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Full Length Research Paper

Biochemical responses of Alfalfa (*Medicago sativa* L.) cultivars subjected to NaCl salinity stress

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This investigation was conducted to determine NaCl salinity effects on antioxidant enzymes activities, reducing sugar contents and lipid peroxidation in two alfalfa cultivars. Plants grown in solution cultures were subjected to 0, 100, 150 and 200 mM solutions of sodium chloride. Yazdi and Diabolourde alfalfa were used as tolerant and sensitive cultivars, respectively, in a germination experiment under similar conditions. Results show that the amount of reducing sugars and the activities of peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) enzymes increased with the increase in salt concentration. However, SOD activities decreased at high salt concentrations. The increase in the activities of antioxidant enzymes in response to salt treatments was higher in the tolerant cultivar. The results also show that salt treatment provoked an oxidative stress in both cultivars, as shown by the increase in lipid peroxidation. However, the level of lipid peroxidation was higher in the sensitive cultivar. The increase in antioxidant activities could also be a response to the cellular damage induced by NaCl. It seems that the tolerant cultivar has a better mechanism to cope with the deleterious effects ROS produced under salt stress.

Key words: Alfalfa, antioxidant enzymes, malondialdehyde, salt stress.

INTRODUCTION

Sodium chloride (NaCl) salinity is one of the major environmental factors that limit plant growth, productivity and distribution (Wang et al., 2003). NaCl salt stress occurs in areas where soils are naturally high in salts and where irrigation, hydraulic lifting of salty underground water or invasion of seawater in coastal areas brings salt to the surface soil where plants inhabit (Zhang et al., 2006). The problem of soil salinity also is becoming even more serious because of high evapotranspiration and improper water management (Wang and Han, 2009). It has been estimated that two-thirds of the potential yield of major crops are usually lost due to adverse effects of salt in the plants environment (Munns, 2002; Bajaj et al., 1999).

Salt stress adversely affects legume production mainly due to the dependency of these plants on symbiotic N_2 fixation for their nitrogen requirements (Elsheikh and Wood, 1995). Some processes affected in such conditions include the rates of host plants growth (Tejera et al., 2004; Tejera et al., 2006), the development of root-nodules (Georgiev and Atkins, 1993) and finally, the nitrogen-fixing capacity (Delgado et al., 1993). The mere presence of different cultivars, genotypes and ecotypes of alfalfa is a valuable source for screening and identifying the tolerant types with regard to environmental stresses such as salinity. In Iran, alfalfa is the most commonly grown forage crop. In 2006, the total area covered by alfalfa crop, its annual yield and annual

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Abbreviations: PUFA, Poly unsaturated fatty acids; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **CAT**, catalase; APX, ascorbate peroxidase; **DHAR**, dehydroascorbate reductase; **MDHAR**, monodehydro ascorbate reductase; **GR**, glutathione reductase; **PPFD**, photo period flow density; **TBA**, thio barbituric acid; **EDTA**, ethylene diamino tetra acetic acid; **TCA**, tri chloro acetic acid.

productivity were 6.161 × 10^5 hectares; 4.762 × 10^6 tones and 8.287 × 10^3 Kg ha^{-1} dry mass, respectively (Anonymous, 2006). As a perennial forage crop, alfalfa (*Medicago sativa L.)* can be cultivated in marginal lands and has high yield and good quality proteins (Ehsanpour and Fatahian, 2003). In addition, Alfalfa's deep-root system can help soils to prevent water loss in semi-dry lands (Moran et al., 1994). Due to the legumes capacity of symbiotic nitrogen fixation, these plants are often used to improve soil organic fertility and nitrogen economy (Howieson and Ballard, 2004).

The widespread use of this forage crop is due to its high yield, nutritional quality, high protein content, presence of different vitamins and digestible materials and its adaptability to different habitats. According to FAO report, 40% of irrigated lands in Iran are exposed to secondary salinization (Pessarakli, 1993). Alkaline lands in this country are about 26.399 × 10^6 hectares and sodic lands are about 6.86 × 10^5 hectares (Abrol, 1988). Therefore, cultivation of tolerant varieties and species of alfalfa is an effective way of making good use of these lands. Studies on salt tolerance of alfalfa are scarce and controversial, which makes it necessary to do more research in the line of finding both more tolerant species and their mechanism of salt tolerance. It is well known that high salinity levels in external media affect many physiological and metabolic processes, leading to low cell growth and development (Ashraf and Harris, 2004). The effects of various environmental stresses such as salinity on plants are known to be mediated, at least partially, by an enhanced generation of reactive oxygen species (ROS) such as superoxide (O^{-2}), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). These ROS species can react with PUFA, forming conjugated dienes (CD) or trienes (CT), lipid peroxyl radicals and lipid hydroperoxides (Smirnoff, 1995). Under these conditions, different biochemical and physiological responses are induced in order to help the plants to survive (Seki et al., 2003). Therefore, understanding the biochemical and physiological mechanisms of salt stress effects is essential for breeding tolerance cultivars and improving environmental conditions to increase crop productivity (Wang and Han, 2009). In order to decrease the oxidative damages, plants employ the enzymatic and non-enzymatic mechanisms to scavenge ROS, which include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases and enzymes involved in the ascorbate-glutathione cycle [ASC-GSH cycle: ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR)] (Foyer and Halliwell, 1976). One of the most common non-enzymatic mechanisms in plants is overproduction of different types of compatible organic solutes (Serraj and Sinclair, 2002), which could fall into three major groups: amino acids (proline), quaternary amines (glycine betaine and dimethylsulfoniopropionate) and polyol/sugars (mannitol and trehalose) (Wang et al., 2003).

The aim of this investigation was to compare the germination rate of different alfalfa cultivars under salt stress and also study some enzymatic, non enzymatic responses of tolerant and sensitive alfalfa plants to salt stress.

MATERIALS AND METHODS

Plant material

Seeds of 11 alfalfa (*M. sativa* L.) cultivars (Silvaneh, Garghalogh, Nikshahri, Dastjerd, Zivar, Sequel, Yazdi, Diabolourde, Bami, Gharayonje and Ghahavand) were provided by the Department of Plant and Seed Research Institute of Karaj, Iran.

Germination experiment

Ten alfalfa (*M. sativa* L.) cultivars seeds were surface-sterilized with 5% sodium hypochloride solution for 5 min. Seeds were then thoroughly rinsed three times with distilled water and germinated in 10 cm petri dishes with one Whatman No. 1 filter papers moistened with the appropriate saline solutions or distilled water for control (0). Salinity levels of 100, 150 and 200 mM NaCl were used. Five replicates of 20 seeds each were used for each treatment. Germination tests were carried out in growth chamber (HPG- 400, Haerbin, China) set at 16 h/8 h day/night photoperiod and a PPFD of around 500 µmol m^{-2} s^{-1} generated by white fluorescent and incandescent lamps. Germinated seeds were kept in petri dishes under light condition for 7 days. The rate of germination was determined by counting the germinated seeds for a period of 7 days at one day intervals. Alfalfa seeds were considered to have germinated when the radicles were visibly protruded from the seed coat by at least 2 mm.

Growth condition and stress treatment

Based on the results obtained from screening experiment, Yazdi and Diabolourde were selected as salt tolerant and salt sensitive cultivars, respectively. Seeds of these two cultivars were surface sterilized as before and were germinated for 48 h in growth chamber under dark condition. Uniform seedlings of alfalfa plants were transferred into pots containing half strength Hoagland solution (6 pots for each treatment and 5 seedlings per pot). The pH of the solutions was adjusted to 7.0 to 7.5 daily, using 200 mM KOH or HCl. Plants were grown for 21 days, followed by another 14 days in the presence of 0, 100, 150 and 200 mM NaCl added to the nutrient solutions. Experiments were conducted in a house chamber with the average temperature of 27°C/18°C day/night. Photosynthetic photon flux density of 500 µmol m^{-2} s^{-1} was supplied by combined fluorescent and incandescent lamps. Plants were harvested at the end of the14 days of salt treatment.

Enzyme extraction

Leaf samples (200 mg each) were ground into fine powder with liquid nitrogen in a pre-chilled mortar and pestle. Further grinding was performed in a solution of 50 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA and 2% (w/v) polyvinyl-polypyrrolidone (PVPP) for APX and CAT assays, and in a

solution of 50 mM potassium phosphate buffer at pH 7.0 containing 0.5 mM EDTA for SOD and POD assays. The homogenates were centrifuged at 14000 × *g* for 15 min at 4°C. The resulting supernatants were centrifuged again and used immediately for enzyme activity assays or stored at -30°C to be used later.

Enzyme assays

Total SOD (EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977) method by monitoring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). Each 3 ml reaction mixture contained 50 mM potassium phosphate (pH 7.8), 13 mM methionine, 2 µM riboflavin, 75 µM NBT, 0.1 mM EDTA and 25 to 100 µl of the enzyme extract. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm. Identical tubes with the same reaction mixtures were kept in the dark and served as blanks. Reactions were carried out in test tubes (10 mm in diameter) at 25°C under illumination supplied by two fluorescent lamps (20 W). The entire reaction assembly was enclosed in a box lined with aluminum foil. Riboflavin was added last and the tubes were shaken before they were placed in the reaction assembly. The reaction was initiated by switching on the light and was run for 15 min, before been stopped by switching off the light while the tubes were still covered by aluminum foil. Under the experimental conditions, the initial rate of reaction, as measured by the difference in increase in absorbance at 560 nm in the presence and absence of leaf extract, was proportional to the amount of enzyme. One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction measured at 560 nm (Martinez et al., 2001).

Activities of CAT and POD were measured by the methods of Chandlee and Scandalios (1984) and Chance and Maehly (1955), respectively. For CAT (EC 1.11.1.6), the decomposition of H_2O_2 was determined by following the decline in absorbance at 240 nm. Reaction mixture of 3 ml contained 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 25 to 100 µl of the enzyme extract. The reaction was initiated by adding the enzyme extract. CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient of 39.4 $mM^{-1}.cm^{-1}$) at 240 nm over a 2 min intervals. For POD (EC 1.11.1.7), the oxidation of guaiacol was measured by the increase in absorbance at 470 nm. The assay mixture of 3 ml contained 30 µl of enzyme extract, and 2970 µl of guaiacol (45 mM) and H_2O_2 (100 mM) which was prepared in 50 mM potassium phosphate buffer pH 7.0 containing 0.5 mM EDTA. POD activity was determined by measuring the oxidation of guaiacol in the presence of H_2O_2 (extinction coefficient of 26.6 $mM^{-1}.cm^{-1}$) at 470 nm over a 2 min intervals. APX activity (EC 1.11.1.11) was determined by following the decrease of ascorbate and measuring the change in absorbance at 290 nm over 2 min intervals. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 50 µl of crude enzyme extract (Nakano and Asada, 1981). The activity of ascorbate peroxidase was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1}).

Lipid peroxidation

The method of Heath and Packer (1968) was used to determine lipid peroxidation. Leaf samples of 0.3 g were homogenized in 4 ml of 1% (w/v) tri chloro acetic acid (TCA), and then centrifuged at 10000 × *g* for 10 min. 1.5 ml of 20% (w/v) TCA containing 0.5% (w/v) TBA was added to 1.5 ml of the supernatant aliquot. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. The mixture was centrifuged at 10000 × *g* for 5 min and their absorbance was read at 532 nm. The values for non-specific absorption at 600 nm were subtracted from the 532 nm reading. The malondialdehyde (MDA) content was calculated using its extinction coefficient of 155 $mM^{-1}.cm^{-1}$ and expressed as µmol/g FW.

Extraction and determination of reducing sugars

Sugars extraction was carried out following the method of Naureen and Naqvi (2010) with some modifications. Leaf sections (250 mg) were frozen in liquid nitrogen and subjected to a triple extraction of ethanol-soluble sugars (ESS) by boiling in ethanol (80%) at final volume of 20 ml in water bath. Chloroform of 5 ml was added to the extractions and mixed with vortex, then centrifuged at 12000 × *g* for 3 min. The supernatant used for the determination of reducing sugars was as described by Nelson (1944).

Statistical analysis

Mean values from five replications were subjected to Duncan's test to discriminate significant differences ($P < 0.05$). Data were shown as the mean ± standard error (SE). Analyses were done using the SAS software; graphs were drawn by Excel 2003.

RESULTS

Germination experiment

The salt effects on alfalfa seeds germination are shown in Figure 1. Except for Sizivar and Silvane, in other cultivars, the highest germination rate occurred in control (no salinity). Thus, under salt treatment, Yazdi and Diabolourde cultivars had the highest and the lowest rate of germination (86.8 and 31.3%, respectively). The rate of germination, decreased with the increase in salinity. At 200 mM NaCl, Yazdi and Diabolourde cultivars had the highest and the lowest rate of germination, respectively (72.5% versus 7.5%). According to these results, salt stress decreased germination rate of alfalfa significantly ($P < 0.05$). Thus, Yazdi and Diabolourde were selected as resistant and sensitive alfalfa cultivars, respectively and were used for biochemical experiments.

Antioxidant enzymes

SOD

The effects of various NaCl concentrations on SOD activities of the two alfalfa cultivars are shown in Figure 2A. In both cultivars, salt increased in a dose-dependent manner and SOD specific activity increased significantly ($P < 0.05$). The rates of increase in SOD activity were slower in Diabolourde than in Yazdi. At 150 mM NaCl, the rates of SOD activity in Diabolourde and Yazdi cultivars

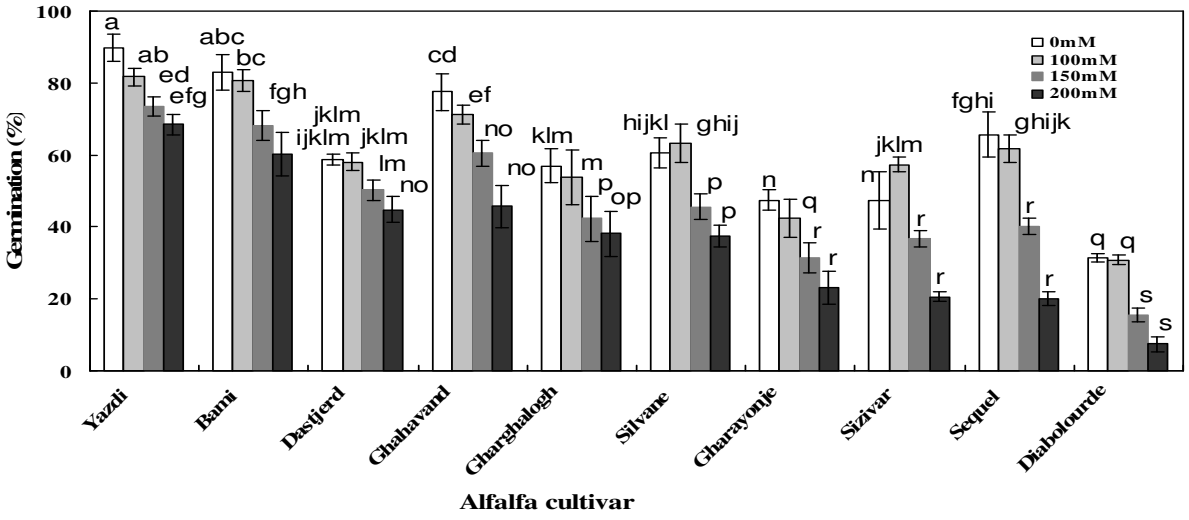

Figure 1. Germination rate of the 11 alfalfa cultivars seeds under 0, 100, 150 and 200 mM NaCl. Values are expressed as means of five independent experiments ± standard error (S.E.). Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's method at $P < 0.05$.

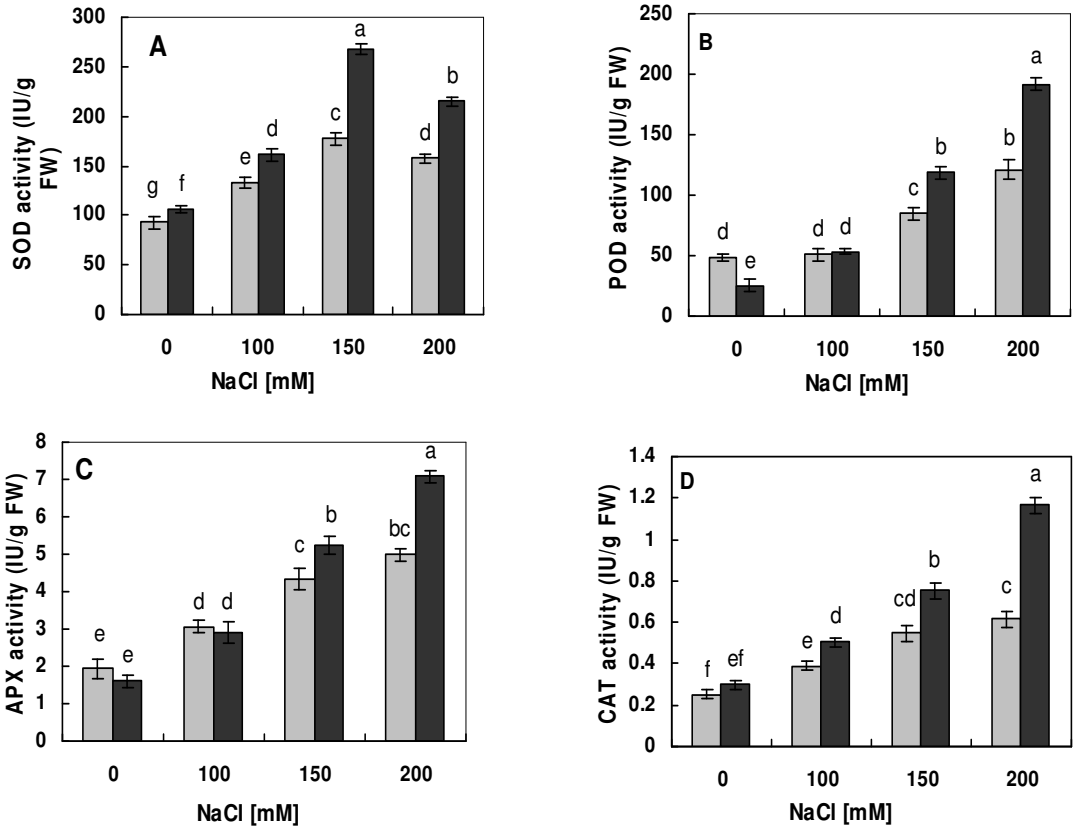

Figure 2. Changes in antioxidant enzymes activities; SOD (A), POD (B), APX (C) and CAT (D) of Gharayonje (grey bars) and Yazdi (black bars) cultivars under 0, 100, 150 and 200 mM NaCl. Values are expressed as means of five independent experiments ± standard error (S.E.). Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's method at $P < 0.05$.

increased by 1.9 and 2.52 folds, respectively, as compared to their controls. However, in 200 mM NaCl solution, the increase in SOD activity in both cultivars were lower than in 150 mM NaCl, which were 2.02 and 1.63 folds in Yazdi and Diabolourde, respectively, when compared with the control.

POD

The rate of POD activity, which catalyzes the decomposition of H_2O_2 produced by SOD, also changed with respect to species and salinization as shown in Figure 2B. Similar to SOD, POD activities in alfalfa cultivars leaves also increased remarkably with the increase in NaCl concentration when compared with control ($P < 0.05$). In the control, POD activity in Yazdi cultivar was lower than that in Diabolourde, but the highest POD activity was observed in Yazdi cultivar in 200 mM NaCl. POD activity in Yazdi cultivar increased by 4.66 and 7.55 folds in 150 and 200 mM NaCl, respectively when compared with the control. However, the corresponding values were 1.76 and 2.52 folds, respectively in Diabolourde.

APX

Salinity effects on APX activity is shown in Figure 4. The activity of APX, which also decomposes H_2O_2 increased significantly in both species with the increase in salinity ($P < 0.05$). Examination of the control groups of Yazdi and Diabolourde indicates that constitutive activity of APX was relatively higher in the latter. However, salt-induced APX activity was significantly higher in Yazdi cultivar than in Diabolourde in 150 and 200 mM. Results show that APX activity increased by 3.26 and 4.4 folds in150 mM and 200mM NaCl, respectively in Yazdi when compared with the control and by 2.25 and 2.58 folds, respectively in Diabolourde (Figure 2C).

CAT

Activity of CAT, as a scavenger of H_2O_2 outside the chloroplasts, was affected by all salinity treatments in both cultivars throughout the experiment (Figure 2D). Salt stress caused a significant increase in CAT activity in both cultivars ($P < 0.05$). However, the rates of increase in CAT activity in Diabolourde cultivar were slower than that in Yazdi cultivar. The activity of CAT in Yazdi cultivar was 2.53 and 3.92 times higher in 150 and 200 mM NaCl, respectively in comparison with the control, but it was 2.18 and 2.45 folds under the same condition in Diabolourde (Figure 2D). However, the activity of CAT was the same in control group of both cultivars.

Lipid peroxidation

Lipid peroxidation levels in leaves of the two alfalfa cultivars, as measured by the content of MDA, are given in Figure 3. In the leaves of both cultivars which were growing under normal growth conditions, a small level of lipid peroxidation was apparent; however, under salt stress, the levels of MDA content in both alfalfa cultivars increased significantly with the increase in NaCl concentration ($P < 0.05$). The MDA accumulation in Diabolourde was considerably greater (10.68 and 12.33 folds) than in Yazdi (6.38 and 9.16 folds), at 150 and 200 mM NaCl, respectively when compared with the control groups. These results indicate a higher degree of lipid peroxidation at 150 and 200 mM salt treatments.

Reducing sugars

The amounts of reducing sugars in leaves of both cultivars are shown in Figure 4. Salt stress increased significantly the reducing sugar levels of both cultivars ($P < 0.05$). As shown in Figure 4, reducing sugar contents show low differences in non saline treatments, but the increase in sugar contents due to high levels of salt was lower in Diabolourde than in Yazdi when compared with the control groups. Precisely, in 100, 150 and 200 mM NaCl, sugar contents in Diabolourde increased by 15.4, 20.1 and 31.2%, respectively when compared with their controls, whereas, the increase in sugar contents in Yazdi cultivar under similar conditions were 31.1, 38.0 and 51.1%, respectively when compared with their control groups.

DISCUSSION

Seed germination is normally limited by the intensity of abiotic stresses, such as high salinity. In this study, a significant difference in the germination rate of 11 alfalfa cultivars treated with different sodium chloride concentrations were observed. Similar observations were made in alfalfa sensitive and tolerant cultivars by Wang et al. (2009) and in *Medicago ruthenica* by Guan et al. (2009) under salt stress. The decrease in germination may be ascribed to an apparent osmotic 'dormancy' developed under salinity stress conditions, which may represent an adaptive strategy to prevent germination under stressful environment (Patanea et al., 2009). The germination rate due to increasing salinity can be correlated to the nature of salinity that reduces imbibitions of water due to lowered osmotic potentials of the medium and causes changes in metabolic activity (Yupsanis et al., 1994). Moreover, salinity perturbs plant hormone balances (Khan and Rizvi, 1994) and reduces the utilization of seed reserves (Ahmad and Bano, 1992).

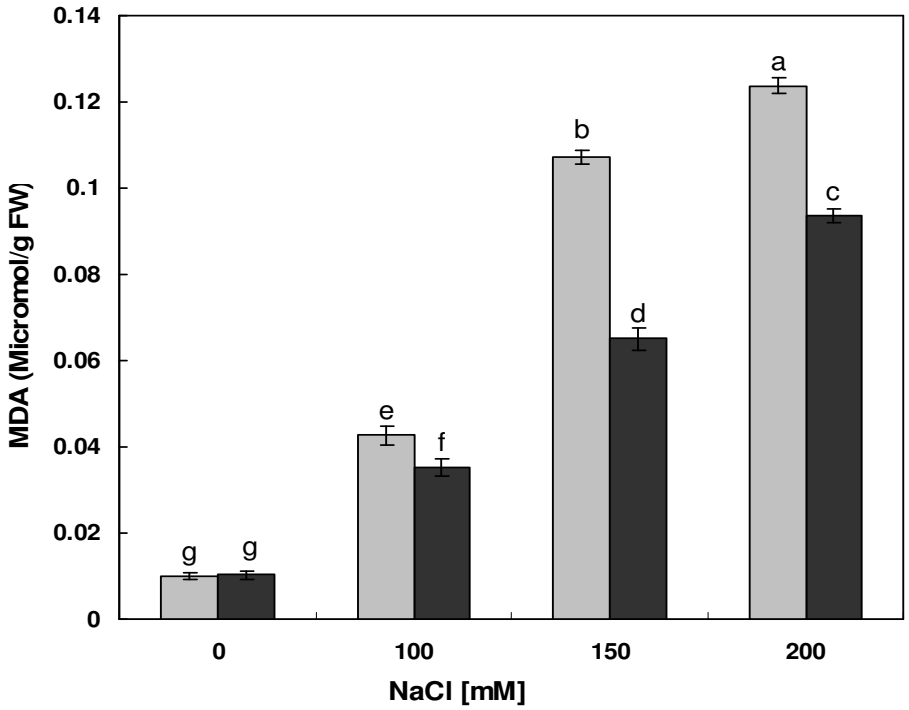

Figure 3. Changes in MDA content of Gharayonje (grey bars) and Yazdi (black bars) cultivars incubated under 0, 100, 150 and 200 mM NaCl. Values are expressed as means of five independent experiments ± standard error (S.E.).Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's method at $P < 0.05$.

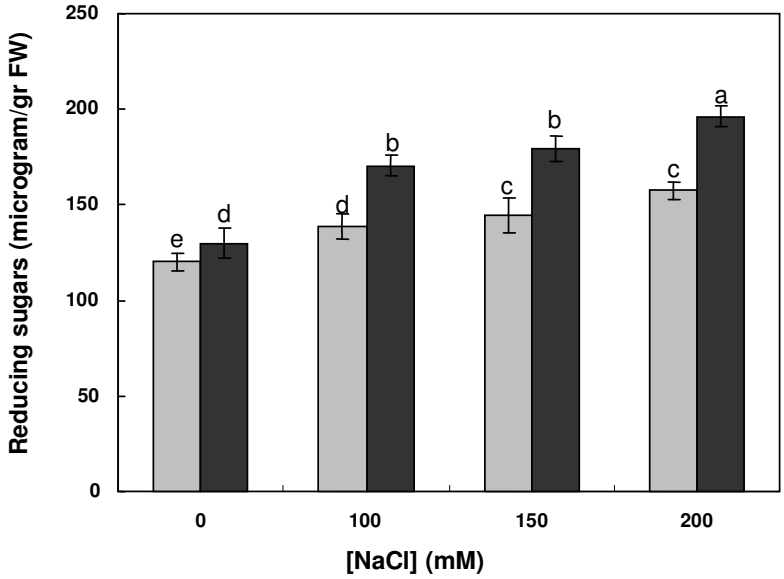

Figure 4. Changes in reducing sugars content of Gharayonje (grey bars) and Yazdi (black bars) cultivars incubated under 0, 100, 150 and 200 mM NaCl. Values are expressed as means of five independent experiments ± standard error (S.E.). Treatments with the same lower-case letters were not significantly different based on mean comparison by Duncan's method at $P < 0.05$.

Salt provoked a dose-dependent increase in SOD activity, which could represent a defense mechanism against NaCl-induced $O_2^{\cdot-}$ generation. SOD catalyzes the conversion of the superoxide anion to H_2O_2. Although the rates of salt-induced SOD activity in Diabolourde were lower than in Yazdi, a comparison of absolute enzymatic activity values at 100,150 and 200 mM NaCl clearly indicates that Yazdi cultivar has a higher dismutating capacity under moderate and high doses of NaCl salinity. These results are in a good agreement with those reported by Wang et al. (2009). Wang and Han (2009) found higher constitutive and induced level of SOD in tolerant alfalfa cultivar under salt stress. Similarly, Shalata and Tal (1998) and Koca et al. (2010) reported higher activities of SOD in wild salt-tolerant tomato species than in the cultivated salt sensitive ones.

Significant roles of POD have been suggested in plant development processes (Gaspar et al., 1985). POD is among the enzymes that scavenge H_2O_2 in chloroplasts, which is produced through dismutation of $O_2^{\cdot-}$ catalyzed by SOD. In tolerant plant species, POD activity was found to be higher, providing a greater protection against the oxidative stress caused by salt stress (Asada and Takahashi, 1987). In this study, POD activity increased significantly in Yazdi and Diabolourde cultivars in 150 and 200 mM treatments. However, induction in POD activity due to salinity was higher in 200 mM NaCl treatment. This may be considered as an indication that Yazdi has a higher capacity in removing H_2O_2 more rapidly. Similarly, increased POD activity has also been reported in salt-tolerant and sensitive species of alfalfa (Wang et al., 2009; Wang and Han, 2009), tomato (Shalata and Tal, 1998; Koca et al., 2010) and rice cultivars (Dionisio-Sese and Tobita, 1998). Increased POD activities in wild salt-tolerant Yazdi and relatively salt sensitive Diabolourde may be attributed to increased activity of POD encoding genes or increased activation of already existing enzymes as suggested (Dionisio-Sese and Tobita, 1998).

High levels of intercellular H_2O_2 induced cytosolic APX activity under salt stress (Lee et al., 2001); APX activity may have an important role in the mechanism of salt tolerance in plants (Gueta et al., 1997). APX and GR, the key enzymes in the Halliwell-Asada pathway, are involved in the reduction of H_2O_2 by using ASC as an antioxidant and NADPH as a reductant, to regenerate ASC (Asada, 1992) and GSH (Foyer and Halliwell, 1976), respectively. Hence, ASC-GSH cycle is well known to be important in the removal of H_2O_2 (Asada and Takahashi, 1987), and thereby protecting plants against oxidative stress (Khan and Ungar, 1984; Foyer and Halliwell, 1976; Guy and Carter, 1984; Wang et al., 1999). These results are in good agreement with those reported by Wang and Han (2009) and Wang et al. (2009) who reported higher inherently and induced levels of APX in wild salt-tolerant alfalfa. Same observation have been reported by Shalata and Tal (1998) and Lopez et al. (1996) who reported higher levels of APX activity in wild salt-tolerant tomato and radish plants, respectively.

CAT eliminates H_2O_2 by breaking it down directly to form water and oxygen. Thus, this enzyme does not require a reducing power and has a high reaction rate but a low affinity for H_2O_2 (Willekens et al., 1997). CAT together with SOD are the most effective antioxidant enzymes in preventing cellular damage (Scandalios, 1993). Olmos et al. (1994) reported that increase in the activity of CAT, a hydrogen peroxide scavenging enzyme located in peroxisomes, is related to the increase in stress tolerance. This might be due to increased levels of H_2O_2 which is formed from dismutation of $O_2^{\cdot-}$ by SOD. Supporting this observation, increase in the activities of CAT, have been reported in alfalfa (Wang and Han, 2009; Wang et al., 2009), soybean (Comba et al., 1998), tobacco (Bueno et al., 1998) and mulberry (Chinta et al., 2001) under salt stress.

MDA is the decomposition product of polyunsaturated fatty acids of plants membranes under stress. The rate of lipid peroxidation level in terms of MDA can therefore be used as an indicator to evaluate plants tolerance to oxidative stress as well as the sensitivity of plants to salt stress (Jain et al., 2001). It is also known that the formation of ROS enhances peroxidation at the cellular level, and that the rate of such enhancement relates to plant species and the severity of stress (Navari-Izzo et al., 1996). Increase in lipid peroxidation level in plants exposed to 1 00, 150 and 200 mM NaCl shows that increased activities of APX, SOD, POD and CAT might have not been enough to prevent the peroxidation of membrane lipids caused by high concentration of salinity. On the other hand, increase in MDA level in different salt treatments might also be correlated with inadequate activities of SOD, POD, APX and CAT to scavenge ROS produced in alfalfa leaves (Wang and Han, 2009; Wang et al., 2009). Variations in MDA contents were found in rice (Tijen and Ismail, 2005) and cotton (Diego et al., 2003) cultivars differing in salt tolerance.

It has been reported that drought and NaCl salt stresses cause an active conversion of starch to sugars, the phenomenon that stresses cause a decrease in starch content and an increase in sugar content (Stewart, 1971). This observation is supported by studies on a variety of plants that demonstrate a water stress-induced conversion of hexoses and other carbohydrates, such as starch, into sugar alcohols (polyols) and proline (Perez-Alfocea and Larher, 1995; Wang et al., 1996). High salt concentrations in soil, reduces the ability of plant to take up water which results in slower plant growth (Munns, 2005). Under stress conditions, plants need to maintain internal water potential below that of soil and maintain turgor and water uptake for growth (Tester and Davenport, 2003). This requires an increase in osmotica, either by uptake of soil solutes or by synthesis of metabolic (compatible) solutes. However, the type of

solutes accumulated varies between plant species. A major category of organic osmotic solutes which consists of simple sugars (mainly fructose and glucose), sugar alcohols (glycerol and methylated inositols) and complex sugars (trehalose, raffinose and fructans) are accumulated (Bohnert and Jensen, 1996). Change of external osmolarity, and osmotic balance which support continued water influx (Hasegawa et al., 2000), protection of cell structures against oxidative damage and maintaining the structure of proteins and membranes are the main functions of compatible solutes (Hajihashemi et al., 2006). Same observation was reported by Sidari et al., (2008) and Thakura and Dev-Sharma (2005) in their study of the effects of salt stress on different varieties of lentil and sorghum, respectively.

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REFERENCES

Abrol DP (1988). Effect of climatic factors on pollination activity of Alfalfa-pollinating subtropical bees *Megachile nana* Bingh and *Megachile flavipes* Spinola (Hymnoptera: Megachilidae). Acta. Oecologia Genera, 9: 371-377.

Ahmad J, Bano M (1992). The effect of sodium chloride on physiology of cotyledons and mobilization of reserved food in *Cicer arietinum*. Pak. J. Bot. 24: 40-48.

Asada K (1992). Ascorbate peroxidase-a hydrogen scavenging enzyme in plants. Physiol. Plant, 85: 235-241.

Asada K, Takahashi M (1987). Production and scavenging of active oxygen in photosynthesis, in: Kyle DJ, Osmond CD, Arntzen, CJ (Eds.), Photoinhibition. Elsevier Sci. Publishers, Amsterdam, pp. 227-287.

Ashraf M, Harris PJC (2004). Potential biochemical indicators of salinity tolerance in plants. Plant Sci. 166: 3-16.

Bajaj S, Targolli J, Liu LF, Ho THD, Wu R (1999). Transgenic approaches to increase dehydration-stress tolerance in plants. Mol. Breed, 5: 493-503.

Bohnert HJ, Jensen RG (1996). Strategies for engineering water-stress tolerance in plants. Trends Biotechnol. 14: 89-97.

Bueno P, Piqueras A, Kurepa J, Savoure A, Verbruggen N, Montagu VM, Inze D (1998). Expression of antioxidant enzymes in responses to abscisic acid and high osmoticum in tobacco BY-2 cell cultures. Plant Sci. 138: 27-34.

Chance B, Maehly SK (1955). Assay of catalase and peroxidase. Methods Enzymol. 2: 764-775.

Chandlee JM, Scandalios JG (1984). Analysis of variants affecting the catalase developmental program in maize scutellum. Theor. Appl. Genet. 69: 71-77.

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

Comba ME, Benavides MP, Gallego SM, Tomaro ML (1998). Relationship between nitrogen fixation and oxidative stress induction in nodules of salt-treated soybean plants. Phyton-International J. Exp. Bot. 60: 115-126.

Delgado MJ, Garrido JM, Ligero F, Lluch C (1993). Nitrogen fixation and carbon metabolism by nodules and bacteroids of pea plants under sodium chloride. Physiol. Plant, 89: 824-829.

Diego AM, Marco AO, Carlos AM, Jose C, (2003). Photosynthesis and activity of superoxide dismutase peroxidase and glutathione reductase in cotton under salt stress. Environ. Exp. Bot. 49: 69-76.

Dionisio-Sese ML, Tobita S (1998). Antioxidant responses of rice seedlings to salinity stress. Plant Sci. 135: 1-9.

Ehsanpour AA, Fatahian N (2003). Effects of salt and proline on Medicago sativa callus, Plant Cell Tissue Org. 73: 53-56.

Elsheikh EAE, Wood M (1995). Nodulation and N_2 fixation in soybean inoculated with salt-tolerant rhizobia or salt-sensitive bradyrhizobia in saline soil. Soil Biol. Biochem. 27: 657-661.

Foyer CH, Halliwell B (1976). Presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta, 133: 21-25.

Gaspar T, Penel C, Castillo FJ, Greppin H (1985). A two step control of basic and acid peroxidases and its significance for growth and development. Plant Physiol. 64: 418-423.

Georgiev GI, Atkins CA (1993). Effects of salinity on N_2 fixation, nitrogen metabolism and export and diffusive conductance of cowpea root nodules. Symbiosis, 15: 239-255.

Giannopolitis CN, Ries SK (1977). Superoxide dismutase: I. Occurrence in higher plants. Plant Physiol. 59: 309-314.

Guan B, Zhou D, Zhang H, Tian Y, Japhet W, Wang P (2009). Germination responses of Medicago ruthenica seeds to salinity, alkalinity, and temperature. J. Arid Environ. 73: 135-138

Gueta DY, Yaniv Z, Zilinskas BA, Ben-Hayyem G (1997). Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in citrus. Planta, 203: 460-469.

Guy CL, Carter JV (1984). Characteristic of partially purified glutathione reductase from cold-hardened and non-hardened spinach leaf tissue Cryobiol. 21: 454-464.

Hajihashemi S, Kiarostami K, Enteshari S, Saboora A (2006). The effects of salt stress and paclobutrazol on some physiological parameters of two salt tolerant and salt sensitive cultivars of wheat. Pak. J. Biol. Sci. 9: 1370-1374.

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000). Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51: 463-499.

Heath RL, Packer L (1968). Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125: 189-198.

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

Jain M, Mathur G, Koul S, Sarin NB (2001). Ameliorative effects of proline on salt stress-induced lipid peroxidation in cell lines of groundnut (*Arachis hypogea* L.). Plant Cell Rep. 20: 463-468.

Khan MA, Rizvi Y (1994). Effect of salinity, temperature and growth regulators on the germination and early seedling growth of *Atriplex griffithii* var, Stocksii. Can. J. Bot. 72: 475-479.

Khan MA, Ungar IA (1984). The effect of salinity and temperature on the germination of polymorphic seeds and growth of *Atriplex triangularis* Willd. Am. J. Bot. 71: 481-489.

Koca H, Ozdemir F, Turkan I (2010). Effect of salt stress on chlorophyll fluorescence, lipid peroxidation, superoxide dismutase and peroxidase activities of cultivated tomato (*L. esculentum*) and its wild relative (*L. pennellii*). Environ. Exp. Bot. (in press).

Lee DH, Kim YS, Lee CB (2001). The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). J. Plant Physiol. 158: 737-745.

Lopez F, Vansuyt G, Casse-Delbart F, Fourcroy P (1996). Ascorbate peroxidase activity not the mRNA level, is enhanced in salt stressed *Raphanus sativus* plants. Physiol. Plant. 97: 13-20.

Martinez CA, Loureiro ME, Oliva MA, Maestri M (2001). Differential responses of superoxide dismutase in freezing resistant *Solanum*

curtilobum and freezing sensitive *Solanum tuberosum* subjected to oxidative and water stress. Plant Sci. 160: 505-515.
Moran JF, Becana M, Iturbe-Ormaetxe I, Frechilla S, Klucas RV, Aparicio-Tejo P (1994). Drought induces oxidative stress in pea plants, Planta, 194: 346-352.
Munns R (2002). Comparative physiology of salt and water stress. Plant Cell Environ. 25: 239-50.
Munns R (2005). Genes and salt tolerance: bringing them together. New Phytol. 167: 645-663.
Nakano Y, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: 867-880.
Naureen G, Naqvi FN (2010). Salt tolerance classification in wheat genotypes using reducing sugar accumulation and growth characteristics. Emir. J. Food Agric. 22(4): 308-317.
Navari-Izzo F, Quatacci MF, Sgherri CLM (1996). Superoxide generation in relation to dehydratation and rehydration. Biochem. Soc. 24: 447-450.
Nelson N (1944). A photometric adaptation of the somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.
Olmos E, Hernandez JA, Sevilla F, Hellin E (1994). Induction of several antioxidant enzymes in the selection of salt tolerant cell line of *Pisum sativum*. J. Plant Physiol.144: 594-598.
Patanea C, Cavallaroa V, Cosentinob SL (2009). Germination and radicle growth in unprimed and primed seeds of sweet sorghum as affected by reduced water potential in NaCl at different temperatures. Ind. Crop Prod. 30: 1-8.
Perez–Alfocea F, Larher F (1995). Effects of phlorizin and p–chloromercuribenzenesulfonic acid on sucrose and praline accumulation in detached tomato leaves submitted to NaCl and osmotic stresses. J. Plant Physiol. 145: 367-73.
Pessarakli M (1993). Handbook of plant and crop stress, first ed. CRC Press, Boca Raton, London, New York.
Scandalios JG (1993). Oxygen stress and superoxide dismutase. Plant Physiol. 101: 7-12.
Seki M, Kamei A, Yamaguchi-Shinozaki K, Shinozaki K (2003). Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr. Opin. Biotechnol. 14: 194-9.
Serraj R, Sinclair TR (2002). Osmolyte accumulation: can it really help increase crop yield under drought conditions?. Plant Cell Environ. 25: 333-341.
Shalata A, Tal M (1998). The effect of salt stress on lipid peroxidation and antioxidants in the cultivated tomato and its wild salt tolerant relative *Lycopersicon pennellii*. Physiol. Planta. 104: 169-174.
Sidari M, Santonoceto C, Anastasi U, Preiti G, Muscolo A (2008). Variations in four genotypes of lentil under NaCl-salinity stress. Am. J. Agric. Biol. Sci. 3(1): 410-416.
Smirnoff N (1995). The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol. 125: 27-58.
Stewart CR (1971). Effect of wilting on carbohydrates during incubation of excised bean leaves in the dark. Plant Physiol. 48: 792-794
Tejera NA, Campos R, Sanjuan J, Lluch C (2004). Nitrogenase and antioxidant enzyme activities in *Phaseolus vulgaris* nodules formed by Rhizobium tropici isogenic strains with varying tolerance to salt stress. J. Plant Physiol. 161: 329-338.
Tejera NA, Soussi M, Lluch C (2006). Physiological and nutritional indicators of tolerance to salinity in chickpea plants growing under symbiotic conditions, Environ. Exp. Bot. 58: 17-24.
Tester M, Davenport R (2003). Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot. 91: 503-527.
Thakur M, Dev-Sharma A (2005). Salt stress and phytohormone (ABA) induced changes in germination, sugars and enzymes of carbohydrate metabolism in *Sorghum bicolor* (L.) Moench Seeds. J. Agric. Soc. Sci. 1(2): 89-93.
Tijen D, Ismail T (2005). Comparative lipid peroxidation, antioxidant defense systems and praline content in roots of two rice cultivars differing in salt tolerance. Environ. Exp. Bot. 53: 247-257.
Wang BW, Kim YH, Lee HS, Kim KY, Deng XP, Kwak SS (2009). Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. Plant Physiol. Biochem. 47: 570-577.
Wang FZ, Wanga QB, Kwon SY, Kwak SS, Su WA (2005). Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. J. Plant Physiol. 162: 465-472.
Wang J, Zhang H, Allen RD (1999). Overexpression of an *Arabidopsis* peroximal ascorbate gene increases protection against oxidative stress. Plant Cell Physiol. 40: 725-732.
Wang W, Vinocur B, Altman A (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 218: 1-14.
Wang XS, Han JG (2009). Changes of proline content, activity, and active isoforms of antioxidative enzymes in two alfalfa cultivars under salt stress. Agric. Sci. China. 8(4): 431-440.
Wang Z, Quebedeaux B, Stutte GW (1996). Partitioning of (^{14}C) glucose into sorbitol and other carbohydrates in apple under water stress. Aust. J. Plant Physiol. 23: 245-51.
Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van-Montagu M, Inze D, Van-Camp W (1997). Catalase is a sink for H_2O_2 and is indispensable for stress defense in C_3 plants. EMBO J. 16: 4806-4816.
Yupsanis T, Moustakas M, Domiandou K (1994). Protein phosphorylation– dephosphorylation in alfalfa seeds germination under salt stress. J. Plant Physiol. 143, 234-240
Zhang J, Jia W, Yang J, Ismail AM (2006). Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Res. 97: 111-119.