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ORIGINAL ARTICLE **OPEN ACCESS**

Catalase and Peroxidase Antioxidant enzyme activities in barley Cultivars seedling under salt stress

Mohammad Behrouzi [1], Mostafa Valizadeh[2], Mohammad Moghaddam Vahed [2]
[1]M.Sc. in Plant Breeding and Biotechnology, Faculty of Agriculture, Tabriz University, Tabriz, Iran
[2]Department of Plant Breeding and Biotechnology, Faculty of Agriculture, Tabriz University, Tabriz, Iran
Email: behrouzi.m6880@gmail.com

ABSTRACT
Activity of catalase (CAT), peroxidase (POX) were studied in ten barley cultivars in seedling stage. A factorial experiment was conducted using three NaCl levels (0, 100 and 200 mM), and three levels of proline (0, 5 and 10 mM) based on completely randomized design with three replications. Electrophoretic analyses were performed, using slab polyacrylamide gels. Enzymatic activities of CAT showed significant difference between cultivars, NaCl treatment and salinity x cultivar, also salinity × cultivar × proline interaction. Enzymatic activities of three isozymes of POX were significant for cultivars, salinity levels and salinity × cultivar effects. Salinity × proline for POX3, also salinity × cultivar × proline interaction for POX2 and POX3 was significant. For POX3, 5 mM proline treatment resulted in modulating salinity stress compared to 200 mM salinity condition. Finally, the authors suggest antioxidant analysis by gel electrophoresis as a useful tool for studying plants tolerance to salt stress.
***Key words**; CAT, POX, ROS, antioxidants, isozyme profile, salt stress.*

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INTRODUCTION

In Iran, barley cultivation bears economical and agricultural importance, since it is grown nationwide for different purposes including human and livestock food, and pharmaceuticals.
Excessive presence of NaCl frequently constrains the production of most economically important crops in many arid and semi-arid regions in the world [1], reducing the plant growth and development through osmotic stress, specific ion (Na^+) toxicity as well as nutritional imbalance [2], physiological and biochemical perturbations [3]. when the plant is experiencing stress condition, the equilibrium is perturbed, therefore, the production of ROS may exceed the capacity of the plant's defense mechanisms, an imbalance in intracellular ROS content is established, which results in oxidative stress, and significant damage to cell structures through oxidation of lipids, proteins and nucleic acids [4][5]. So far, many researchers have done experiments addressing the molecular and chemical systems involved in the mechanisms of tolerance in barely and other crops [6][7]. These experiments proved molecular approaches as potential selection criteria through identifying candidate enzymes, and genetic mechanisms involved. To protect themselves against ROS damage, plant cells and its organelles employ some indigenous enzymatic and non-enzymatic antioxidant defense systems [8]. It has been reported that a significant correlation exists between the activities of antioxidative enzymes and the salt tolerance of plants [9][10][11]. This system consists of low-molecular-weight antioxidants, such as proline, ascorbate and glutathione, as well as several enzymes such as superoxide dismutase (SOD) which convert O-2 to H2O2 and peroxidase (POX), catalyzing the breakdown of H2O2 [12][13][14][15]. In a previous study, barley seedlings were subjected to 200 mM salinity, and researchers observed a significant increase in the production of CAT, among antioxidant enzymes studied [16]. They found a significant relationship between H2O2 accumulation in roots and increased activity of CAT, indicating the CAT involvement in scavenging of hydrogen peroxide under salinity stress. Jin and the colleagues [17] studied POX activities in two types of barely genotypes, one tolerant and one sensitive, subjected to salinity stress. They found that, when exposed to salt stress, the salt-tolerant genotypes showed a higher enzymatic activity than the sensitive ones. Both genotypes, however, had significantly higher peroxidase activities in the salt-stressed plants than the control. They concluded that the amount of POX activity depended on both genotype and

salinity level. Similar increases in the activities of CAT and POX have been reported in rice [18], alfalfa half [19], and tomato [20].

Proline can also be added to the group of non-enzymatic antioxidants that in different organisms neutralize the inhibitory effects of ROS. A number of studies have elucidated the protective function of Proline against environmental stresses. For example, it is now well established that Proline content increases dramatically following high salinity, drought and heavy metal stress [21].

In this research, we investigated the effects of different combination of salinity and proline treatments on some antioxidant enzymes profile in barley seedlings using horizontal electrophoresis.

MATERIALS AND METHODS

Plant growth and NaCl treatment

This experiment was conducted using 10 Iranian germplasm of barley cultivars (*Hordeum vulgare* L.) obtained from the Institute of Research center for Seed and Seedling, in Karag, Iran. These cultivars were as follows: Aras, Bahman, Yusof, Kavir, Sahra, Karoon, Makoii, Nosrat, Torsh, Jonub,. The experiment was performed in a factorial experiment based on completely randomized design with three replicates. Uniform seeds of cultivars were surface-sterilized in 5% sodium hypochlorite and ethanol, rinsed with water and then planted in disposable plates in Lab condition. For the first 5 days, plants were irrigated with distilled water. After five days, plants were subjected to nine treatment combinations of 0, 100 and 200 mM NaCl and 0, 5 and 10 mM proline [22].

Enzyme Extraction and Electrophoresis

For enzyme extraction, the mixed leave samples from each experimental plot were homogenized separately with mortar and pestle in a buffer pH 7.5 (containing tris 50 mM, sucrose 5%, ascorbic acid 50 mM, sodium metabisulfite 20 mM, PEG 2% and 2ME 0.1% freshly before use [23] with a ratio of 0.5 mg per µl (1W:2V), then centrifuged at 4ºC and 10,000g for 10 minutes.

The supernatants were immediately absorbed onto 3×5 mm wicks cut from Whatman 3 MM filter paper and loaded onto 8% horizontal slab acrylamide gel (0.6×15×10 cm) according to poulik gel buffer [24], using TBE (Tris-Borate-EDTA) electrode buffer (pH= 8.8).

Electrophoretic separation was performed at 4 °C for 3 hours (constant current of 26 mA, and voltage of 180V).

Staining of POX isozymes was performed according to Anderson et al [25], and CAT enzyme according to Soltis and Soltis [26]. Detected isoforms on each gel were designated numerically, with 1 given to the most anodally migrating isoform and so on.

Statistical Analysis

An image analysis program (MCID Analysis Evaluation 7.0) was used to quantify optical density× area (D×A) parameter for each isozymic band on gels. Mean comparison following The ANOVA was carried out by Duncan test by using SPSS 16.0 software. To demonstrate significant interactions, the charts were drawn using EXCEL software.

RESULTS AND DISCUSSION

The results for banding pattern of CAT and POX are illustrated in Fig. 1. There was only one monomorphic band for CAT (Fig. 1 A), similar to other studied crop plants [19] and three bands for POX, namely POX1, POX2 and POX3, based on their migration rates in barley seedling shoots (Fig. 1 B).

Analysis of variance for activity of CAT and three POX isozymes in barley are presented in Table 1. The analysis of variance of data for CAT showed a significant difference for cultivars ($P < 0.01$), salinity levels ($P < 0.05$), salinity x cultivar interaction ($P < 0.01$) and salinity x proline x cultivar interaction. (Table-1).

For POX significant differences for cultivar ($P < 0.01$) salinity levels and salinity x cultivar interaction in all isozymes were observed. In addition, there were significant differences for salinity x cultivar interaction in POX2 and POX3 ($P < 0.01$) and salinity x cultivar x proline interaction in POX2 ($P < 0.05$).

In order to explain the cultivar x proline x salinity interaction for CAT, dual interactions were studied as follows (Fig. 2 A, B and C).

The observed cultivar x salinity interaction (Fig. 2 A) was of change-in-order type. Salinity of 100 mM led to a significantly reduction in CAT enzyme activity in Yusof and Nosrat cultivars as opposed to control (0 mM).). Salinity of 200 mM treatment in Sahra cultivar caused a significant decrease, but Aras, Yusof, Karoon and Nosrat a significant increase CAT activity compared to normal and/or 100 mM salinity conditions. Aras, Yusof and Nosrat cultivars, however, showed highest enzymatic activity under 200 mM salinity conditions. The exogenous application of 5 mM proline did not significantly affect the CAT activity compared to control. Applying 10 mM proline led to a significantly increment in CAT activity in Aras and Karoon cultivars, but it significantly reduced the enzymatic activity in Yusof cultivar compared to other two levels (Fig. 2. B). Salinity x proline interaction showed that the proline was ineffective on CAT activity

under any salinity condition in barely seedlings (Fig. 2 C). Considering the lack of apparent distinction between cultivar x proline interaction and salinity x proline interaction, it could be concluded that the distinctively cultivar x proline x salinity interaction can be attributed to the significant cultivar x salinity interaction. In fact, cultivars were affected much significantly by salinity than by proline.

Mean enzymatic activity of POX1 for combination of cultivars and salinity levels is shown in Fig. 3. Salinity of 100 mM led to a significantly reduction in POX1 enzyme activity in Sahra, Yusof and Karoon compared to the control. On the other hand, 200 mM salinity in Aras, Yusof, Makoii and Nosrat cultivars has increased POX_1 activity significantly, compared to normal and/or 100 mM salinity conditions. In one cultivar (Sahra cultivar), however, 200 mM NaCl has caused a significant reduction in POX_1 activity. For this isoform, Makoii cv. showed highest densitometric enzymatic activity under salinity conditions.

Investigating of salinity x cultivar x proline interaction for POX2 (Fig. 4. A) showed that since proline x cultivar interaction and of proline x salinity carried non-significant differences for POX2 activity, the significant cultivar x salinity x proline interaction must be derived mainly from the significant cultivar x salinity interaction as for CAT activity.

The mean activity of POX3 in cultivars under different salinity levels (Fig. 4. D) revealed that 100 mM salinity significantly reduced enzymatic activity in Sahra, Karoon and Nosrat cultivars compared with the control. Nevertheless, 200 mM salinity resulted in significant increment in all of the cultivars except Jonub, Sahra and Kavir compared to control and 100 mM.

Investigating the effects of cultivar x proline interaction on POX3 activity (Fig. 4. E) found that 5 mM proline treatment in Bahman and Torsh cultivars a significantly declining effect on the isozyme's activity compared to control. Likewise, 10 mM proline treatment significantly reduced enzyme activity in Bahman and Sahra cultivars compared to control. However, this treatment significantly increased enzyme activity in Torsh cultivar compared to 5 mM proline.

The mean activity of POX3 under combinations of salinity and proline levels is shown in Fig. 4 F. Only under 200 mM salinity could 5 mM proline lead to a significant reduction in enzyme activity, compared to 100 mM salinity of treatment.

Exposure of plants to unfavorable environmental conditions, as salinity stress among others, can increase or decrease the production of radical and non-radical oxygen intermediates known as reactive oxygen species (ROS). To protect themselves against ROS damage, plant cells and its organelles employ some indigenous enzymatic and non-enzymatic antioxidant defense systems [8]. For example, some studies conducted on the involvement of antioxidant enzymes in salt tolerance, supplemented by transgenic plants, have found a reduced [14][27][28][29] or an increased [30] expression of CAT. Salinity reduces CAT activity in leaves and roots, due to the fact that CAT associates loosely with its substrates, which allows salinity to limit protective activity of CAT and, eventually, inactivate it [31]. CAT is an anti-oxidant, scavenging enzyme, with less sensitivity to oxidative stress than peroxidase, which is mainly present in peroxisomes and prevents oxidative damage by scavenging H_2O_2. Excess of H2O2 in cells may prevent peroxidase activities; therefore, CAT activity is likely to be essential for sustaining peroxidase activity under salinity stress [32]. Investigating antioxidant defense system, Hafsi and the colleagues [33] found an increase in catalase activity by 100 mM NaCl in comparison to the control, which could explain the absence of changes in H2O2 content under the treatment applied. They suggested that antioxidant defense system plays a vital role in salt tolerance ability in barely. In the present study, we found that some barley cultivars (cultivars Aras, Yusof and Nosrat) might possess a higher ability to counteract salinity-induced damages, by expressing more amounts of CAT than less tolerant cultivars.

Table 1. Analysis of variance for one CAT and three POX isozymes activities for barley seedling in different levels of NaCl salinity and exogenous proline application

		Mean squares of enzymatic activity ($*10^5$)			
S.O.V	df	CAT	POX_1	POX_2	POX_3
Cultivar (C)	9	67.9936**	475.2017**	1658.9352**	3823.8641**
Salinity (S)	2	3.7505*	148.5673**	793.2567**	1525.0151**
Proline (P)	2	0.1255^{ns}	19.4671^{ns}	99.4968^{ns}	75.0065^{ns}
C*S	18	2.4629**	47.6156*	183.1350*	259.8306**
C*P	18	1.3574^{ns}	12.3561^{ns}	118.4970^{ns}	32.3278^{ns}
S*P	4	1.8833^{ns}	11.2900^{ns}	129.8539^{ns}	127.9890**
S*C*P	36	1.6675*	18.3514^{ns}	144.7890*	64.5938**
Error	180	1.0320	26.6224	97.8110	26.6247

* and ** stand for significant at probability level of 0.5 and 0.1 present and non-significant, respectively

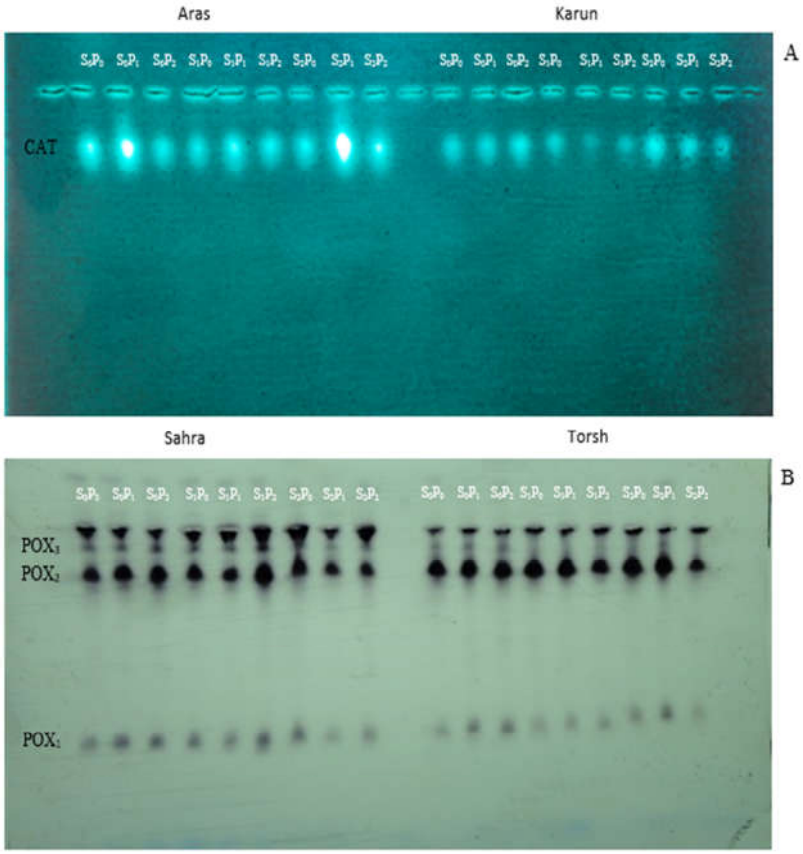

Fig. 1. Banding patterns of one CAT isozyme in two cultivars (Fig. 1. A) and three POX isozymes in two other cultivars (Fig. 1. B) subjected to nine different combinations of treatment including three levels of salinity (0, 100, and 200 mM presented by S_0, S_1 and S_2) and three levels of proline (o, 5 and 10 mM presented by P_0, P_1 and P_2). Izosymes were named according to their speed of migration towards the anode pole.

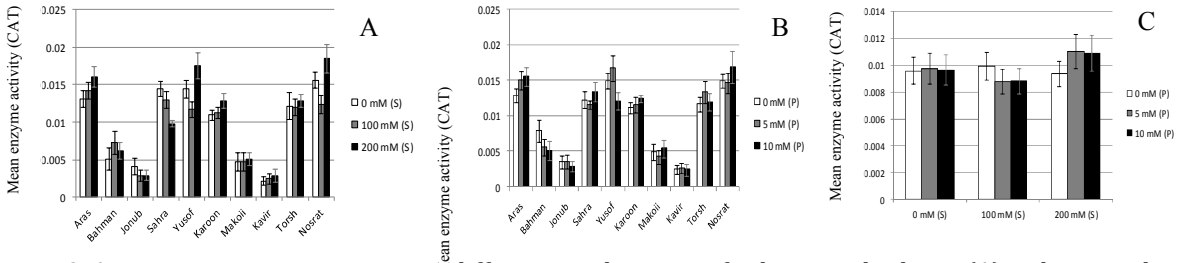

Fig. 2. Average enzymatic activities in different combination of cultivar and salinity (A), cultivar and proline (B) and salinity and proline levels (C) for CAT.

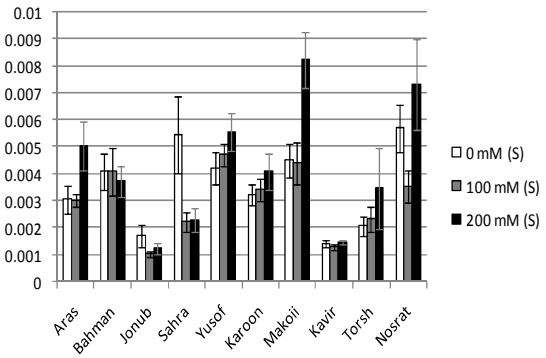

Fig. 3. Average densitometric activities of barley seedling of POX1 in different combination of cultivar and salinity.

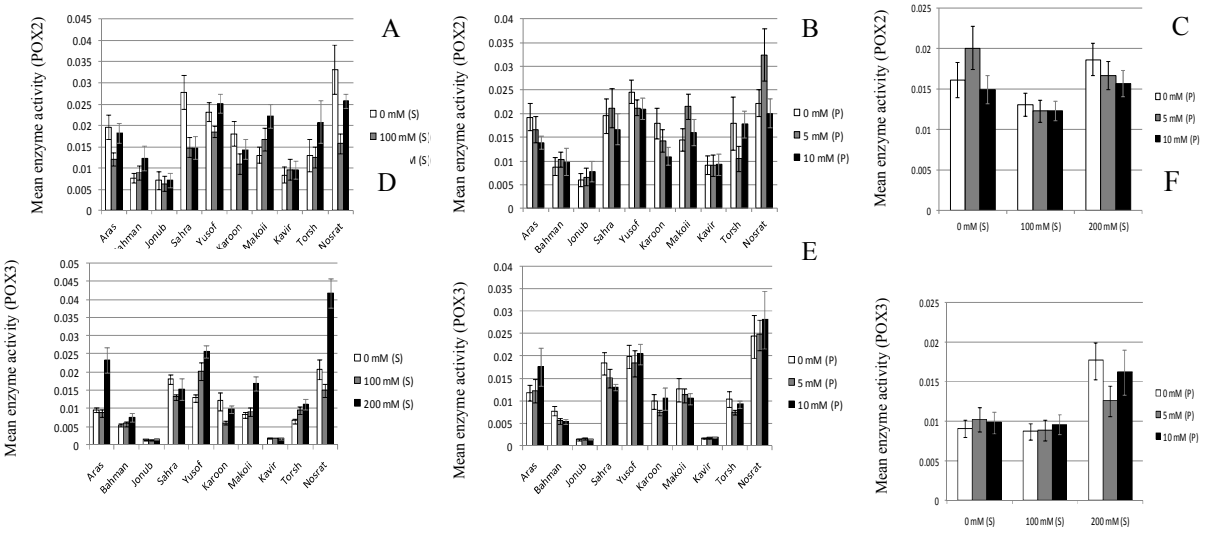

Fig. 4. Average enzymatic activities in different combination of cultivar and salinity, cultivar and proline and salinity and proline levels for POX2 (A, B, C), and POX3 (D, E, F).

Peroxidases are present in cytosol and most organelles of plant cells, and mainly involved in oxidative-induced H2O2 dissociation. It has been suggested that the rise in POX activity under drought condition is probably due to H2O2 accumulation [34]. Yildiz and Terzi [35] subjecting two tolerant and sensitive cultivars of barely to salinity stress (levels of 0, 100, 200 and 300 mM), found a significant positive correlation between increase of salinity levels with increased activity of POX, with tolerant showing more increase than the sensitive one. They concluded that increased activity of the said enzyme could be the cause of tolerance in the former. In the present study, the highest densitometric activity of POX1 was observed in Aras cultivar and POX3 in Nosrat cultivar, and therefore, we can draw the conclusion that these two cultivars could possess highest tolerance to salinity stress among ten cultivars studied.

Stress- induced proline accumulation indicates a multifunctional defensive system, which in fact, indicates the plant's general response to unfavorable environmental conditions during growth. Plant's treatment with osmolytes like proline, betaine and trehalose can remarkably improve their tolerance to stress condition [36]. Treatment of barley embryo culture with exogenous application of proline under salinity condition resulted in reduction of Na^+ and Cl^- ions accumulation as well as improving growth [37]. In present study, addition of 5 mM proline significantly reduced POX3 activity by 200 mM of salinity. This is probably because application of 5 mM proline reduces the stress impact, hence, reducing POX3 production significantly. Proline modulating effects have been reported in several researches. For example, investigating the effects of proline on antioxidant defense system in the presence of NaCl, researchers found that treatment with 150 mM NaCl, applied 2 days after planting, increased, while mix applying of 150 mM NaCl and 10 mM proline, applied 2 days after planting, decreased POX activity significantly, which is further consistent with our findings [38].

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