Bangladesh J. Bot. **43**(2): 191-196, 2014 (September)

EFFECTS OF SALT STRESS ON LIPID PEROXIDATION AND ANTIOXIDATIVE ENZYMES OF ALFALFA (*MEDICAGO SATIVA* L.) CULTIVARS

ENSIEH ASHRAFI, MORTEZA ZAHEDI* AND JAMSHID RAZMJOO

Department of Agronomy and Plant Breeding, College of Agriculture, Isfahan University of Technology, Isfahan, Iran

Key words: Antioxidative enzymes, Lipid peroxidation, *Medicago sativa*, Salt stress

Abstract

The effect of salt stress on enzyme activities of nine alfalfa cultivars at germination and seedling stage was studied. The activities of SOD, GR, POX and APOX were higher in salt tolerant and lower in salt sensitive cultivars. Results of the effect of salt stress on the SOD, GR, POX, APOX activities and MDA content may be used to select salt tolerance cultivars at the germination and seedling stages. SOD, GR, POX, APOX and MDA may play an important role in salt tolerant mechanisms in alfalfa.

Introduction

Alfalfa (*Medicago sativa* L.) is moderately tolerant to salinity (Howieson and Ballard 2004) but there is high variation between and within cultivars of alfalfa relative to center of diversity (Howieson and Ballard 2004). Therefore, selection of more salt tolerant cultivar at these stages is possible for production of this crop. In addition information on mechanism tolerance of these cultivars at this stage under salt stress is important for selection or screening of salt tolerance cultivars at this stage.

Torabi *et al.* (2011) reported that the cultivars *Nik-Shahri, Rehnani* and *Gharegozloo* were more salt tolerance at germination stage. Reports on the mechanism of salt tolerance in alfalfa cultivars at germination stage are none.

Salt stress can stimulate formation of active oxygen species (AOS) such as superoxide, hydrogen peroxide and hydroxyl radicals. These activated oxygens injure the cellular components of proteins, membrane lipids and nucleic acids (Sudhakar *et al.* 2001). Malondialdehyde (MDA) is the decomposition product of polyunsaturated fatty acids of membranes and shows greater accumulation under salt stress (Sudhakar *et al.* 2001). Plants that produce high levels of antioxidants are able to provide better resistance to damage induced by salinity (Bor *et al.* 2003).

The objective of this study was to evaluate the effect of salt stress on antioxidant enzymes activities in nine alfalfa cultivars to elucidate the possible physiological mechanism of the stress during germination and seedling stages of alfalfa.

Materials and Methods

Seeds of alfalfa were provided by Agricultural Research Center of Isfahan. Early seedling growth of nine alfalfa (*Medicago sativa* L.) cultivars (Tolerant cultivars: Rehnani, Esfahani and Gharehyonje. Moderate cultivars: Ranjer, Hamedani and Yazdi. Sensitive cultivars: Nikshahri, Pioneer and Bami) was studied using distilled water and three salinity solutions. Three replicates of 100 seeds of each cultivar were placed between double layered rolled anchor germination papers with 10 ml of relevant experiment solutions. The papers were replaced every 2 days in

*Author for correspondence: <mzahedi@cc.iut.ac.ir>.

order to avoid salt accumulation. Seeds were allowed to germinate in a growth chamber at 25±1ºC in the 16/8 dark and light for 7 days. For dry weight determination, samples were oven dried at 75-80ºC for 48 hrs.

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in the seedling tissues according to Madhava Rao and Sresty (2000).

Fresh samples of seedling tissue first ground with mortar and pestle with liquid nitrogen. 0.5 g of each powdered sample was then homogenized in ice-cold 50 mM sodium phosphate extraction buffer (pH 6.8) containing 1mM Na_2-EDTA and 2% (W/V) PVPP. The entire extraction procedure was carried out at 4ºC. The homogenate was centrifuged at 13,000 × *g* for 40 min at 4 °C and the supernatant was used to assay the activity of superoxide dismutase (SOD), Peroxidase (POX), ascorbate peroxidase (APOX), and glutathione reductase (GR). The enzymatic activity of SOD was determined in terms of its ability to inhibit photochemical reduction of nitrob-blue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich 1971). The activity of POX was determined in 3,3′ - diaminobenzidine-tetrahydrochloride dihydrate (DAB) solution containing 50% (W/V) gelatin and 0.15 M Na-phosphate-citrate buffer (pH 4.4) and 0.6% H_2O_2 (Herzog and Fahimi 1973). The activity of APOX was assayed as the decrease in absorbance at 290 nm following the $H_2O_2^-$ mediated oxidation of ascorbate (Nakano and Asada 1981). The activity of GR was measured according to Foyer and Halliwell (1976).

The laboratory tests were a two-way factorial (4 × 9) arranged in a completely randomized design with three replications. Data were subjected to analysis of variance (ANOVA) using GLM procedure of SAS statistical program (ver.9.1) and MSTAT-C procedures. Treatment means were separated using the least significant difference (LSD) test ($P < 0.05$).

Results and Discussion

The shoot and root lengths and shoot and root dry weights in all cultivars had decreased as salinity level increased (Table 1). The highest decrease in root and shoot length under all salinity levels was in cultivars *Esfahani* and *Rehnani* (Table 1). The average reductions in shoot dry weight at saline treatments from 17 - 19% in *Rehnani*, *Esfahani* and *Gharehyonje* were distinctly lower than those of other cultivars, and 30 - 57% in *Hamedani*, *Ranjer*, *Yazdi*, *Bami*, *Nikshahre*, and *Pioneer* (Table 1). In this study, based on root and shoot lengths and weights, *Rehnani* and *Esfahani* were the most salt tolerant and *Pioneer* and *Bami* were the least tolerant among the tested cultivars. These results are in line with the results reported by Torabi *et al.* (2011) and Peel *et al.* (2004). Thus, the cause of the reduction of shoot and root growth was due to salinity induced ionic imbalance (Howieson and Ballard 2004, Jaleel *et al.* 2008).

Salinity caused 3.05-, 2.21- and 2.13-folds increase in SOD in *Rehnani*, *Gharehyonje*, and *Esfahani* than those of other cultivars. Similarly, increase in SOD by 1.75 - 1.32-folds in Bami, Pioneer, Yazdi, Nikshahre, *Hamedani*, and *Ranjer*. Among cultivars, *Rehnani* had the highest SOD at 180 mM of NaCl treatment (Table 2). Higher SOD activity in salt-tolerant cultivars of alfalfa was also reported (Wang *et al.* 2009, Wang and Han 2009).

Salinity (60 - 180 mM) increase the activity of POX, by 3.77 folds in Ranjer, followed by 2.68-, 2.22-, 2.19-, 1.81-, 1.58-, 1.43-, 1.41- and 1.38-folds in *Hamedani*, *Nikshahre*, *Yazdi*, *Rehnani*, *Pioneer*, *Bami*, *Gharehyonje*, and *Esfahani,* respectively (Table 2).

Salinity (6- - 180 mM) increase the activity of APOX, by 4.39 - 2.23-folds in *Pioneer*, *Bami*, *Yazdi*, *Nikshahre*, *Gharehyonje*, *Rehnani*, *Ranjer*, *Hamedani*, and *Esfahani* (Table 2).

Table 1. Effect of salinity on the growth parameter of alfalfa cultivars.

Cultivar	NaCl (mM)	Root length (cm)	Shoot length (cm)	Root dry weight (mg)	Shoot dry weight (mg)
Rehnani	0	10.1^{c†}	14.2^{b†}	10.0^{a†}	144^{a†}
	60	9.5^{e}	13.4^{c}	9.2^{b}	134^{b} (-6)
	120	8.9^{g}	12.7^{d}	8.0^{c}	124^{cd} (-13)
	180	8.6^{hi}	11.7^{e}	6.4^{e}	99^{f} (-31)
Esfahani	0	11^{a}	15.1^{a}	9.0^{b}	129^{bc}
	60	10.5^{b}	14.5^{b}	7.4^{d}	119^{d} (-7)
	120	9.8^{d}	13.6^{c}	6.4^{e}	109^{e} (-15)
	180	9.1^{f}	12.8^{d}	5.6^{f}	89^{g} (-31)
Gharehyonje	0	8.7^{h}	12.4^{d}	8.3^{c}	119^{d}
	60	8.2^{jk}	11.3^{e}	7.2^{d}	109^{e} (-8)
	120	7.7^{m}	10^{f}	6.3^{e}	99^{f} (-16)
	180	7.2^{o}	9.3^{g}	5.5^{f}	79^{h} (-33)
Ranjer	0	8.3^{j}	11^{e}	8.2^{c}	99^{f}
	60	8^{l}	10.4^{f}	6.6^{e}	79^{h} (-20)
	120	7.5^{n}	9.9^{f}	5.6^{f}	59^{j} (-40)
	180	6.8^{q}	8.9^{g}	4.8^{h}	29^{m} (-70)
Hamedani	0	6.5^{r}	8.4^{h}	5.8^{f}	79^{h}
	60	6.2^{s}	8.1^{h}	5.0^{gh}	69^{i} (-12)
	120	5.5^{u}	7.1^{i}	4.1^{i}	59^{j} (-25)
	180	4^{x}	6^{j}	3.3^{k}	39^{l} (-50)
Yazdi	0	7^{p}	9.1^{g}	5.4^{fg}	59^{j}
	60	6.2^{s}	8.3^{h}	4.7^{h}	49^{k} (-16)
	120	5^{v}	7^{i}	3.8^{ij}	39^{l} (-33)
	180	4.5^{w}	6^{j}	2.9^{kl}	19^{n} (-67)
Nikshahri	0	6.9^{pq}	9^{g}	3.1^{k}	39^{l}
	60	6^{t}	7.9^{h}	2.4^{m}	29^{m} (-25)
	120	4.5^{w}	6.1^{j}	1.9^{mno}	21^{n} (-46)
	180	4^{x}	5^{k}	1.6^{nop}	9^{op} (-76)
Pioneer	0	5^{v}	5.8^{j}	2.0^{mn}	19^{n}
	60	4^{x}	4.7^{k}	1.4^{op}	11^{o} (-42)
	120	3.4^{y}	4^{l}	0.9^{pq}	8^{op} (-57)
	180	2.5^{z}	3^{m}	0.4^{rs}	4^{p} (-78)
Bami	0	5.6^{u}	7.2^{i}	1.6^{nop}	9^{op}
	60	4.5^{w}	6.8^{j}	0.9^{pq}	8^{op} (-11)
	120	4^{x}	5.7^{j}	0.8^{qr}	5^{p} (-44)
	180	3.4^{y}	4.9^{k}	0.3^{s}	4^{kl} (-55)
LSD		1.71	0.6	0.5	5

†Mean within columns with the same letters is not significantly different at 5% level.

Table 2. Effect of salinity on activities of enzymes and lipid peroxidation of alfalfa cultivars.

Cultivar	NaCl (mM)	Activities of enzymes				MDA content
		SOD	POX	APOX	GR	
		unit mg/protein				nmol g/FW
Rehnani	0	$39^{uv\dagger}$	$3.7^{fghi\dagger}$	$1.9^{lmn\dagger}$	$0.56^{lmn\dagger}$	$6.2^{v\dagger}$
	60	59^{qr}	4.5^{ef}	2.8^{gh}	0.7^{hijk}	6.4^{uv}
	120	119^{e}	6.9^{cd}	4.3^{e}	1.08^{de}	6.7^{tuv}
	180	179^{a}	8.7^{ab}	6.5^{bc}	1.33^{b}	6.9^{tu}
Esfahani	0	55^{r}	4.2^{fg}	2.6^{hijk}	0.57^{klmn}	6.6^{tuv}
	60	72^{kl}	4.9^{ef}	3.9^{ef}	0.82^{gh}	6.8^{tuv}
	120	109^{fg}	5.7^{de}	5.7^{c}	1.19^{cd}	6.9^{tu}
	180	164^{b}	6.8^{cd}	7.8^{a}	1.48^{a}	7.2^{t}
Gharehyonje	0	49^{st}	4.8^{ef}	1.7^{mno}	0.48^{nop}	7.9^{s}
	60	72^{mn}	5.7^{de}	2.8^{hij}	0.67^{ijkl}	8.6^{r}
	120	99^{h}	6.9^{cd}	3.9^{ef}	0.98^{ef}	9.4^{q}
	180	154^{c}	7.7^{bc}	5.8^{c}	1.22^{bc}	10.1^{p}
Ranjer	0	84^{kj}	1.9^{kl}	2.3^{ijkl}	0.35^{qrst}	10.9^{o}
	60	92^{i}	4.7^{ef}	3.6^{f}	0.53^{mno}	11.8^{mn}
	120	112^{f}	7.6^{bc}	5.1^{d}	0.68^{ijkl}	12.7^{kl}
	180	129^{d}	9.2^{a}	6.9^{b}	0.89^{fg}	13.4^{hij}
Hamedani	0	79^{kl}	2.8^{hijk}	1.4^{nop}	0.41^{opqr}	11.3^{no}
	60	89^{ij}	5.2^{ef}	2.2^{jkl}	0.58^{klmn}	12.1^{lm}
	120	104^{gh}	7.7^{bc}	3.4^{fg}	0.76^{ghi}	13^{ijk}
	180	119^{e}	9.6^{a}	4.9^{d}	0.98^{ef}	13.9^{gh}
Yazdi	0	59^{qr}	1.2^{l}	0.77^{qr}	0.24^{tu}	12.8^{jk}
	60	66^{op}	1.7^{kl}	1.3^{op}	0.33^{rst}	13.7^{h}
	120	84^{jk}	2.4^{ijkl}	2.7^{ij}	0.57^{lmn}	14.6^{ef}
	180	99^{h}	3.8^{fghi}	3.9^{ef}	0.73^{hij}	15.4^{cd}
Nikshahri	0	54^{rs}	1.5^{kl}	0.97^{pqr}	0.18^{uvw}	13.6^{hi}
	60	63^{pq}	2.1^{jkl}	1.1^{opq}	0.29^{stu}	14.4^{fg}
	120	75^{lm}	3.2^{ghij}	3^{gh}	0.48^{nop}	15^{def}
	180	89^{ij}	4.7^{ef}	4.2^{e}	0.62^{jklm}	16.2^{b}
Pioneer	0	36^{v}	2.7^{hijk}	0.47^{r}	0.08^{w}	13.6^{hi}
	60	44^{tu}	3.2^{ghij}	0.98^{pqr}	0.19^{uvw}	14.4^{fg}
	120	54^{rs}	3.9^{fgh}	2.1^{klm}	0.37^{pqrs}	15.2^{de}
	180	77^{lm}	5.7^{de}	3.4^{fg}	0.48^{nop}	16^{bc}
Bami	0	29^{w}	4^{fgh}	0.37^{r}	0.11^{vw}	14.4^{fg}
	60	34^{vw}	4.7^{ef}	0.67^{qr}	0.23^{tuv}	15.2^{de}
	120	49^{st}	5.8^{de}	1.4^{nop}	0.46^{nopq}	16^{bc}
	180	69^{no}	6.7^{c}	2.8^{gi}	0.58^{klmn}	16.9^{a}
LSD		5.7	1.4	0.59	0.12	0.6

†Mean within columns with the same letters is not significantly different at 5% level.

Salinity increase of GR activity by 4.33-folds in *Pioneer*. followed by 3.85 - 1.86-folds in *Bami*, *Nikshahre*, *Yazdi*, *Esfahani*, *Ranjer*, *Gharehyonje*, *Hamedani*, and *Rehnani* (Table 2). GR activity was higher in salt tolerant cotton cultivars (Meloni *et al.* 2003). However, the enhancement of APX activity was greater in salt tolerant than in salt sensitive alfalfa cultivars (Wang *et al.* 2009; Babakhani *et al.* 2011). The positive relation between APX and salt tolerance has also been reported in wild beet (Bor *et al.* 2003). The H_2O_2 produced by SOD may also be detoxified in the ascorbate-glutathione pathway (Wang *et al.* 2009), which involves the oxidation of ascorbate by APX and reduction of glutathione by GR (Wang *et al.* 2009). On the other hand, the increase in APX and GR activities appears to be an indication of salt tolerance in tested alfalfa cultivars. APX is a component of the ascorbate-glutathione pathway, which plays a key role in scavenging H_2O_2 (Wang and Han 2009). The activities of APX and GR were higher in salt tolerance cultivars as compared to sensitive cultivars suggesting that these enzymes may play an important role in salt tolerant mechanisms in alfalfa.

MDA content which is an indicator of lipid peroxidation of alfalfa seedlings was significantly enhanced under saline treatments (Table 2). The average increase in MDA content under saline treatments increase from 5.55 - 18.6% in *Esfahani, Rehnani*, *Bami*, *Pioneer*, *Yazdi*, *Nikshahre*, Hamedani, Ranjer, and *Gharehyonje* (Table 2). Similar results were observed in salt tolerant and salt sensitive cultivars of alfalfa (Wang *et al.* 2009, Wang and Han 2009, Babakhani *et al.* 2011) and barley (Liang *et al.* 2003). Lipid peroxidation is an identified cell membrane damage mechanism, and is used as an indication of oxidative stress caused by various stresses including salt stress (Babakhani *et al.* 2011). The result suggested that ROX was increased under salt stress, however, increased in antioxidant enzymes activities suppressed the damages caused by ROX in tolerant cultivars.

Changes in seeds and seedlings growth parameters of the cultivars under salt stress showed that *Rehnani* and *Esfahani* were most salt tolerant while *Bami* and *Pioneer* were the most salt sensitive cultivars. The activities of SOD, GR, POX and APOX were higher in salt tolerant and lower in salt sensitive cultivars. The MDA content was lower in salt tolerance cultivars as compared to sensitive cultivars. These results showed that the SOD, GR, POX, APOX activities and MDA content at the germination and seedling stages may be used to select salt tolerance cultivars.

References

Babakhani B, Khavari-Nejad RA, Hassan Sajedi R, Fahimi H and Saadatmand S 2011. Biochemical responses of Alfalfa (*Medicago sativa* L.) cultivars subjected to NaCl salinity stress. African J. Biotechnol. **10:** 11433-11441.

Beauchamp CO and Fridovich I 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Ann. Biochem. **44:** 276-287.

Bor M, Ozdemir F and Turkan I 2003. The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. Plant Sci. **164:** 77-84.

Foyer CH and Halliwell B 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Plantarum **133:** 21-25.

Herzog V and Fahimi H 1973. Determination of the activity of peroxidase. Ann. Biochem. **55:** 554-562.

Howieson J and Ballard R 2004. Optimising the legume symbiosis in stressful and competitive environments within southern Australia: some contemporary thoughts. Soil Biol. Biochem. **36:** 1261-1273.

Jaleel CA, Lakshmanan GMA, Gomathinayagam M and Panneerselvam R 2008. Triadimefon induced salt stress tolerance in *Withania somnifera* and its relationship to antioxidant defense system. South African J. Botany **74:** 126-132.

Liang Y, Chen Q, Liu Q, Zhang W and Ding R 2003. Exogenous silicon (Si) increases antioxidant enzyme activity and reduces lipid peroxidation in roots of salt-stressed barley (*Hordeum vulgare* L.). J. Plant Physiol. **160:** 1157-1164.

Madhava KV and Sresty TVS 2000. Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* (L.) Millspaugh) in response to Zn and Ni stresses. Plant Sci. **157:** 113-128.

Meloni DA, Oliva MA, Martinez CA and Cambraia J 2003. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ. Exp. Bot. **49:** 69-76.

Nakano Y and Asada K 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. **22:** 867-880.

Peel MD, Waldron BL, Jensen KB, Chatterton NJ, Horton H and Dudley LM 2004. Screening for Salinity Tolerance in Alfalfa: A Repeatable Method. Crop Sci. **44:** 2049-2053.

SAS Institute Inc 2007. *SAS OnlineDoc 9.1.3.* Cary, NC: SAS Institute Inc.

Sudhakar C, Lakshmi A and Giridarakumar S 2001. Changes in the antioxidant enzymes efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Sci. **161:** 613-619.

Torabi M, Halim RA, Sinniah UR and Choukan R 2011. Influence of salinity on the germination of Iranian alfalfa ecotypes. African J. Agric. Res. **6:** 4624-4630.

Wang F, Chu R, Yang J, Gong Y and Zhu X 2009. Cadmium accumulation and antioxidant enzyme activity in response to cadmium stress in radish (*Raphanus sativus* L.). pp. 1-4. Proc. 3rd Int. Conf. Bioinformatics and Biomedical Engineering, June 11-13, 2009. Beijing, China.

Wang XS and Han JG 2009. Changes of proline content, activity, and active isoforms of antioxidative enzymes in two alfalfa cultivars under salt stress. Agric. Science China **8:** 431-440.

(Manuscript received on 25 September, 2013; revised on 26 February, 2014)