ANTIOXIDANT RESPONSE OF TWO SALT-STRESSED BARLEY VARIETIES IN THE PRESENCE OR ABSENCE OF EXOGENOUS PROLINE

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Summary. The antioxidant response to salt stress as well as the effect of proline metabolism on the activity of antioxidant enzymes were investigated in two barley varieties (*Hordeum vulgare* L. var. Sahand and var. Makoui) after exposure to salt stress and exogenous proline. The MDA content of Makoui plants grown under different salt regimes remained nearly constant but it strongly increased in Sahand plants under the same conditions. There was a linear and significant increase in CAT, APX, DHAR, and GR activities in Makoui plants in response to increased salt concentration. The strong and positive correlation between antioxidant enzymes and salt concentrations may account for the MDA level in Makoui plants remaining constant in response to different salt regimes. The MDA level in Sahand plants grown at 150 and 200 mM NaCl decreased significantly in response to exogenous proline. This decrease was independent of SOD and APX activities. At the same time, there was a significant decrease in DHAR activity and an increase in GR activity under the same conditions. These results show that proline is able to quench oxygen radicals not only chemically but also by its effect on the activity of DHAR and GR. The PDH activity in Makoui variety decreased significantly in response to 150 mM NaCl and disappeared in plants grown at 200 mM NaCl. The PDH activity in Sahand plants was very high under all salt regimes. Sahand plants may have an active proline cycle, though highly active proline cycling in plants is usually detrimental during stressful

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conditions. Some other plants accumulate proline in stressful conditions, as in Makoui variety, through an increase in its synthesis concomitant with the inhibition of its catabolism, which subsequently leads to higher tolerance in salt stress condition.

Key words: salt stress, oxidative stress, antioxidant enzymes, proline, barley.

Abbreviations: APX - ascorbate peroxidase, CAT - catalase, DHA - dehydroascorbic acid, DHAR - dehydroascorbate reductase, GR - glutathione reductase, GSH - reduced glutathione, GSSG - oxidized glutathione, MDA - malondialdehyde, PDH - proline dehydrogenase, ROS - reactive oxygen species, SOD - superoxidedismutase.

INTRODUCTION

Salt-induced oxidative stress is one of the most important factors that affect plants. Chloroplasts are a major source of ROS in plants (Asada and Takahashi, 1987; Foyer et al., 1994). Superoxide radicals (O_2^-) are the first activated O_2 molecules that are formed by photo reduction of O_2 at PSI and PSII in chloroplasts. These O_2^- radicals are converted to H_2O_2 by SOD catalyzing dismutation. Mitochondria and peroxisomes are also potential sources of superoxide radicals. Electron transport and enzymatic reactions generate most of the O_2^- in these subcellular compartments (del Rio et al., 1992). Energy transferred to O_2 from triplet excited state chlorophyll produces singlet O_2. Hydroxyl radicals (OH^o) that can originate, in turn, by the metal-catalyzed Harber-Weiss reaction from superoxide and H_2O_2, are very harmful and can rapidly react with all types of bio-molecules, such as DNA, proteins and lipids, leading to radical chain processes, cross linking, peroxidation, membrane leakage, production of toxic compounds and finally cell death (Halliwell and Gutteridge, 1989; Davies, 1995). MDA that originates as a result of lipid peroxidation has been widely used to assess the free radicals level in living cells (Kunert and Edere, 1985). To keep these harmful reactions to a minimum, plant cells should be able to remove these activated O_2 radicals promptly. Plants use different enzymatic and non-enzymatic antioxidant defense systems to protect biomolecules against oxygen radicals (Bowler, and Van Montagu, 1992; Willekens et al., 1997; Asada, 1999). SOD, which catalyzes the dismutation of O_2^- radicals to molecular O_2 and H_2O_2, plays a key role in this defense mechanism (Fridovich, 1986). Catalase and various peroxidases remove H_2O_2 (Asada, 1994). The first step of the ascorbate-glutathione cycle, which removes H_2O_2, is catalyzed by APX (Foyer and Halliwell, 1976; Asada, 1994). DHAR also participates in the cycle (Foyer et al., 1994). Finally, GR which catalyzes the reduction of GSSG to GSH, plays a crucial role in the ascorbate-glutathione cycle and in producing more GSH

which is an important compound in stress tolerance in plants. In higher plants GR has been suggested to be a rate-limiting enzyme in the anti-oxidative defense system (Foster and Hess, 1980; May and Leaver, 1993; Gossett et al., 1994a; Tanaka, 1994; Gossett et al., 1996).

Proline accumulates in higher plants in response to various biotic and abiotic stresses, such as water deficit and salinity stress (Stewart and Lather, 1980; Thompson, 1980; Stewart, 1981; Hanson and Hitz, 1982; Rhodes, 1987; Delauney and Verma, 1993; Samaras et al., 1995; Taylor, 1996; Rhodes et al., 1999). Proline plays a major role in the anti-oxidative stress as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989; Matysik et al., 2002), regulation of NAD^+/NADH ratio (Alia and Saradhi, 1993), and as a protein-compatible hydrotrope (Srinivas and Balasubramanian, 1995). Proline also protects membranes and proteins against the effects of high concentrations of inorganic ions (Gibson et al., 1984; Rudolph et al., 1986). It has been reported that the higher accumulation of proline could be due to inhibition of proline catabolizing enzymes, such as proline oxidase and proline dehydrogenase (PDH), (Kandpal et al., 1981; Delauney and Verma, 1993). Proline dehydrogenase may act as pyroline-5-carboxylate reductase in the synthesis of proline and catalyzes the reaction with the same reactants and co-enzymes, but operating in an opposite direction (Huang and Cavalieri, 1979). On the other hand, proline accumulation may not be regarded as a marker for salt tolerance as it accumulates under various conditions of stress, such as high temperature, drought and starvation (Storey and Wyn-Jones, 1975), although it has been widely noted that proline accumulation can be used as a salt stress parameter (Ramanjulu and Sudhakar, 2001; Madan et al., 1995). In many plants under salt stress its level decreases (Naik and Joshi, 1983; Siddiqui and Krishnamoorthy, 1987). Some researchers believe that there is no any appreciable increase in free proline content (Dix and Pearce, 1981; Jain and Dhawan, 1987) whereas others have shown that enhanced proline level merely reflects a stress effect rather than being a cause of stress to tolerance (Moftah and Michel, 1987). Recently, it has been reported that proline accumulation appears to be due to a reaction to salt stress damage and not a mechnism for increased salt tolerance (de Lacerda et al., 2003). Therefore, the role of proline accumulation and its metabolism in respect of tolerance to salinity needs to be critically considered. In this research, the activities of anti-oxidative enzymes as well as the effect of exogenous proline on the activities of these enzymes in two barley varieties under different salinity regimes were studied.

MATERIALS AND METHODS

Plant material and treatments

Barley (*Hordeum vulgare* L. var. Sahand and var. Makoui) seeds were surface sterilized and grown in pots containing pre-washed sand and silica mix. Plants were watered every second day using one-half-strength Hoagland nutrient solution without and with different NaCl concentrations (100, 150, and 200 mM) in a growth room. Approximately 20 days after sowing, plants were divided randomly into six groups subjected to different treatments for 14 days. Leaves to be used for biochemical determinations were frozen and stored in liquid nitrogen immediately after harvest until enzyme extraction. For exogenous proline treatments, plants were grown in the same nutrient solution additionally supplemented with 5 mM L-proline.

Measurements of proline and MDA contents

Free proline concentration was determined according to the procedure used by Bates et al. (1973). MDA content was measured using a 2-thiobarbituric acid reaction (Heath and Packer, 1968).

Enzyme extraction and assay

Samples were prepared for SOD, CAT, APX, and GR analyses by the method of Foster and Hess (1980) as modified by Gossett et al. (1994b). DHAR was extracted using to the method of Dalton et al. (1993). Catalase activity was determined by monitoring the disappearance of H_2O_2 using the method of Beers and Sizer (1952). Total SOD activity was measured by determining the amount of enzyme required to produce 50% inhibition of the reduction of Cyt c by superoxide generated by xanthine oxidase, as described by Forman and Fridovich (1973). GR activity was determined by monitoring the glutathione-dependent oxidation of $NADPH^+H$, as described by Schaedle and Bassham (1977). APX activity was assayed by monitoring the ascorbic acid dependent reduction of H_20_2, as described by Anderson et al. (1992). DHAR was determined using the method of Dalton et al. (1993) and the reaction rate was corrected for the nonenzymatic reduction of DHA by GSH. For CAT APX, 1 unit of enzyme was defined as the amount necessary to decompose 1 nmol substrate/min at 25°C; 1 unit of GR and DHAR was defined as the amount of enzyme required to reduce 1 nmol substrate/min at 25°C; and 1 unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of Cyt c by 50%. Data presented are the means from a minimum of three replicates. All data were subjected to ANOVA test and significance was determined at the 95% confidence limits. Proline dehydrogenase was extracted in 100 mmol/l phosphate buffer (pH 8.0) containing 1.0 mmol/l cystine and 0.1 mmol/l EDTA and was assayed by following NAD^+ reduction in 1.0

ml of reaction medium containing 100 mmol/l $Na_2CO_3/NaHCO_3$ buffer (pH 10.3), 20 mmol/l l-proline and 10 mmol/l NAD^+ (Rena and Splittstoesser, 1975).

RESULTS

Proline and MDA contents

Proline accumulated with increasing the concentration of NaCl in the nutrient solution from 0 (control) to 200 mM in both varieties, the increase in Sahand being higher than in Makoui. In the presence of 150 mM NaCl proline level in Sahand plants was two times higher than in Makoui plants. Proline content of Sahand increased more than 10-fold and 13.6-fold in response to 150 and 200 mM NaCl, respectively. Proline accumulation in Makoui increased about 3-fold and 7-fold at 150 and 200 mM NaCl, respectively (Fig. 1).

MDA content in Sahand plants increased 2-fold in response to 150 and 200 mM NaCl. This was about 74% more than MDA content in Makoui plants grown at the same NaCl level, however, MDA production in Makoui grown at 0 and 100 mM

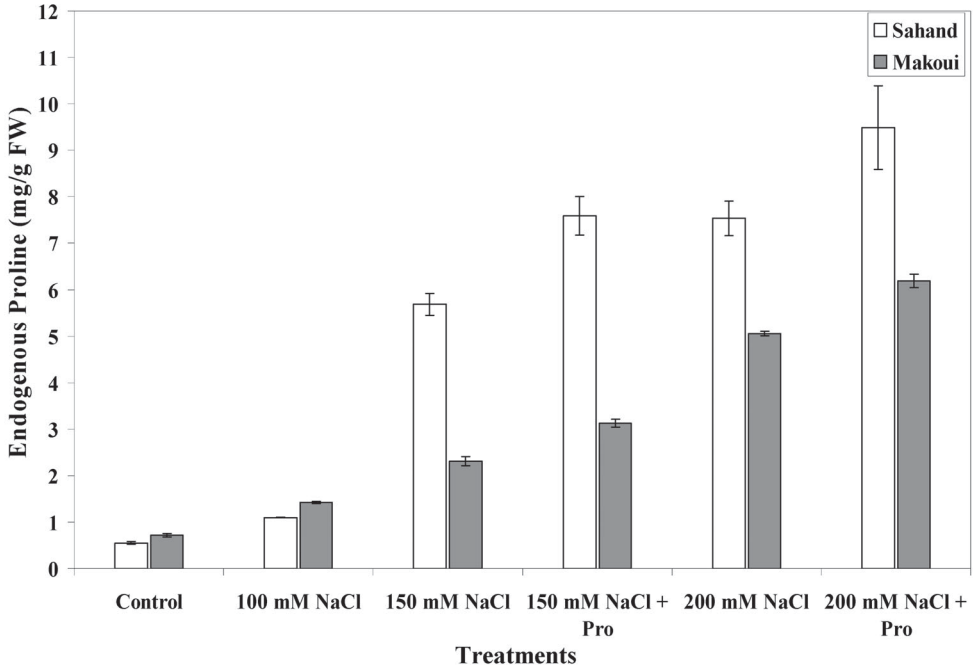

Figure 1. The effect of salt stress and exogenous proline (5 mM) on the endogenous proline content of barley plants. Values presented are the averages of three independent samples.

NaCl was significantly higher than in Sahand grown under the same conditions (Fig. 2). A decline in shoot fresh weight of Sahand was observed under different NaCl regimes. The degree of decline was dependent on the severity of stress (20% and 30% FW decrease at 150 and 200 mM NaCl, respectively). However, Sahand produced more shoot fresh weight compared to Makoui even at 200 mM NaCl (30% more FW at 200 mM NaCl). MDA content and shoot fresh weight of Makoui grown at different NaCl concentrations remained nearly constant (Fig 2, 3).

Activity of antioxidant enzymes

Both varieties grown at various NaCl concentrations showed similar changes in SOD activity. SOD activity increased significantly in both varieties in response to 150 mM NaCl and the increase was greater in Makoui (less than 1.9-fold in Sahand and more than 2-fold in Makoui). Both varieties grown at 100 mM NaCl exhibited a slight but significant decrease in SOD activity, however, severe salt stress (200 mM NaCl) had no significant effect on the enzyme activity.

In Sahand plants CAT activity remained constant following various NaCl treatments but APX activity increased more than 3-fold when NaCl concentration increased from 0 to 200 mM. CAT activity in Makoui plants increased significantly

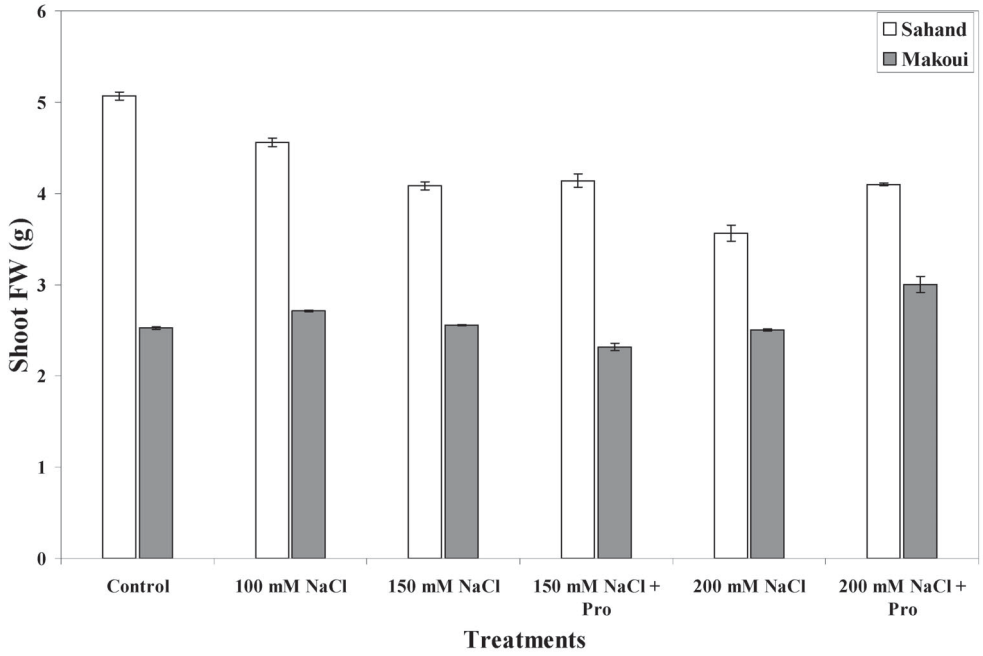

Figure 2. The effect of salt stress and exogenous proline (5 mM) on MDA content of barley plants. Values presented are the averages of three independent samples.

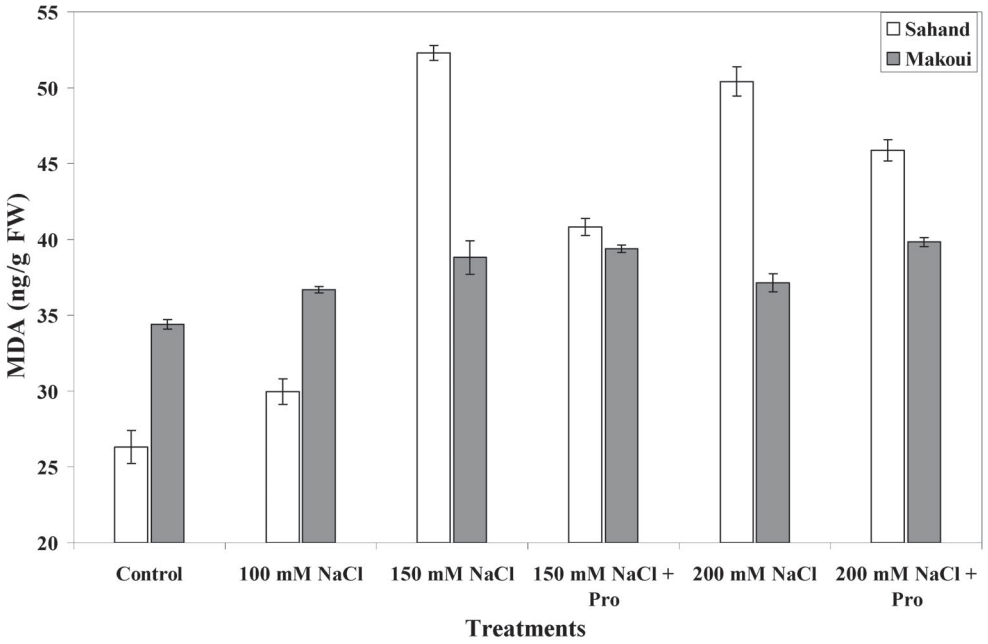

Figure 3. The effect of salt stress and exogenous proline (5 mM) on the shoot fresh weight of barley plants. Values presented are the averages of three independent samples each containing about 5 plants.

with increasing NaCl concentration and plants grown at 150 mM NaCl showed higher CAT activity (more than 2-fold increase compared to control).

There was a slight increase in APX activity of Makoui plants in response to increasing NaCl concentrations and its activity was 1.9-fold higher in the presence of 200 mM NaCl compared to control. This treatment showed the lowest CAT activity compared with the other salt treatments applied.

A linear increase in the activity of the ascorbate-glutathione cycle enzymes, DHAR and GR was observed in Makoui at all stress levels and the increase in the activity of these enzymes was proportional to the severity of stress. Further, the increase was higher in Makoui plants grown at 200 mM NaCl (more than 8-fold and 2.5-fold reduction in DHAR and GR activity, respectively). Activity of DHAR and GR was also significantly elevated in Sahand in response to NaCl, but there was no correlation between the activity of these enzymes and the stress level. A more pronounced increase was observed in Makoui plants. In Sahand plants the highest and the lowest increase in DHAR was observed at 100 and 150 mM NaCl, respectively. Plants grown at 200 mM NaCl showed less increase (less than 20% increase compared to control). The highest reduction in GR activity in Sahand plants was observed at 150 mM NaCl (more than 2-fold) (Fig. 4).

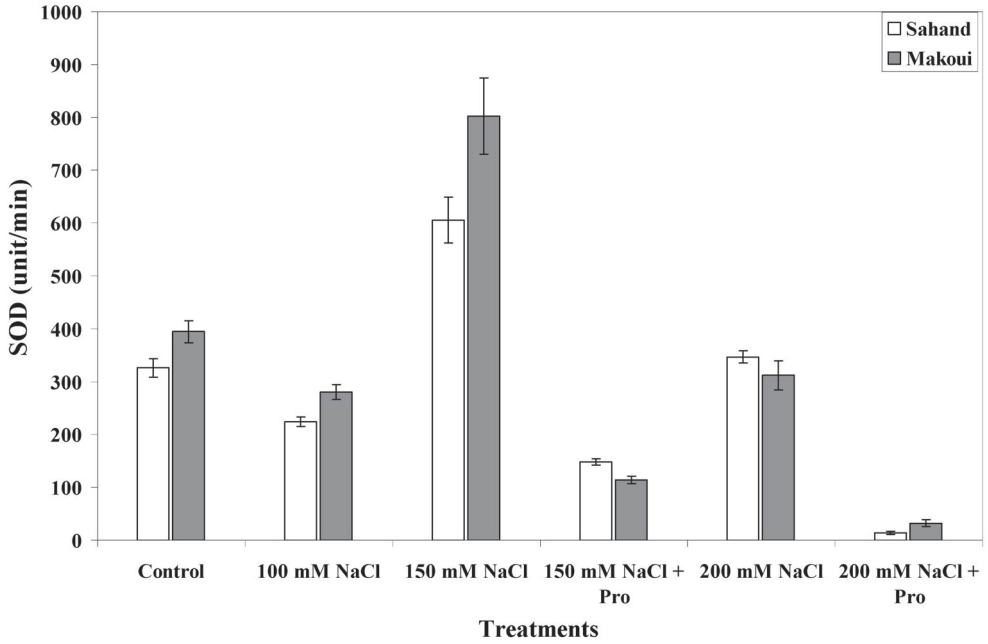

Figure 4a.

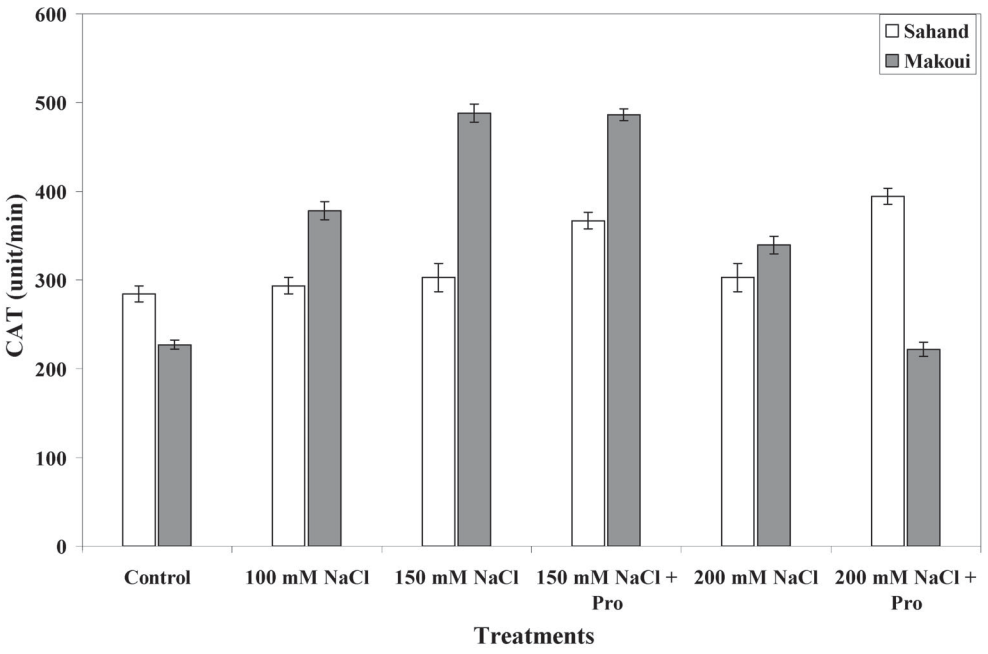

Figure 4b.

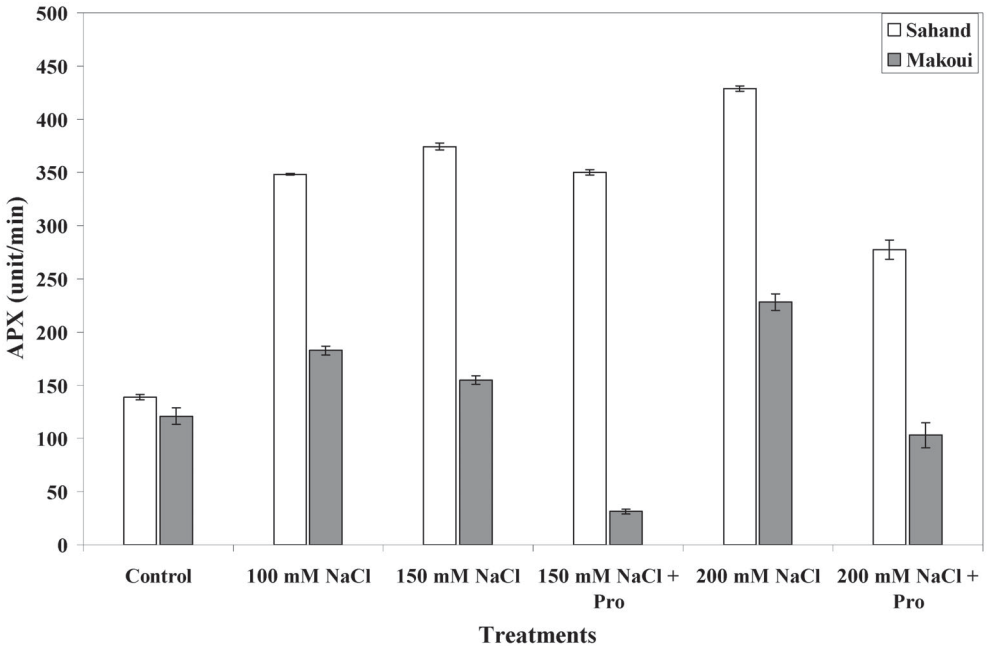

Figure 4c.

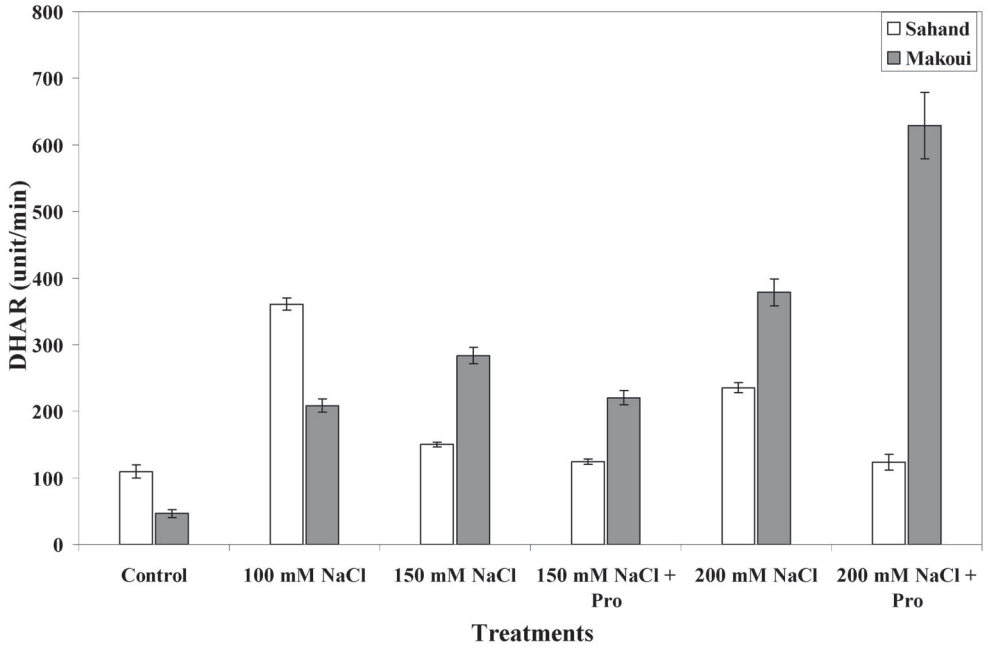

Figure 4d.

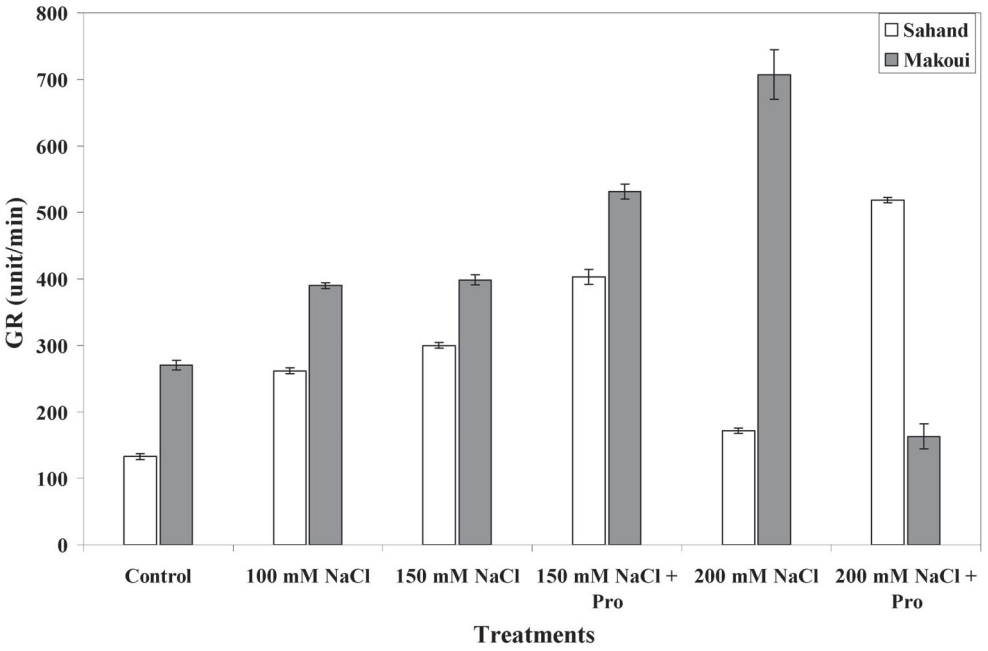

Figure 4e. The effect of salt stress and exogenous proline (5 mM) on the activity of antioxidant enzymes of barley plants. Values presented are the averages of three independent samples.

PDH activity

No PDH activity was found in Sahand plants under optimum growing conditions (control), but its activity strongly increased in response to 100 mM NaCl and remained at high levels at the different salt concentrations applied. PDH was already active in Makoui plants under control conditions. Its activity increased slightly in response to 100 mM NaCl, but it decreased 5-fold and then disappeared in the presence of 150 and 200 mM NaCl, respectively (Fig, 5).

The effect of exogenous proline on proline and MDA contents

Plants grown at 150 and 200 mM NaCl, either with or without 5 mM exogenous proline were used to study the effect of exogenous proline on oxidative stress and the activities of anti-oxidative enzymes. Proline content of both varieties grown at 150 and 200 mM NaCl with exogenous proline was significantly higher than the control (plants grown at same salt level without exogenous proline). There was 33% and 26% increase in proline content in response to exogenous proline in Sahand plants grown at 150 and 200 mM NaCl, respectively. In Makoui plants grown under the same conditions this increase was 36% and 22%, respectively (Fig. 1).

Exogenous proline did not show any significant effect on growth rate of both varieties with 150 mM NaCl, but there was a slight increase in growth rate of both of

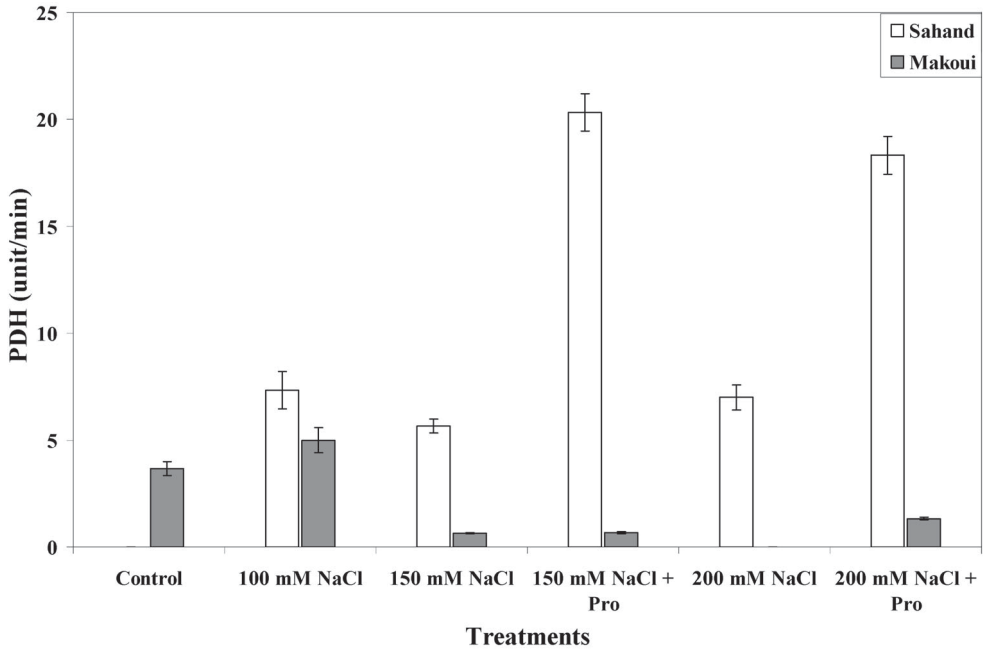

Figure 5. The effect of salt stress and exogenous proline (5 mM) on the activity of PDH of barley plants. Values presented are the averages of three independent samples.

them when grown on 200 mM NaCl with exogenous proline (15% and 20% in Sahand and Makoui, respectively) (Fig. 3). Exogenously applied proline affected MDA production in salt stressed Sahand plants. The addition of proline resulted in 22% and 9% decreases in MDA production in Sahand plants grown at 150 and 200 mM NaCl levels, respectively. No significant difference in MDA content was observed when proline was exogenously applied to Makoui plants grown at 150 mM NaCl. MDA production slightly increased (7%) when Makoui plants grown at 200 mM NaCl were treated with proline (Fig. 2).

The effect of exogenous proline on the activities of antioxidant enzymes

Exogenous proline had a strong effect on SOD activity in both varieties. In response to exogenous proline SOD activity in Sahand plants grown at 150 and 200 mM NaCl decreased to 25% and 4%, respectively. There was a similar decline in SOD activity in Makoui plants (14% and 10%) under the same conditions. In Sahand plants grown at 150 mM NaCl exogenous proline had a moderate effect on CAT (21% increase) and APX (7% decrease) activities. Under severe stress conditions (200 mM NaCl) it was more effective on CAT (31% increase) and APX (35% decrease) activities compared to their respective controls. No significant difference in CAT activity was observed in Makoui plants grown at 150 mM NaCl in response to exogenous proline,

but under these conditions APX activity decreased to 20%. In plants grown at 200 mM NaCl the addition of proline caused a decrease in CAT and APX activities by 34% and 55%, respectively.

The addition of proline caused a decrease in DHAR activity in Sahand and Makoui plants by 20% and 22%, respectively at 150 mM NaCl. However, under these conditions GR activity in both varieties significantly increased (by 34% and 33% in Sahand and Makoui, respectively). DHAR activity in Sahand plants grown at 200 mM NaCl decreased to 48% when they were subjected to exogenous proline in contrast to a 3-fold increase observed in GR activity under these conditions. On the other hand, there was an increase in DHAR activity (66%) and a greater decline in GR activity (4.3-fold) in Makoui plants at the same NaCl concentration and proline treatments (Fig. 4).

The effect of exogenous proline on PDH activity

Exogenous proline induced strongly the activity of PDH in Sahand plants by 3.5-fold and 2.5-fold in the presence of 150 and 200 mM NaCl, respectively. There was no change in the PDH activity in Makoui plants grown at 150 mM NaCl in response to exogenous proline. The activity of PDH in Makoui plants grown at 200 mM NaCl was very low in the presence of exogenous proline (Fig. 5).

DISCUSSION

Abiotic stresses like drought and salinity cause osmotic stress and ionic imbalance in addition to inducing oxidative stress (Hasegawa et al., 2000). Plants use their available defense system to neutralize oxidative stress by scavenging excess ROS. Several reports demonstrate an enhanced activity of various antioxidant enzymes under stressful conditions like salt, drought and heavy metal damage (Jiang and Zhang, 2002; Patsikka et al., 2002; Demiral and Türkan, 2005). On the other hand, the accumulation of low molecular weight solutes and/or compatible osmolytes may help to maintain the relatively high water content necessary for plant growth by balancing the osmotic pressure of cytosol with that of vacuole and external environment. The most conspicuous of these osmolytes are amino acids in general and proline in particular (Gadallah, 1999; Hellebust, 1976; Greenway and Munns, 1980).

The present study aimed at investigating the response of Hordeum vulgare L. var. Sahand and var. Makoui to salt stress. In moderate salt stress condition (100 mM NaCl) the MDA level of Sahand was lower than in Makoui. Under these conditions APX and DHAR activities in Sahand plants were not only higher than the control but were also higher than the respective activities in Makoui. Sahand plants possibly responded to moderate salt stress by elevating APX, DHAR, and GR activities. However, when the salt level increased to 150 mM, APX and GR activities of Sahand did

not show significant changes and DHAR activity decreased meaningfully compared to the activities at 100 mM NaCl. As a result of these changes in the enzyme activities, MDA production in Sahand plants dramatically increased in response to 150 mM NaCl. Severe salt stress (200 mM NaCl) did not cause significant changes in MDA production in Sahand, but resulted in a moderate increase in APX activity and a significant induction in DHAR activity compared to 150 mM NaCl.

There was a linear and significant increase in CAT, APX, DHAR, and GR activities in Makoui plants in response to increased salt concentration. The strong and positive correlation between antioxidant enzymes and salt concentrations may account for the MDA level in Makoui plants remaining constant in response to different salt regimes. These results suggest that the correlated increase in the activities of anti-oxidative enzymes is very important in salt tolerance. In the present work, lower MDA induction observed in Makoui variety suggested better protection against oxidative damage compared to Sahand variety. The higher level of protection in Makoui variety seemed to result from the more effective antioxidative system, whereas significant increases in MDA levels in Sahand variety appeared to be derived from decreased activities of the enzymes. There is a good agreement between our findings and the results of Shalata and Tal (1998) and Bor et al. (2003). They found a correlation between decreased lipid peroxidation and increased antioxidant enzyme activities in salt tolerant tomato and wild beet, respectively, under salt stress conditions. In addition, significantly greater constitutive levels of catalase and NaCl-induced levels of APX and GR were present in leaves of NaCl tolerant cultivars. Callus from these salt-tolerant cultivars showed significant increases in SOD, catalase, APX, and GR activities in response to salt stress (Gossett et al., 1994a,b). Therefore, they suggested that a strong correlation exists between antioxidant levels and NaCl tolerance. SGH is one of the most important antioxidants in plants and plays a crucial role in stress tolerance and defense against active oxygen toxicity (Gossett et al., 1996; Tanaka, 1994). Maintaining sufficient GSH pools is very important in oxidative stress tolerance. The GSH pool is controlled by GR and DHAR activities. It is possible that during NaCl –induced oxidative stress ascorbate levels increase at the upper end and glutathione levels decrease at the lower end of the ascorbate-glutathione cycle. The elevated GR activity keeps the GSH levels favorable for ascorbate reduction by DHAR (Gossett et al., 1996).

MDA level of Sahand plants grown at 150 and 200 mM NaCl decreased significantly in response to exogenous proline. In addition, there was a significant and similar decrease in SOD and APX activities in both Sahand and Makoui plants. A moderate increase in CAT activity was observed in Sahand plants whereas in Makoui plants a decline in its activity was registered (200 mM NaCl). These results showed that in the presence of exogenous proline the production of oxygen radicals decreased significantly and this decrease was independent from SOD and APX activities (as their activities decreased under proline treatment). Proline accumulates to high

amounts in plants under stress. Its high capability to quench singlet oxygen and hydroxy radicals can well be understood by its chemical properties. The pyrrolidine ring of proline has a remarkably low ionization potential (IP) and is therefore capable of quenching singlet oxygen effectively by forming a charge-transfer complex. Proline reacts with $OH^{\bullet}$ under hydrogen abstraction by forming the most stable radical, which carries the spin on the C-5 atom (Matysik et al., 2002).

Exogenous proline had different effects on DHAR and GR activities in both varieties. In response to exogenous proline there was a significant decrease in DHAR activity and an increase in GR activity in Sahand plants grown at 150 and 200 mM NaCl. The same response was observed in Makoui plants grown at 150 mM NaCl. MDA production in Makoui and Sahand plants beneficially decreased under these conditions. Only in Makoui plants grown at 200 mM NaCl, GR activity largely decreased and DHAR activity increased in the presence of exogenous proline. MDA levels slightly increased under these conditions. A decrease in DHAR activity and an increase in GR activity will possibly increase the GSH levels. These high levels of GSH help plants to deal with stress conditions (Tanaka, 1994; Gossett et al., 1996). Proline regulates the ratio of reduced and oxidized forms of pyridine nucleotide in plant cells (Alia and Saradhi, 1993). Exogenously applied proline possibly changes this ratio by changing proline metabolism in plant cells. This result is in agreement with other researches showing that exogenous application of proline caused a decrease in shoot Na^+ and Cl^- accumulation and thereby enhanced growth under saline conditions in cultured barley embryos (Lone et al., 1987). Proline concentration in many salt tolerant plants has been found to be higher than in salt sensitive ones. Petrusa and Winicov (1997) found that salt-tolerant alfalfa plants rapidly doubled their proline content in the roots whereas in salt sensitive plants the increase was slow. Similar results were also reported in alfalfa by Fougere et al. (1991). However, in some reports, the role of proline in osmoregulation and salt tolerance has generally been questioned (Lutts et al., 1996; Colmer et al., 1995) or has pointed to an opposite role for exogenous proline (Garcia et al., 1997).

The PDH activity of both varieties was measured under different conditions to determine whether there is a difference in proline catabolism between these two varieties. PDH activity in Makoui plants remarkably decreased in response to 150 mM NaCl and disappeared in the plants grown at 200 mM NaCl. This result agrees with previous studies that have shown a higher decrease in the PDH activity in the NaCl tolerant cultivars compared to sensitive plants (Sudhakar et al., 1993; Ashraf, 1994; Mattioni et al., 1997 and Giridara Kumar et al., 2003). The PDH activity in Sahand plants was very high under all salt regimes and they had higher proline levels compared to Makoui. The PDH activity in Sahand plants under salt stress conditions was significantly elevated in response to exogenous proline and the oxidative damage in these plants decreased. Previous studies on PDH showed that the addition of exogenous proline increased survival rates of salt-stressed PDH-S plants (e.g. in transgenic

Arabidopsis plants with high levels of PDH) by 30% (Mani et al., 2002). In the present study, there was no significant increase in PDH activity in Makoui plants in the presence of exogenous proline.

Under optimum growth conditions Sahand plants may have an active proline cycle. Active proline synthesis in chloroplasts and its catabolism in mitochondria by PDH can enable plants to use proline as a sink for energy to regulate redox potentials resulting in better growth under optimum conditions and during recovery after stress. However, it seems that a highly active proline cycle in plants is usually detrimental during stress conditions. In these cases, exogenous proline helps the plant to deal with the stress. It is possible that in the presence of exogenous proline plants can maintain proline metabolism at high levels without using more endogenous energy sources for proline synthesis. As in Makoui variety, some other plants accumulate proline in stress conditions through an increase in its synthesis, concomitant with inhibition of its catabolism (Giridara Kumar et al., 2003) and they are more tolerant under stress conditions. In plants where proline accumulates in lower amounts by repressing PDH, the radical scavenging and other properties of proline are more useful.

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