Olson and Varner (1993). The gels were fixed and scanned immediately after staining.

Statistical Analysis

An image analysis program (MCID software) was used to measure D×A (optical density×area) parameter for each isozymic band to evaluate the activity onto gels. For statistical analysis and correlation estimates between isozyme markers and morphologic traits SPSS 16.0 software was used.

RESULTS AND DISCUSSION

The analysis of variance of data to assess the effect of salinity on alfalfa forage yield and related attributes showed a highly significant difference for families, salinity and familles×salinity interaction (Table 2).

According to Table 2, there were significant differences among families for leaf weight and plant height. The effect of salinity was significant for dry weight, leaf weight, stem weight and plant height ($P< 0.01$). Familes×salinity interactions for three attributes were significant ($P< 0.01$). Treatment means comparisons for these three attributes are shown in Figures 1-a, b and c. Except for Ranger an improved variety and Zaghl-aghaj and Taze-kand which are originated as landraces from saline plain of Tabriz regions, in other families, the higher

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Table 2. ANOVA of the effects of salinity, families, and their interaction on studied traits of alfalfa half-sib families.

S.V	df	MS			
		Dry weight	Leaf weight	Stem weight	Plant height
Families	11	5.609 ^{ns}	2.870 *	2.142 ^{ns}	10.726 **
Salinity	1	335.054**	71.732 **	61.935**	748.501**
Families×Salinity	11	10.312 **	1.680 ^{ns}	3.213 **	20.887 **
Error	96	2.973	1.266	1.231	2.832
CV (%)		23.80	29.41	32.34	12.18
Mean	Normal	8.9±0.30	4.59±0.19	4.14±0.17	16.31±0.32
	Salinity	5.57±0.19	3.05±0.11	2.71±0.13	11.31±0.26
Decreasing percentage		37.5	33.7	34.7	31.7

^{ns}; *, **: Non significant and significant differences at 5 and 1% probability respectively.

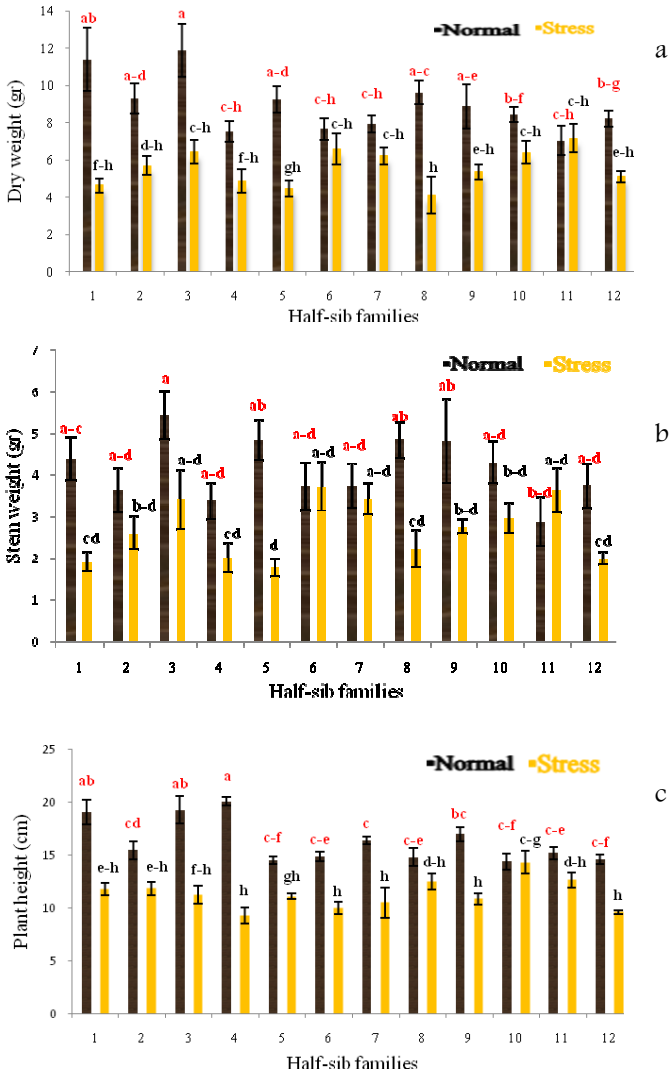

Figure 1. Dry weight(a) Stem weight (b) Plant height (c)means of 12 half-sib families grown under normal and salt stress conditions.

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dry weight and stem weight were obtained in normal condition. Therefore, under salt treatment, the above mentioned three cultivars and landraces had the highest whereas Maman-famenin and Selvana had the lowest agronomic traits.

Activity analysis of the same half-sib families under both normal and salt stressed conditions stained onto the same gels showed a substantial increment of SOD and POX antioxidant isozymes, but no increment for EST isozymes (Figures 2a, b and c). According to statistical analyses of all data obtained from all replications and gels, it has been shown that there were significant differences in POX-2, POX-4 and POX-5 among families. Under the salinity condition, there were significant differences

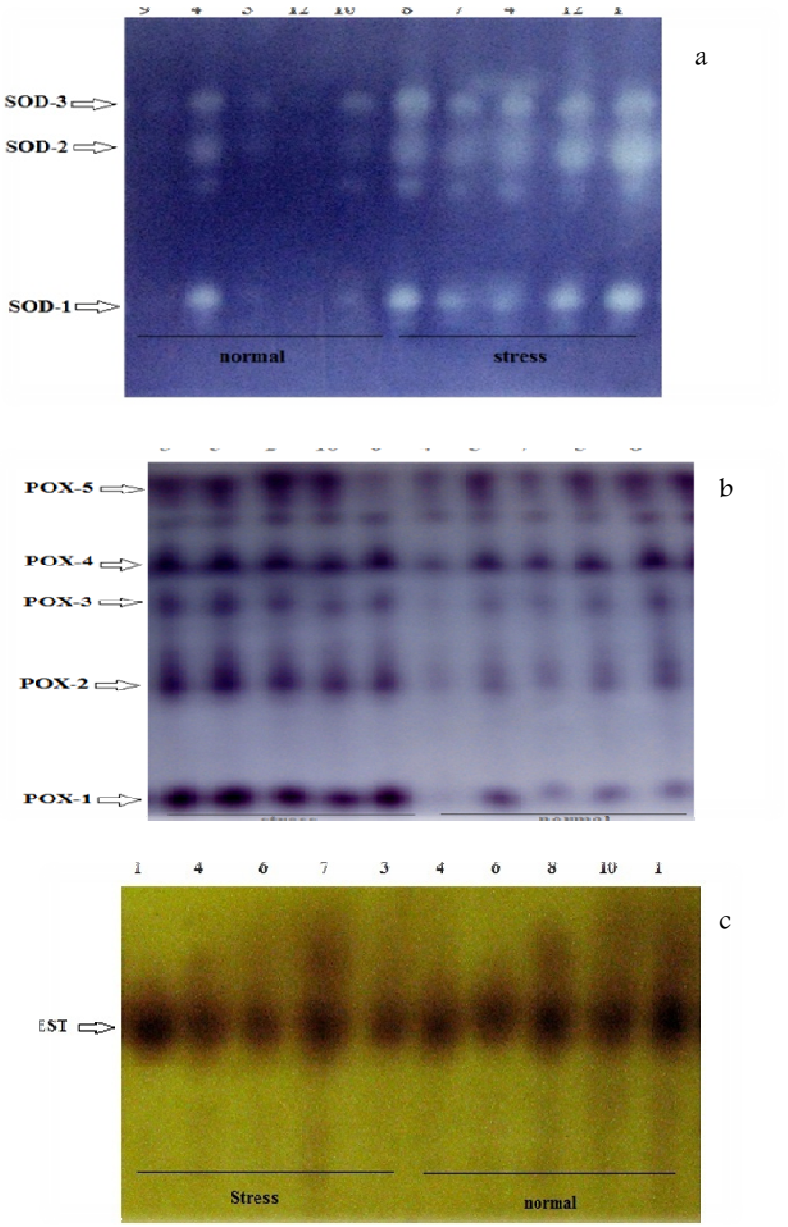

Figure 2. Example of superoxide dismutase (a) example of peroxidase (b) example of esterase (c) banding pattern for normal and stress conditions in alfalfa (The numbers refer to half-sib family, Tab. 1).

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for POX-1, POX-2, POX-3 and POX-4 (Table 3, Figure 5). For families×salinity interactions there were no significant differences for their isozymic activities. Also, there were no significant differences among families and families×salinity interactions for SOD. Under the salinity condition, there were significant differences only in SOD-1 (Table 3, Figure 4). This result indicates that in salt stress, mean activities of SOD and POX isozymes are significantly higher than normal conditions.

Correlations between average isozyme activities and mean values of agronomic traits related to forage attributes are presented in Table 4. Most of POX and SOD isozymes showed positive and significant correlations for plant height. This means that plant height was affected by salinity stress as antioxidant enzyme activities. However, dry weight and stem weight had negative and significant correlations with SOD-3 isozyme activity alone.

In alfalfa breeding programs for improved yield, knowledge of the quality of the new variety and its relationships with forage yield is very important. Yield and yield components are two major characters of plants selected for salt tolerance. Metwali *et al*. (2011) reported that using quantitative trait loci analysis became important to study genetic control of yield under a range of environments with more salt. Bhardwaj *et al*. (2010) stated that increase in salinity level showed an inverse relation with the plant height in alfalfa. Munns (2002) suggested that excessive quantities of salt enter the plants and eventually rise to toxic levels in transpiring leaves causing senescence. Inhibition of growth and a decrease in water content induced by water stress have been universally observed even in tolerant plants (Bartels and Salamini, 2001; Mittler *et al*., 2001). One of the most crucial functions of plant cells is their activity to respond to fluctuation in their environment. SOD is an important antioxidant enzyme and is the first line of defense against oxidative stress in plants. It plays an important part in determining the concentration of O_2^- and H_2O_2 in plants and hence performs a key role in the defense mechanism against free-radical toxicity (Bowler *et al*., 1992). The induction of POX activity under water and salt stress is well documented and some commonly positive relationships have been found between its upregulation and stress tolerance (Hernandez *et al*., 2000; Hamilton and Heckathorn, 2001; Shalata *et al*., 2001; Ushimaru *et al*., 2001; Cruzde, 2008). In order to eliminate intra and inter family isozymic diversity, we used bulked (five individuals in each family sample) extraction of enzymes in this research. Thus, possible isozymic polymorphisms were eliminated for different bands as shown in Figures 4, 5 and 6. In addition, using more than three replications (analyzed gels) for each half-sib family could be useful for estimating mean activity of each isozyme. These approaches, along with no enzymatic changes of EST in salt treatment allowed us to obtain our results with more accuracy, compared to other studies where only spectrophotometric data are used.

Table 4. Correlations between means of agronomic traits and isozymes activities in 12 half-sib families.

Enzyme data	Isozye	Dry weight (gr)	Leaf weight (gr)	Stem weight (gr)	Plant height (cm)
Densitomtric (D×A)	POX-1	0.28^{ns}	0.37^{ns}	0.15^{ns}	0.78^{**}
	POX-2	0.25^{ns}	-0.53^{ns}	0.11^{ns}	0.42^{ns}
	POX-3	0.56^{ns}	-0.54^{ns}	0.50^{ns}	0.74^{**}
	POX-4	-0.19^{ns}	0.24^{ns}	-0.24^{ns}	0.56^{ns}
	POX-5	0.25^{ns}	0.01^{ns}	0.10^{ns}	0.61^{*}
	SOD-1	0.41^{ns}	0.37^{ns}	0.39^{ns}	0.60^{*}
	SOD-2	-0.52^{ns}	-0.23^{ns}	-0.57^{ns}	0.11^{ns}
	SOD-3	-0.68^{*}	0.08^{ns}	-0.70^{*}	0.14^{ns}

^{ns}; *, **: Non significant and significant correlations at 5 and 1% probability, respectively.

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CONCLUSION

Evaluation of effect of relatively high salinity (9±0.2 ds m^{-1}) on some alfalfa yield related traits along with analysis of antioxidant and non-antioxidant enzymes by native polyacrylamide gel electrophoresis showed that significant correlations exist only between changes on antioxidant isozymes activities and variation of yield characteristics, which are consistent with previous findings in several plants. Therefore, study of antioxidant enzymes activities by gel electrophoresis and subsequent data analyses would be useful complementary tools for their spectrophotometric studies.

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تغییر پروفیل فعالیت برخی از آنزیم‌های آنتی اکسیدان در خانواده‌های ناتنی یونجه تحت تنش شوری

م. ولیزاده، س. محرم نژاد، م. احمدی و ح. محمد زاده جلالی

چکیده

به منظور ارزیابی خانواده‌های ناتنی یونجه (*Medicago sativa* L.)، آزمایشی در قالب فاکتوریل با طرح پایه کاملاً تصادفی با ۲۰-۲۵ (گلدان) در شرایط مزرعه‌ای ایستگاه تحقیقاتی دانشگاه تبریز پیاده شد. تجزیه‌های الکتروفورزی با استفاده از ژل‌های پلی آکریلامید ۷/۵ درصد انجام شدند. دو سیستم آنزیمی آنتی اکسیدان شامل سوپر اکسید دیسموتاز (SOD) و پراکسیداز (POX) با یک سیستم ایزوزیمی رایج به‌نام استراز (EST) رنگ‌آمیزی شدند و برای هر نوار در روی ژل میزان " مساحت × شدت" به‌عنوان فعالیت آنزیمی با نرم افزار MCID ارزیابی و یادداشت گردید. مواد گیاهی شامل ۱۲ خانواده برادر-خواهر ناتنی بودند که چند سال پیش از یک آزمون نتاج در خزانه پلی کراس در همان ایستگاه به‌دست آمده بودند. سطح تنش شوری به‌کار رفته ($9\pm0/2$ ds m^{-1}) موجب کاهش ارتفاع گیاه، وزن خشک، وزن برگ و وزن ساقه به‌ترتیب به‌میزان ۳۱/۷، ۳۷/۵، ۳۳/۷ و ۳۴/۷ درصد گردید. بین ارتفاع گیاه و فعالیت برخی ایزوزیم‌های آنتی اکسیدان همبستگی‌های معنی دار به‌دست آمد. تنش شوری فعالیت برخی از ایزوزیم‌های SOD و POX را افزایش داد ولی روی فعالیت EST تاثیری نداشت. بین خانواده‌های ناتنی یونجه رقم اصلاح شده رنجر و ارقام بومی زغال-آغاج و تازه-کند که ارتفاع بیشتر و ویژگی‌های بالاتری نیز داشتند بیشترین افزایش فعالیت ایزوزیم‌های SOD و POX را نشان دادند. این نتایج نشان می‌دهند که تجزیه فعالیت آنزیم‌های آنتی اکسیدان با الکتروفورز در ژل پلی آکریلامید می‌تواند ابزار مفیدی برای مطالعات تحمل به شوری در نظر گرفته شود.

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