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Effect of Salt Stress and Exogenous Application of Proline on some Antioxidant Enzymes Activity in Barley Cultivars Seedling

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ABSTRACT: In order to investigate the effect of salt stress and exogenous proline treatment on catalase and peroxidase activity, on ten barley cultivars at seedling stage, a factorial experiment on the basis of completely randomized experimental design in three replications was performed using NaCl levels (0, 100 and 200 mM), and three levels of proline (0, 5 and 10 mM). Electrophoretic analyses were performed by using 8% slab polyacrylamide gels. Two antioxidant enzymes including catalase (CAT) and peroxidase (POX) were stained and for each isozymic band the "density × area" scores onto gels were evaluated by MCID software as enzymatic activity. The analysis of variance of data for CAT showed a significant difference for cultivars, salinity levels, salinity × cultivar interaction and salinity × proline × cultivar interaction. For POX, significant differences for cultivars, salinity levels and salinity × cultivar interaction in all isozymes were observed. In addition, there were significant differences for salinity × proline interaction in POX3 and salinity × cultivar × proline interaction in POX2 and POX3. In present study, treatment of 5 mM proline significantly reduced POX3 activity which resulted in modulating salinity stress compared to200 mM salinity condition.

Keywords: Antioxidant enzymes, Barley, Proline, Salt stress.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is a major cereal grain with economical and agricultural importance grown in temperate climates globally but excessive presence of salt (NaCl) cause reduction of most crops including barley (Baik & Ullrich, 2008). This environmental stress affects plant growth and development through osmotic stress, specific ion (Na^+) toxicity as well as nutritional imbalance (Bartels & Sunkar, 2005), physiological and biochemical characteristics (Hasegawa *et al*, 2000).

Exposure of plant to stress could result in the production of reactive oxygen species (ROS) and the ROS content may exceed the capacity of the plant's defense mechanisms, which in turn results in oxidative stress, and significant damage to cell structures through oxidation of lipids, proteins and nucleic acids (Pastori & Foyer, 2002; Gill & Tuteja, 2010).

The mechanisms of defense against ROS in a molecular level or morphological state have been studied by many researchers in barely and other crops (Seckin *et al*, 2010; Ahmed *et al*, 2013). The very goal of these experiments is to provide potential selection criteria through identifying candidate enzymes, and genetic mechanisms according to tolerant and/or sensitive cultivars.

Plants trigger various biochemical pathways by non-enzymatic antioxidant defense systems and employ different enzymes to protect themselves against ROS damage (Manchania *et al*, 1999). Various reports indicate a significant correlation between the activities of some antioxidative enzymes and salt tolerance of plants (Chinta *et al*, 2001; Diego *et al*, 2003; Nader *et al*, 2005). Low-molecular-weight antioxidants, such as proline as an osmoprotectant, ascorbate and glutathione, as well as several enzymes such as superoxide dismutase (SOD) which convert O^{-2} to H_2O_2 and peroxidase (POX), catalyzing the breakdown of $H_2O_2$are some examples of Components of the defense systems in plants against stress derived ROS (Mittler, 2002; Thomas *et al*, 2005; Turkan *et al*, 2005; Gaber, 2010).

For instance, Barley seedlings were treated with 200 mM salinity and a significant increase in the production of CAT, among antioxidant enzymes was observed (Kim *et al*, 2005). Another observation was a significant relationship between H_2O_2 accumulation in roots and increased activity of CAT, which introduces CAT - as scavenging of hydrogen peroxide - as another important component of stress defense system.

POX activity has been studied under salinity stress condition in two tolerant and sensitive genotypes of barely (Jin *et al*, 2009). In this study, a higher enzymatic activity was observed in tolerant genotype rather than sensitive one, and both sensitive and tolerant genotypes had significantly higher peroxidase activities than the control. In conclusion, 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 (Khan & Panda, 2008), alfalfa half (Valizadeh *et al*, 2013), and tomato (Dogan, 2012).

One of the most common stress responses in plants is overproduction of different types of compatible organic solutes such as proline and glycine betaine (GB) (Serraj & Sinclair, 2002). The organic solutes have been proven to be helpful in osmoregulation, (Rhodes & Hanson, 1993), enzyme activity (Mansour, 2000), detoxification of reactive oxygen species (Greenway & Munns, 1980; Ashraf, 1994; Ashraf & Wu, 1994), and protection of membrane integrity, (Bohnert & Jensen, 1996). There, Proline has the ability to neutralize the inhibitory effects of ROS.

The aim of this study is to investigate the effects of different combination of salinity and proline treatments on CAT and POX profile in barley seedlings by horizontal gel electrophoresis.

MATERIALS AND METHODS

A. 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 (Radyukina *et al*, 2008).

B. 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 (Valizadeh *et al*, 2011) 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 (Poulik, 1957), 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* (Anderson *et al*, 1995), and CAT enzyme according to Soltis and Soltis (Soltis & Soltis, 1990). Detected isoforms on each gel were designated numerically, with 1 given to the most anodally migrating isoform and so on.

C. 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. 1A), similar to other studied crop plants (Valizadeh *et al*, 2013) and three bands for POX, namely POX1, POX2 and POX3, based on their migration rates in barley seedling shoots (Fig. 1B).

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 × cultivar interaction ($P < 0.01$) and salinity × proline × cultivar interaction ($P < 0.05$) which is mentioned in Table 1. For POX, significant differences for cultivars ($P < 0.01$), salinity levels ($P < 0.01$) and salinity × cultivar interaction [POX1 and POX2 ($P < 0.05$), POX3 ($P < 0.01$)] in all isozymes were observed (Table 1).

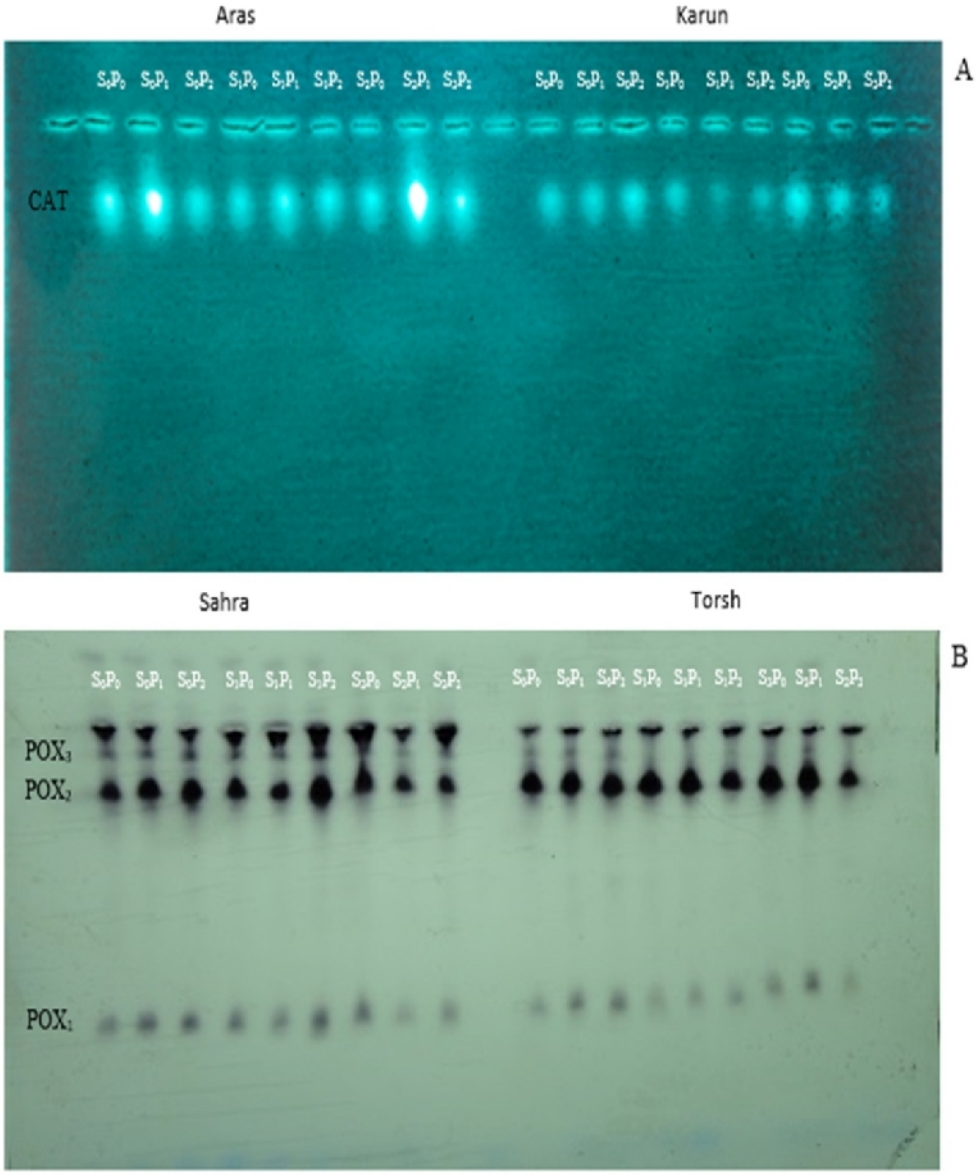

Fig. 1. Banding patterns of one CAT isozyme in two cultivars (Fig. 1. A) and three POX isozymes in two other cultivars (Fig. 1. B) subjected to nine different combinations of treatment including three levels of salinity (0, 100, and 200 mM presented by S0, S1 and S2) and three levels of proline (o, 5 and 10 mM presented by P0, P1 and P2). Izosymes were named according to their speed of migration towards the anode pole.

In addition, there were significant differences for salinity × proline interaction in POX3 ($P < 0.01$) and salinity × cultivar × proline interaction in POX2 ($P < 0.05$) and POX3 ($P < 0.01$).

In order to explain the cultivar × proline × salinity interaction for CAT, dual interactions were studied as follows (Fig. 2A, B and C). The observed cultivar × salinity interaction (Fig. 2A) 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 it significantly increased the enzymatic activity in Aras, Yusof, Karoon and Nosrat compared to the normal and/or 100 mM salinity conditions. Aras, Yusof and Nosrat cultivars, however, showed highest enzymatic activity under 200 mM salinity conditions.

Table1: Analysis of variance forone 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**	**POX1**	**POX2**	**POX3**
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.The numbers in the table presented 105 times bigger than they are.

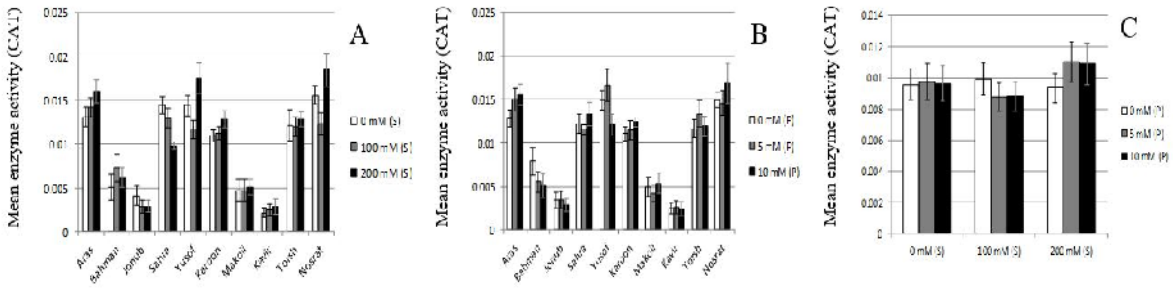

Fig. 2. Average enzymatic activities in different combination of cultivar and salinity (A), cultivar and proline (B) and salinity and proline levels (C) for CAT.

The exogenous application of 5 mM proline did not significantly affect the CAT activity compared to the control. Applying 10 mM proline led to a significantly increase in CAT activity in Aras and Karoon cultivars to the control, but it significantly reduced the enzymatic activity in Yusof cultivar compared to other two levels (Fig. 2B). Salinity × proline interaction showed that the proline was ineffective on CAT activity under any salinity condition in barely seedlings (Fig. 2C). Considering the lack of apparent distinction between cultivar x proline interaction and salinity × proline interaction, it could be concluded that the distinctively cultivar × proline ×salinity interaction can be attributed to the significant cultivar × 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 Jonub, Sahra and Nosrat compared to the control. On the other hand, 200 mM salinity in Aras, Yusof, Makoii and Nosrat cultivars has increased POX1 activity significantly, compared to the normal and/or 100 mM salinity conditions. In one cultivar (Sahra), however, 200 mM NaCl has caused a significant reduction in POX1 activity compared to the control. For this isoform, Makoii cultivarshowed highest densitometric enzymatic activity under salinity conditions.

Investigating of salinity x cultivar x proline interaction for POX2 (Fig. 4A, B and C) showed that since cultivar x proline interaction (Fig. 4B) and of salinity x proline interaction (Fig. 4C) 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 (Fig. 4A) as for POX2 activity.

The mean activity of POX3 in cultivars under different salinity levels (Fig. 4D) revealed that 100 mM salinity significantly reduced enzymatic activity in Sahra, Karoon and Nosrat cultivars compared to the control, but it significantly increased the enzymatic activity in Yusof and Torsh cultivars compared to the control. Nevertheless, 200 mM salinity resulted in significant increase in all of the cultivars except Jonub, Sahra and Kavir compared to the control and/or 100 mM salinity conditions. For this isoform, Nosrat cv. showed highest densitometric enzymatic activity under salinity conditions.

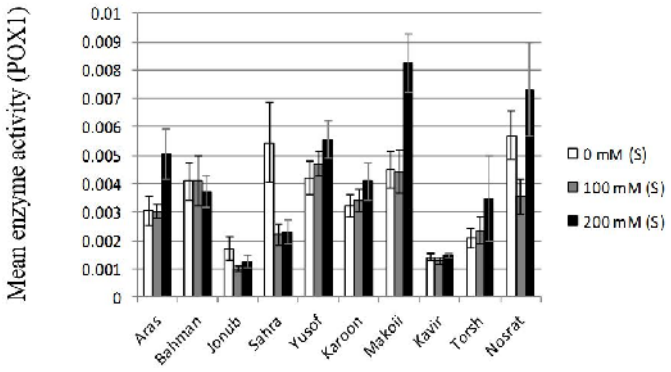

Fig. 3. Average densitometric activities of barley seedling of POX1 in different combination of cultivar and salinity.

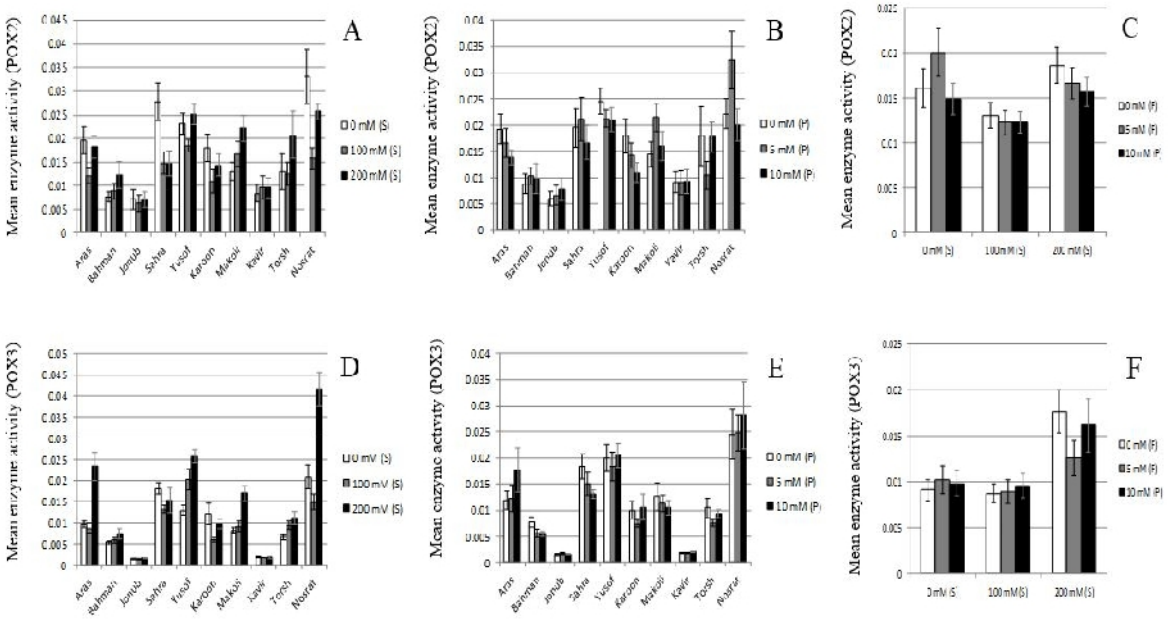

Fig. 4. Average enzymatic activities in different combination of cultivar and salinity, cultivar and proline and salinity and proline levels for POX2 (A, B, C), and POX3 (D, E, F).

Investigating the effects of cultivar × proline interaction on POX3 activity (Fig. 4E) found that 5 mM proline treatment in Bahman, Karoon and Torsh cultivars a significantly declining effect on the isozyme's activity compared to the control. Likewise, 10 mM proline treatment significantly reduced enzyme activity in Bahman and Sahra cultivars compared to the control. However, this treatment significantly increased enzyme activity in Torsh cultivar compared to the 5 mM proline. The mean activity of POX3 under combinations of salinity and proline levels is shown in Fig. 4F. Only under 200 mM salinity, treatment of 5 mM proline lead to a significant reduction in enzyme activity, compared to the 100 mM salinity 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 (Manchania *et al*, 1999).

For example, some studies conducted on the involvement of antioxidant enzymes in salt tolerance, supplemented by transgenic plants, have found a reduced (Fu & Huang, 2001; Turkan *et al*, 2005; Noreen & Ashraf, 2009; Aydin *et al*, 2011) or an increased (Rubio *et al*, 2002) 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 (Cramer, 2002). 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 H_2O_2 in cells may prevent peroxidase activities; therefore, CAT activity is likely to be essential for sustaining peroxidase activity under salinity stress (Cruz de Carvalho, 2008). Investigating antioxidant defense system, Hafsi and the colleagues (Hafsi *et al*, 2010) found an increase in catalase activity by 100 mM NaCl in comparison to the control, which could explain the absence of changes in H_2O_2content 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.

Peroxidases are present in cytosol and most organelles of plant cells, and mainly involved in oxidative-induced H_2O_2 dissociation. It has been suggested that the rise in POX activity under drought condition is probably due to H_2O_2 accumulation (Jebara *et al*, 2005). Yildiz and Terzi (2013) 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 can 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 (Ashraf & Foolad, 2007). 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 (Lone *et al*, 1987). 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 effect, because of significant reduction in POX3 production. Proline modulating effects have been reported in several researches (Hare and Cress, 1997). 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 (Öztürk & Demir, 2002).

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