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THE EFFECT OF SALT STRESS ON ANTIOXIDANT ENZYMES' ACTIVITY AND LIPID PEROXIDATION ON THE WHEAT SEEDLING

Ezatollah Esfandiari[1], Fariborz Shekari[1], Farid Shekari[2], Manouchehr Esfandiari[1]

[1] Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Maragheh, Maragheh, Iran, e-mail: esfand1977@yahoo.com
[2] Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Zanjan, Zanjan, Iran; Corresponding author: Fb_Shekari@yahoo.com

Abstract. Salt stress as a major adverse factor can lower leaf water potential, leading to reduced turgor and some other responses, and ultimately lower crop productivity in arid and semi arid zones. Wheat is one of the main crops occupying a large area in Iran, where salt stress is the most limiting factor. Clearly, plant salt stress tolerance requires the activation of complex metabolic activities including antioxidative pathways, especially reactive oxygen species (ROS) and scavenging systems within the cells which can contribute to continued growth under water stress. In the work reported in this paper, the seeds of two local wheat cultivars (Alvand and Sardari) were grown hydroponically. Seedlings were subjected to Hoagland's solution as control, and 50, 100, 150 and 200 mM NaCl for 10 days. As a result, SOD (superoxide dismutase) increased in Sardari with the increase of salt stress, while in the case of Alvand, SOD showed constant activity at all salt stress levels. Meanwhile, CAT and GR exhibited the same trends in the two cultivars of wheat in salt stress conditions. Results indicated that in the case of Sardari, the scavenging of ROS by the scavenging system especially by SOD, CAT and GR was done well and damage to membranes or MDA was controlled. But in the case of Alvand, damage to membranes increased with the rise of stress levels. It can be concluded that all three antioxidant enzymes were limiting factors for this cultivar. Also these reasons led to the sensitivity of Alvand to salt stress.

Key words: antioxidant enzyme, malondialdehyde, salt stress, wheat

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important crops in Iran, which plays a special role in people's nutrition. But unfortunately abiotic stresses, such as salinity, decrease wheat growth and productivity by reducing water uptake and cause nutrient disorders and ion toxicity in this region.

Reactive oxygen species (ROS) are regarded as the main source of damage to cells under biotic and abiotic stresses (Candan and Tarhan, 2003; Bor et al., 2003; Gara at al., 2003; Mittler, 2002; Vaidyanathan et al., 2003). ROS's are partially reduced forms of atmospheric oxygen, which are produced in vital processes such as photorespiration, photosynthesis and respiration (Mittler, 2002; Uchida et al., 2002). To produce water in these processes, four electrons are required for perfect reduction of oxygen. But ROS typically results from the transference of one, two and three electrons, respectively, to O_2 to form superoxide ($O_2^{\bullet-}$), peroxide hydrogen (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) (Mittler, 2002). These species of oxygen are highly cytotoxic and can seriously react with vital biomolecules such as lipids, proteins, nucleic acid, etc, causing lipid peroxidation, protein denaturing and DNA mutation, respectively (Breusegem et al., 2001; Scandalios, 1993; Quiles and Lopez,

2004). Evidence suggests that membranes are the primary sites of salinity injury to cells and organelles (Candan and Tarhan, 2003) because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma or intracellular organelles (Karabal et al., 2003; Stewart and Bewley, 1980). Peroxidation of plasmalemma leads to the leakage of cellular contents, rapid desiccation and cell death. Intracellular membrane damage can affect respiratory activity in mitochondria, causing pigment to break down and leading to the loss of the carbon fixing ability in chloroplasts (Scandalios, 1993).

Fortunately, plants have developed various protective mechanisms to eliminate or reduce ROS, which are effective at different levels of stress-induced deterioration (Beak and Skinner, 2003).The enzymatic antioxidant system is one of the protective mechanisms including superoxide dismutase (SOD: EC 1.15.1.1), which can be found in various cell compartments and it catalyses the disproportion of two $O_2^{\cdot-}$ radicals to H_2O_2 and O_2 (Scandalios, 1993). H_2O_2 is eliminated by various antioxidant enzymes such as catalases (CAT: EC 1.11.1.6) (Kono and Fridovich, 1983; Scandalios, 1993) and peroxidases (POX: EC 1.11.1.7) (Gara et al., 2003; Jablonski and Anderson, 1982) which convert H_2O_2 to water. Other enzymes that are very important in the ROS scavenging system and function in the ascorbate-glutathione cycle are glutathione reductase (GR: EC1.6.4.2), monodehydro ascorbat reductase (MDHAR: EC 1.6.5.4) and dehydroascorbate reductase (DHAR: EC 1.8.5.1) (Candan and Tarhan, 2003; Yoshimura et al, 2000). Moreover, ROS are inevitable by-products of normal cell metabolism (Martinz et al., 2001). But under normal conditions production and destruction of ROS is well regulated in cell metabolism (Mittler, 2002). When a plant faces harsh conditions, ROS production will overcome scavenging systems and oxidative stress will burst. In these conditions, ROS attack vital biomolecules and disturb the cell metabolism and ultimately the cell causes its own death (Sakihama et al., 2002).

As mentioned before, salinity has the most important role in decreasing wheat growth and performance in Iran. On the other hand, wheat has a significant role in food security in our country. These reasons indicate the importance of research about salt stress tolerance mechanisms especially ROS scavenging systems in wheat. In this research two wheat cultivars were selected (Alvand and Sardari), which were cultivated in an extensive area in the aforementioned region, and were grown hydroponically. Wheat seedlings were treated with Hoagland's solution and NaCl treatments of 50, 100, 150 and 200 mM were applied, together with a control, for a period of 10 days. With the elapse of this period, the activity level of superoxide dismutase, catalase and glutathione reductase and lipid peroxidation was measured.

MATERIAL AND METHODS

Plant growth conditions

Seeds of wheat (*T. aestivum* L.) from two cultivars, Alvand and Sardari, were disinfected with 10% H_2O_2 for 20 min, washed thoroughly and then imbibed in distilled water for one day. After the imbibitions, approximately 20-25 seeds were planted onto plastic trays covered with cheesecloth containing half-strength Hoagland's solution. They were grown in a greenhouse (approximately 14h light/10 h dark) providing white fluorescent light (Pars, Iran) and natural light with an irradiance of 250 $\mu mol.m^{-2}.s^{-1}$, day/night temperature of 25±2°C/15±2°C and 60±5% relative humidity. The seedlings were grown in normal growth conditions until 4-5 leaf stage. These solutions were permanently aerated and renewed 2-3 times a week to minimize a pH shift and nutrient depletion. Then, seedlings were treated with Hoagland's solution containing 50, 100, 150 and 200 mM NaCl and maintained for 10 days in

these conditions. Control seedlings were kept in Hoagland solution without NaCl. After treatment for 10 days, the wheat seedlings were sampled and transferred to liquid nitrogen and maintained at -70°C until the measurement of variables under study.

Enzyme extraction

For SOD, CAT and GR extraction, leaf samples (0.5g) were homogenized in ice cold 0.1 M phosphate buffer (pH=7.5) containing 0.5 mM EDTA with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in Beckman refrigerated centrifuge for 15 min at 15000×g. The supernatant was used for enzyme activity assay (Esfandiari et al., 2007).

Enzyme activity assay

SOD activity was estimated by recording the decrease in absorbance of superoxide-nitro blue tetrazolium complex by the enzyme (Sen Gupta et al., 1993). About 3 ml of reaction mixture, containing 0.1ml of 200mM methionine, .01ml of 2.25mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1ml distilled water and 0.05 ml of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 ml riboflavin (60 μM) and placing the tubes below a light source of two 15 W florescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal colour. A non-irradiated complete reaction mixture which did not develop colour served as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

CAT activity was measured according to Aebi (1984). About 3 ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH=7), 0.5 ml of 75 mM H_2O_2, 0.05 ml enzyme extraction and distilled water to make up the volume to 3 ml. Reaction started by adding H_2O_2 and decrease in absorbance recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed.

APX activity was measured according to Yoshimura et al. (2000) by monitoring the rate of ascorbate oxidation at 290 nm ($E=2.8mM^{-1}cm^{-1}$). The reaction mixture contained 25 mM phosphate buffer (pH=7), 0.1 mM EDTA, 1 mM H2O2, 0.25 mM AsA and the enzyme sample. No change in absorption found in the absence of AsA in the test medium.

GR activity was assayed by recording the increase in absorbance in the presence of oxidized glutathione (GSSG) and 5,5-dithiobis-2-nitrbenzoic acid (DTNB) (Siram et al., 2002). The reaction mixture contained 1 ml of 0.2 M potassium phosphate buffer (pH=7.5) containing 0.1 mM EDTA, 0.5 ml of 3 mM DTNB in 0.01 M potassium phosphate buffer (pH=7.5), 0.1 ml of 2 mM NADPH, 0.1 ml enzyme extract and distilled water to make up a final volume of 2.9 ml. Reaction initiated by adding 0.1 ml of 2 mM GSSG. The increase in absorbance at 412 nm recorded at 25°C over a period of 5 min on a spectrophotometer.

Protein content of samples was determined by the Bradford method. Bovine serum albumin was used as a standard (Bradford, 1976).

Malondialdehyde (MDA) was measured by colorimetric method (Stewart and Bewley, 1980). 0.5 g of leaf samples were homogenized in 5ml of distilled water. An equal volume of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid solution was added and the sample incubated at 95°C for 30 min. The reaction stopped by putting the reaction tubes in the ice bath. The samples then centrifuged at 10000×g for 30 min. The supernatant removed, absorption read at 532 nm, and the amount of nonspecific absorption at 600 nm read and

subtracted from this value. The amount of MDA present calculated from the extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Enzyme activity and MDA content of samples were recorded with duplication. The data were analyzed with MSTATC and means compared with the LSD method.

RESULTS

SOD activity had different patterns in two cultivars. In Sardari, with the increase of NaCl content to Hoagland's solution, SOD activity increased at 50, 100 and 200 mM NaCl in comparison with the control. In this cultivar, there was a significant difference (P<5%) between the control and 50 mM NaCl at other levels. In Alvand, SOD activity at 50 mM increased severely in comparison with control. But its activity decreased in higher levels of NaCl content, namely 100, 150 and 200 mM. Between these levels, there was not a significant difference (P<5%) with that of control (Figure 1).

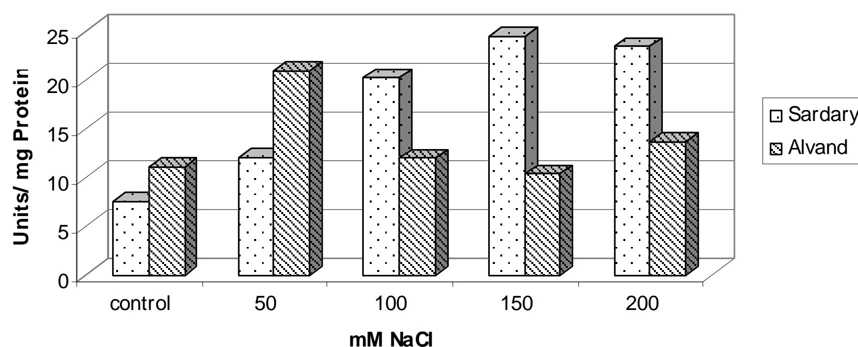

fig. 1. Effects of salt stress on superoxid dismutase activity in two wheat cultivars(LSD5% = 6.38).

Catalase almost had a similar pattern in two cultivars, Alvand and Sardari. Catalase activity was significantly increased in two cultivars at 50 mM NaCl in comparison with the control. But at higher levels of NaCl, i.e., 100, 150 and 200mM, catalase activity significantly decreased (P<5%) in comparison with 50 mM NaCl. Alvand had higher activity of catalase enzyme than Sardari (Figure 2) in addition to the one at 200 mM NaCl level,.

GR activity started to decrease with the rise of NaCl content in comparison with the control in Alvand. However, at 150 and 200 mM, NaCl showed significant difference (P<5%) with the control. Moreover, GR activity fallen at 50 and 100 mM NaCl was not significant (P<5%) relative to control. GR activity had the highest activity at 50 mM NaCl in Sardari. But at 100, 150 and 200 mM, GR activity decreased in comparison with the control and 50 mM NaCl. Furthermore, there is a significant difference (P<5%) between 50 mM with 150 and 200 mM NaCl (Figure 3).

The trends of MDA content in two cultivars of wheat were completely disparate (Figure 4). The amount of MDA rose with the increase in salt stress level in Alvand (Figure 4). However, the difference between control and 50 mM NaCl was not significant in Alvand

(P<5%). But lipid peroxidation remarkably increased when salt stress rose to 100, 150 and 200 mM NaCl. Furthermore, there was a significant difference (P<5%) between control and 50 mM NaCl with other levels of salt stress in Alvand. On the other hand, in this cultivar, MDA content at levels of 100, 150 and 200 mM NaCl showed a significant difference (P<5%) (Figure4). But Sardari had the same amount of MDA at all salt stress levels. Moreover, a significant difference between cultivars was observed at the level of up to 100 mM NaCl (p<0.5%) (Figure 4).

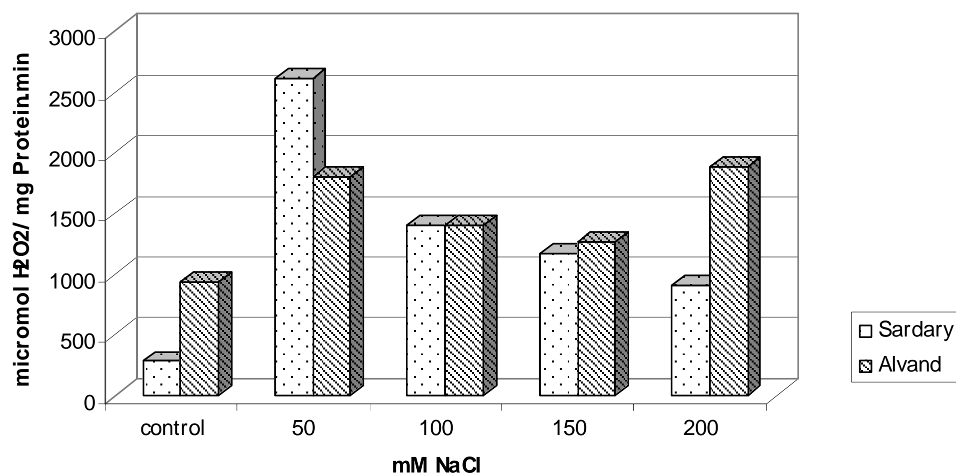

fig. 2. Effects of salt stress on catalase activity of two wheat cultivar (LSD5%=392.1).

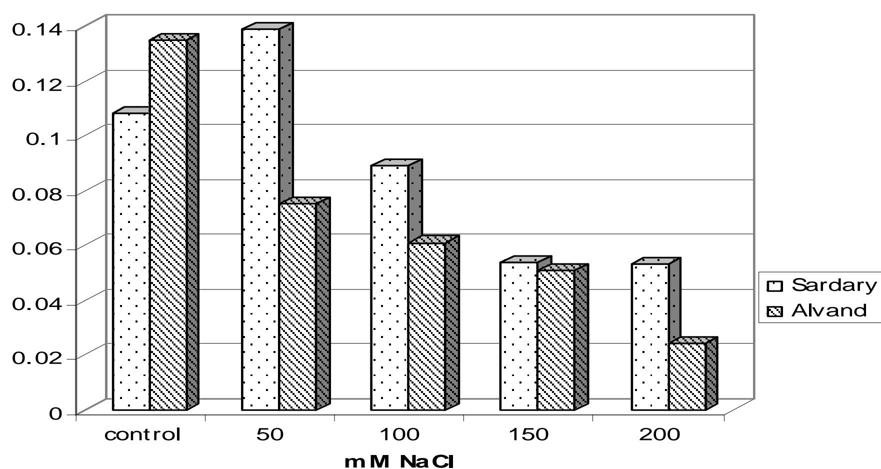

fig.3. Effects of salt stress on glutathion reductase activity in two wheat cultivars(LSD5% = 0.081)

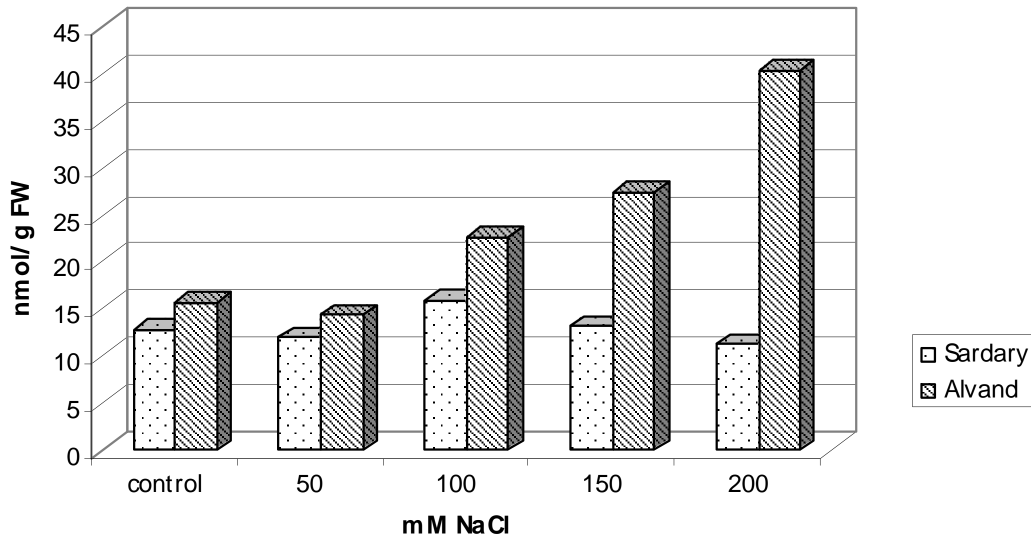

fig.4.Effects of salt stress on malon dialdehydein two wheat cultivars(LSD5% = 4.86).

DISCUSSION

The result indicated that there was a negative relationship between SOD activity and lipid peroxidation or MDA content. As indicated in Fig 1 and 4, in Sardari, SOD activity increased with increasing salt stress levels. When SOD activity was high, ROS, especially superoxide radical, scavenging was done properly and thus, damage to membranes and oxidative stress decreased, leading to the increase of tolerance to oxidative stress. Salt stress increased the superoxide level in cells. If this radical is not scavenged by SOD, it disturbs vital biomolecules (Mittler, 2000). Moreover, it inactivates antioxidant enzymes which are very important for H_2O_2 scavenging such as catalases (Kono and Fraidovich, 1983) and peroxidases (Fridovich, 1989). In Alvand, on the other hand, superoxid radical production increases with the increase of salt stress. But SOD activity was constant at all salt stress levels. For this reason scavenging of this dangerous radical was not done perfectly. Consequently, this radical attacks vital biomolecules that mentioned before and damage to membranes happens in this cultivar. Esfandiari et al, (2007) Candan and Tarhan (2003), Martinez et al (2001), Scandalios (1993), Sen Gupta et al. (1993) and Zhao et al (2006) had similar findings and expressed that the increase in SOD activity and decrease in oxidative damage were closely related.

CAT is another important antioxidant enzyme that converts H_2O_2 to water in the peroxysomes (Fridovich, 1989; McCord and Fridovich, 1969). In this organelle, H_2O_2 is produced from β-oxidation of fatty acids and photorespiration (Morita et al., 1994). Higher activity of CAT and APX decrease H_2O_2 level in cell and increase the stability of membranes and CO_2 fixation because several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2. A high level of H_2O_2 directly inhibits CO_2 fixation (Yamazaki et al., 2003). Both cultivars had similar trends as salt concentration increased. Also CAT activity at 200 mM NaCl, in alvand was higher than Sardari and exhibited a significant difference

(P<5%). Furthermore at 100 and 150 mM NaCl, there was no difference between cultivars. It seems that in Alvand, CAT activity was not adequate. Although its activity increased with the increase of salt stress, it was not sufficient for the complete scavenging of H_2O_2. While the CAT activity increases in Sardari was 3-9 folds compared to that of control, this increase in Alvand was 1.35-2.02 folds (data was not shown). Consequently, there was a negative relationship between CAT activity and MDA (Figs 2 and 4). Esfandiari et al (2007) and Shao et al. (2005 a, b and c) confirmed this relationship, too. In plant cells, the cooperation from antioxidant enzymes is essential for the scavenging of ROS. When the activity of antioxidant enzymes such as SOD and CAT is low, superoxid radical and H2O2 content will increase, the two reacting together to produce hydroxyl radical. This radical attacks all biomolecules and disturbs cell metabolism.

GR activates in glutathione-ascorbate cycle and coverts GSSG to reduced glutathione (GSH) (Asada, 2000; Vega et al., 2003). In addition, GR regulates GSH/GSSG ratio and supplies GSH for GPX and DHAR, which convert H_2O_2 to H_2O and reduce oxidized ascorbate, respectively. Although GR acquires the reduction power from NADPH, H^+ , it dissipates this power and, in turn, increases $NADP^+$/NADPH, H^+ ratio. Results indicated that in Sardari, however, GR activity decreased with the rise of salt stress, but there was no significant difference (P<5%) between the control and other treatments. In Alvand, GR activity decreased upon salt stress treatments as well. In this cultivar, however, there was a significant difference (P<5%) between the control and 100, 150 and 200 mM NaCl. On the other hand, MDA results indicated that lipid peroxidation started to increase at 100 mM NaCl (Figure 4). Furthermore, there was no difference between GR activities of the two cultivars. However, in Alvand, the highest GR activity appeared in the control and as mentioned before, there were significant differences between the control and 100, 150 and 200 mM NaCl. On the other hand, the rate of GR activity decreased with increasing salt stress was quicker than Sardari. These reasons lead to the limitation of glutation-ascorbat cycle and NADP+/NADPH,H+ ratio in Alvand and damage membranes (Figs 3 and 4). Consequently, in Alvand GR activity was sensitive to salt stress and the decrease of its activity influenced ROS scavenging systems.

MDA is regarded as a marker for evaluation of lipid peroxidation or damage to plasmalemma and organelle membranes that increases with environmental stresses. Lipid peroxidation is linked to the activity of antioxidant enzymes e.g. with the increase of SOD, APX, GPX, CAT, etc, oxidative stress tolerance is enhanced and MDA is decreased. As shown in Figure 4, the amount of MDA in Alvand increased with the increase of salt levels, but it was a constant ratio in Sardari. According to this experiment data, there was no significant difference in GR and CAT reactions between cultivars. Therefore, the increase in the concentration of MDA in higher salt levels due to the low activity of SOD and GR or CAT was not a critical factor for the damage of oxidative stress.

CONCLUSIONS

For successful scavenging of ROS by a scavenging system, some antioxidant enzymes must cooperate with each other. Moreover, there was a negative relationship between antioxidant enzymes' activity such as SOD, CAT and GR and MDA. Furthermore, CAT and GR showed the same trends in salt stress in two cultivars. But Alvand was sensitive to salt stress and damage to membranes was higher than that in Sardari. Also, all three enzymes were limitation factors in Alvand.

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REZUMAT

EFECTUL STRESULUI SALIN ASUPRA ACTIVITĂȚII ENZIMELOR ANTIOXIDANTE ŞI A LIPIDELOR PEROXIDANTE LA GRÂU

Presiunea determinată de sare, ca factor de stres, poate scădea potențialul apei în frunze, reduce turgescența plantelor, şi determină scăderea accentuată a producției în zonele aride şi semiaride. Grâul este una dintre principalele specii agricole, ocupând zone largi de cultură în Iran, țară în care stresul salin este un factor limitativ de producție. Toleranța plantelor la stresul cauzat de sare presupune activarea unor sisteme metabolice complexe, care includ căi antioxidante, în special implicarea unor specii reactive la oxigen (ROS) şi sisteme de curățare a celulelor, care pot să contribuie la continuarea creşterii plantelor în lipsa apei. În acest studiu, semințele a două soiuri locale de grâu (Alvand and Sardari) au fost cultivate hidroponic. Plantele au fost expuse în soluție Hoagland ca şi control şi 50, 100, 150 şi 200 mM NaCl pentru 10 zile. Ca şi rezultat, SOD (superoxide dismutase activity) a crescut la soiul Sardari, odată cu creştere sării, în timp ce în cazul soiului Alvand, SOD a avut activități constante la toate nivelurile de sare. În acelaşi timp, CAT şi GR au avut aceeleaşi tendințe la ambele cultivaruri de grâu, la toate nivelurile de sare. Rezultatele indică faptul că în cazul soiului Sardari, sistemele de curățare prin reacții de oxidare şi prin sistemele speciale SOD, CAT şi GR au dat rezultate bune şi pierderile membranelor celulelor au fost controlate. În cazul soiului Alvand, pierderile pereților celulari au crescut odată cu creşterea nivelului presiunii. În concluzie, cele trei enzime antioxidante au fost un factor limitativ la acest cultivar, Alvand putând fi considerat un soi sensibil la stresul determinat de sare.