Oxidative markers in five Iranian alfalfa (*Medicago sativa* L.) cultivars under salinity stress

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Abstract

Five alfalfa (*Medicago sativa* L) cultivars from different areas of Iran were evaluated for oxidative markers under salinity conditions. Plants were grown in hydroponic condition by Hoagland nutrient solution containing different amounts of NaCl (control, 50 and 100 mM). Relative growth rate, membrane stability, lipid peroxidation, proline, hydrogen peroxide and relative water contents were determined. Results indicated that salinity decreased membrane stability, relative water content and growth parameters and increased lipid peroxidation, proline and hydrogen peroxide contents. Important variation was observed for all traits by increasing salinity. There were significant differences between cultivars in amounts of decrease or increase in the measured traits. In general, low membrane stability was observed in Sahand ava cultivar. Regarding salt stress, Yazdi cultivar was successful in maintaining membrane stability and relative growth rate.

Keywords: Medicago sativa L.; growth; membrane stability; hydrogen peroxide; salinity

Abbreviations:
AOS: activated oxygen species; MSI: membrane stability index; RGR: relative growth rate; RWC: relative water content; TBARS: thiobarbituric acid reactive substances

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Introduction

Medicago sativa L., commonly known as lucerne or alfalfa is endemic to Mediterranean region and is one of the most important crops. There are large areas in the world where economical cultivation of alfalfa is constrained by environmental stresses. Investigation of diversity is important for breeding of stress-tolerant crops. The genetic diversity of *Medicago sativa* has been estimated using various markers (Monirifar and Barghi, 2009; Guines et al., 2003).

Soil salinity is an issue that affects approximately 20% of irrigated agricultural land and is a major constraint to food production (Chinnusamy, 2005). By dysfunction of the photosynthetic machinery, salt stress could lead to accumulation of activated oxygen species (AOS) and generating oxidative stress (Munns and Tester, 2008). AOS, then cause oxidative damage

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to the membrane lipids, proteins and nucleic acids (Xiong and Zhu, 2002). The capacity of plants to scavenge AOS and reduce their damaging effects correlates with salt tolerance in many plant species (Nazar et al., 2011). Cell membranes are one of the first targets of many plant stresses and their integrity and stability under stress conditions is a major component of tolerance in plants (Farooq and Azam, 2006). It is demonstrated that electrolyte leakage of membranes is correlated with several physiological and biochemical parameters such as antioxidative enzyme activity (Liu and Huang, 2000) and membrane acyl lipid concentrations (Lauriano et al., 2000).

There are numerous reports on alfalfa response to abiotic stresses (Wang and Han, 2009; Zhou et al., 2008; Peng et al., 2008). The purpose of this research was to study salt stress physiology and evaluate the cultivars of alfalfa using oxidative markers including growth, cell membrane injury, and oxidants under salinity conditions to screen tolerant ones.

Materials and Methods

Plant materials

Seeds of five alfalfa cultivars (Chalashter, Ghareh ghozlo, Hamadani, Sahand ava and Yazdi) were surface sterilized with sodium hypochlorite solution before they were germinated in pots containing washed sand in a greenhouse with dim light, 60 % ± 3 % air humidity, and an ambient temperature 25 ± 2 °C. 10 days after sowing, the seedlings were thinned and salt treatments were applied by adding NaCl to the Hogland solution to obtain 50 and 100 mM concentrations. Hogland solution without NaCl served as control. Measurements carried out 25 days after the beginning of the treatments.

Growth analysis

For growth analysis, relative growth rate (RGR) was calculated using the following equation:

$$RGR = (\ln W_2 - \ln W_1)/ (t_2-t_1)$$

where ln = natural logarithm, t_1 = stress starting time, t_2 = harvesting time, W_1 = Dry weight of plant at starting stress and W_2 = Dry weight of plant at harvesting.

Membrane stability index

Membrane stability index (MSI) was assayed by estimating the ions leaching from leaf tissue into distilled water according to Sairam et al. (2002). Aliquots of fresh leaves dipped in 10 ml of double distilled water in two sets. The first set was subjected to 32 °C for 120 min and its conductivity was recorded using a conductivity meter (C_1). The second set was autoclaved for 15 min at 121 °C and its conductivity was measured after cooling down to room temperature (C_2). MSI was calculated as below:

$$MSI = (1- (C_1/C_2)) \times 100.$$

Hydrogen peroxide content determination

For hydrogen peroxide content determination, aliquots of fresh leaves were homogenized in 50 mM potassium phosphate buffer, pH 6.5, and centrifuged at 10000 × g for 25 min. The solution was mixed with 1% titanium chloride (in concentrated HCl) and then centrifuged at 10000 × g for 15 min. The absorbance of the supernatant was measured at 410 nm. H_2O_2 content was calculated using 0.28 $\mu M^{-1} cm^{-1}$ as extinction coefficient (Chaparzadeh, et al., 2004).

TBARS content determination

For determination of thiobarbituric acid reactive substances (TBARS) content, aliquots of fresh leaves were homogenized in 20% trichloroacetic acid containing 0.5% thiobarbituric acid and incubated at 95 °C in water bath for 30 min. Then, the mixture was quickly cooled in an ice-bath and centrifuged at 10000 × g for 15 min. The absorbance of supernatant was measured at 532 nm and corrected for nonspecific absorbance at 600 nm. TBARS content was calculated using 155 $mM^{-1} cm^{-1}$ as extinction coefficient (Chaparzadeh et al., 2004).

Proline content determination

Free proline content in the leaves was determined following the method of Bates et al. (1973). Aliquots of fresh leaves were homogenized in 10 ml of 3% aqueous sulphosalycylic acid and the homogenate was filtered. 2 ml of extract was reacted in the test tube with 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent. The reaction mixture was boiled in water bath at 100 °C for 60 min. After cooling on ice, 4 ml of toluene was added and thorough mixing, the toluene phase was separated and absorbance determined at 520 nm against toluene blank.

RWC estimation

Leaf relative water content (RWC) was estimated by recording the turgid weight of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven until constant weight was achieved. RWC was calculated as below:

RWC = [(Fresh weight - dry weight) / (Turgid weight - dry weight)] × 100.

Statistical analysis

The experiment was carried out using a factorial design based on completely randomized design (CRD) with three replications. Data means were separated by Fisher's protected least significant difference (LSD) test, P≤0.05 in SPSS.

Results

Effects of salinity on growth

Salt treatment significantly (P < 0.01) affected the RGR of alfalfa plants (Table 1). Large value of RGR was recorded for Sahand ava and Yazdi cultivars (Table 2). RGR decreased in cultivars Ghareh ghozlo, Sahand ava and Chalashter with increasing salinity levels but did not change in Hamadani and Yazdi cultivars (Table 4). The analysis of variance revealed the significant effects of salinity stress on growth (p < 0.01) (Table 3). RGR was more affected by 100 mM NaCl stress level compared with 50 mM.

Effects of salinity on MSI

The effect of salinity on the MSI,

Table 1
Effect of salinity concentration on means of measured parameters for all alfalfa cultivars

NaCl (mM)	H_2O_2 (μmol g^{-1} fw)	TBARS (μmol g^{-1} fw)	MSI (%)	Proline (μg g^{-1} fw)	RWC (%)	RGR (g kg^{-1} day^{-1})
0 (Control)	12.04±0.61^{c}	0.0027±0.0002^{c}	0.59±0.036^{a}	27.57±2.19^{c}	71.51±2.91^{a}	0.126±0.0026^{a}
50 (S1)	14.78±0.93^{b}	0.0037±0.0003^{b}	0.51±0.030^{b}	38.83±2.44^{b}	57.36±1.61^{b}	0.121±0.0017^{b}
100 (S2)	20.78±0.73^{a}	0.0043±0.0004^{a}	0.43±0.037^{c}	51.24±3.08^{a}	49.48±2.32^{c}	0.116±0.0019^{c}
Salt effect	**	**	**	**	**	**

**, significant difference at 0.01 probability

Table 2
Effect of alfalfa cultivar kinds on means of measured parameters for all treatments

cultivars	H_2O_2 (mmol g^{-1} fw)	TBARS (μmol g^{-1} fw)	MSI (%)	Proline (μg g^{-1} fw)	RWC (%)	RGR (g kg^{-1} day^{-1})
Ghareh-ghozlo	15.48±1.54^{bc}	0.0026±0.0001^{c}	0.51±0.049^{b}	32.12±3.58^{b}	66.01±5.05^{a}	0.113±0.0025^{c}
Sahand ava	17.43±1.54^{ab}	0.0057±0.0004^{a}	0.34±0.022^{c}	37.29±4.52^{ab}	60.21±4.58^{ab}	0.130±0.0032^{a}
Chalashter	18.7±1.23^{a}	0.0028±0.0002^{c}	0.65±0.039^{a}	46.05±4.10^{a}	53.06±2.91^{b}	0.119±0.0023^{b}
Hamadani	13.15±1.5^{c}	0.0039±0.0005^{b}	0.52±0.046^{b}	35.25±4.80^{b}	57.60±3.60^{b}	0.118±0.0021^{bc}
Yazdi	14.57±1.58^{c}	0.0029±0.0002^{c}	0.52±0.023^{b}	45.36±4.98^{a}	60.38±4.58^{ab}	0.126±0.0016^{a}
Sig.	***	***	*	**	**	***

*,**,***, significant difference at 0.05, 0.01 and 0.001 probability, respectively.

estimated as electrolyte leakage, was statistically significant based on means of all cultivars (Table 1) while, significant effect of salinity was found only in Ghareh ghozlo (Table 4). There were differences in means of MSI in alfalfa cultivars (Table 2). Chalashter and Sahand ava cultivars had the highest and lowest MSI values, respectively. MSI decreased only in Ghareh ghozlo cultivar at all salinity levels (Table 4).

Effects of salinity on H_2O_2 content

In the base of means of all cultivars, salt stress resulted in the accumulation of H_2O_2 in leaves (Tables 1 and 3). Different cultivars of alfalfa showed significant difference of H_2O_2 accumulation in leaves (Tables 2 and 3). The highest effect was at 100 mM NaCl for all cultivars. Under high salinity conditions, Chalashter and Hamadani cultivars showed higher and lower amount of H_2O_2 content, respectively (Table 4).

Effects of salinity on TBARS content

TBARS content of leaves increased significantly with increasing salinity treatments (Tables 1 and 3). The highest effect was at 100 mM NaCl for all cultivars. Under high salinity conditions, Sahand ava and Ghareh ghozlo cultivars showed higher and lower amounts of TBARS production at 100 mM NaCl level, respectively (Tables 2 and 4).

Effects of salinity on proline

As salt stress increased, so did the proline production (Table 1). Cultivars had significantly different amounts of proline contents (Table 2). In addition, analysis of variance revealed the significant effects of salinity and cultivar on

Table 3
Analysis of variance (mean of squares) for five alfalfa cultivars under salinity stress

Source of variation	df	Proline	H_2O_2	TBARS	MSI	RWC	RGR
Salinity	2	2102.942**	300.170**	0.000010**	0.089**	1869.658**	0.00041**
Cultivar	4	347.283*	44.409**	0.000015**	0.107**	199.627*	0.00040**
Salinity × Cultivar	8	17.300^{ns}	1.661^{ns}	0.000001^{ns}	0.010^{ns}	126.234*	0.00004^{ns}
Error	30	90.710	6.200	0.000001	0.009	55.397	0.00003

ns, *,**, non-significant, significant difference at 0.05, 0.01 probability, respectively.

Table 4
Effect of salinity concentration and alfalfa cultivar kinds on means of measured parameters for each treatment

Cultivar/Salinity		H_2O_2 (mmol g^{-1} fw)	TBARS (μmol g^{-1} fw)	MSI (%)	Proline (μg g^{-1} fw)	RWC (%)	RGR (g kg^{-1} day^{-1})
Ghareh-ghozlo	Control	11.2±0.8^{b}	0.0025±0.0003^{a}	0.64±0.02^{a}	21.8±1.5^{b}	85.2±2.4^{a}	0.12±0.002^{a}
	S1	14.5±1.4 ^{b}	0.0026±0.0001^{a}	0.53±0.01^{b}	31.4±0.5^{ab}	55.3±4^{b}	0.11v0.005^{ab}
	S2	20.7±1.6^{a}	0.0028±0.0001^{a}	0.35±0.07^{b}	43.07±6.1^{a}	57.4±2.4^{b}	0.10±0.001^{b}
LSD		4.675	0.00075	0.163812	12.7826	10.62703	0.011516
Sahand ava	Control	13.3±1.08^{b}	0.004±0.0003^{b}	0.36±0.04^{a}	27.03±6^{b}	74.9±2^{a}	0.140±0.005^{a}
	S1	16.3±1.9^{b}	0.005±0.0007^{ab}	0.34±0.01^{a}	34.09±5.1^{ab}	60.7±0.95^{b}	0.128±0.002^{ab}
	S2	22.6±1.3^{a}	0.006±0.0003^{a}	0.33±0.06$_{a}$	50.74±5.8^{a}	44.9±4.8^{c}	0.122±0.002^{b}
LSD		5.142	0.00177	0.155477	19.708	10.61067	0.013135
Chalashter	Control	14.6±0.08^{c}	0.002±0.0001^{b}	0.74±0.03^{a}	35.2±5.5^{b}	54.9±2.6^{a}	0.125±0.003^{a}
	S1	18.4±0.72^{b}	0.003±0.0003^{a}	0.64±0.07^{a}	46.9±4.2^{ab}	57.8±6.5^{a}	0.122±0.001^{a}
	S2	23.04±0.37^{a}	0.003±0.0002^{a}	0.58±0.07^{a}	56.01±6.7^{a}	46.4±4.05^{a}	0.112±0.001^{b}
LSD		1.642	0.00086	0.225848	19.3732	16.33562	0.00881
Hamadani	Control	8.9±0.34^{b}	0.002±0.0007^{b}	0.64±0.01^{a}	23.3±3.5a	69.07±1.4^{a}	0.120±0.005^{a}
	S1	12.02±1.2^{b}	0.004±0.0008^{ab}	0.50±0.05^{a}	36.3±5.5a	56.48±3.7^{b}	0.119±0.001^{a}
	S2	18.4±1.2^{a}	0.005±0.0005^{a}	0.43±0.1^{a}	45.9±10.2a	47.24±4.4^{b}	0.115±0.003^{a}
LSD		3.674	0.00239	0.231952	24.3642	11.91486	0.013286
Yazdi	Control	12.02±1.4^{a}	0.002±0.0002^{b}	0.55±0.04^{a}	30.3±5.2b	73.4±6^{a}	0.127±0.003^{a}
	S1	12.59±2.9^{a}	0.003±0.0002^{ab}	0.54±0.02^{a}	45.3±6ab	56.4±2.9^{a}	0.125±0.002^{a}
	S2	19.09±2^{a}	0.003±0.0003^{a}	0.48±0.05^{a}	60.4±2.8a	51.3±8.5^{a}	0.125±0.003^{a}
LSD		7.697	0.00101	0.140806	17.0227	21.71047	0.011104

proline content (Table 3). Yazdi and Ghareh ghozlo cultivars had the highest and the lowest amount of proline at 100 mM NaCl level, respectively (Table 4).

Effects of salinity on RWC

RWC decreased under salinity in all cultivars (Tables 1 and 4). However, Chalashter cultivar had the least decrease in RWC among the cultivars under study at 100 mM NaCl. More decline in RWC took place in Sahand ava cultivar (Table 4). Changes in RWC of Yazdi and Chalashter cultivars were not significant during salinity conditions compared with control plants (Table 4).

Discussion

Salinity is one of the important abiotic stresses, which affects crop productivity. Reduction in plant growth under salinity stress is often associated with salt-induced osmotic effect, nutrient deficiency or ion toxicity (Munns, 2002). Numerous papers reported that plant cultivars notable for initially high antioxidant activity were more resistant to oxidative injury under stresses, including salinization stress (Mitteler, 2002). Although alfalfa is characterized as a moderate salt tolerant plant, there are large areas that economical cultivation of this plant is constrained by environmental stresses, such as salinity and drought (Garnett et al., 2004). On the other hand, genetic variability within a species offers a valuable tool for studying mechanisms of salt tolerance. One of these mechanisms depends on the bypass capacity for second oxidative stress that allows growth to continue under saline conditions. AOS induced lipid peroxidation is a reflection of stress induced damage to cell membranes.

H_2O_2 is a toxic molecule that has deleterious effects on plant tissue (Dogan et al., 2010). In this study, salinity treatments caused significant increase in H_2O_2 and TBARS, an indicator of lipid peroxidation, which were higher in Chalashter and Sahand ava, respectively. Increase in H_2O_2 and lipid peroxidation during salt stress has been reported by e.g., Markovska et al. (2009). In most studies, TBARS content, extent of the oxidative stress, was utilized as biomarker for lipid peroxidation (Mitteler, 2002). In this study, H_2O_2 content and lipid peroxidation increased in most of the cultivars under salt stress.

Most studies have reported MSI decrease (membrane permeability increase) under salinity stress (Bhutta 2011; Sairam et al. 2005). In these studies, MSI exhibited a positive correlation with osmotic potential, K^+ concentration, osmotic adjustment, and/or relative water contents, parameters that are influenced by salinity stress (Munns, 2002). MSI has been used as marker of salt injury and salt tolerance in plants. It suggested that decrease in membrane stability reflects the extent of lipid peroxidation caused by reactive oxygen species (Sairam et al., 2002). In our study under salinity conditions, MSI had no significant decrease in any of the cultivars, while Ghareh ghozlo and Sahand ava had the least MSI level.

Accumulation of proline under stress protects the cell by balancing the osmotic strength of cytosol with that of vacuole and external environment (Gadallah, 1999). This solute may interact with cellular macromolecules such as enzymes and stabilize the structure and function of such macromolecules (Smirnoff and Cumbes, 1989). The capacity of some crop plants to accumulate proline in response to environmental stresses may be highly variable from one species to another and even between some varieties of the same crop plant (Quarrie, 1980). Under salt stress, most plant species exhibit a remarkable increase in their proline content (Dasgan et al., 2009). In this study, high salinity caused a significant increase of proline content in all of the cultivars under study and Yazdi cultivar showed higher amount of proline accumulation, about twice as much, in comparison with control. Numerous experiments have shown that under salt stress, higher concentration of proline is accumulated in sensitive plants than in tolerant genotypes (Parvaiz and Satyawati, 2008).

We found that salt stress also affected RWC. RWC decreased significantly in three cultivars under salinity stress. The cultivars of Yazdi and Chalashter had the least and not significant decline among the studied plants. Many important physiological and morphological processes such as leaf enlargement, stomatal

opening and associated leaf photosynthesis can be directly affected by the reduction of leaf turgor potential, which accompanies the loss of water from leaf tissue (Jones and Turner, 1978). These same researchers reported that with a decrease in RWC, leaf osmolality increased and the slow development of water deficits resulted not only in osmotic adjustment, but also in a decrease in leaf tissue elasticity. There is a similar trend in the results of other authors (Bhutta 2011).

In conclusion, NaCl at high concentrations leads to oxidative stress and causes changes in plant physiology. Although, salinity reduced plant growth in all cultivars, we found salt dependent cultivar variation in alfalfa plants. On the basis of the amount of changes in physiological parameters measured in the present study, Yazdi cultivar was marked as tolerant among the five studied cultivars and designated for further studies.

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