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# The effects of salinity on antioxidant enzymes activity in the leaves of two contrast rice (*Oryza sativa* L.) cultivars

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**Abstract**

The aim of this research was to assess the effects of salt stress on antioxidant enzymes activity in rice leaves. Experiment design was completely randomized design in a factorial arrangement with two genotypes (improved cultivar of Ghaem and traditional cultivar of Sangejo) and four salinity levels (0, 40, 80 and 120 mM NaCl) with three replicates. Salt stress treatments were initiated at 16 days after planting, and then activities of some antioxidant enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and superoxide dismutase (SOD) were determined. Results of variance analysis showed that the interaction of salinity and the cultivar for CAT, POD, APX ($\alpha$= 0.01) and SOD ($\alpha$= 0.05) were statistically significant. Activity of all studied enzymes in Sangejo cultivar increased with increasing the salt stress treatments whereas in Ghaem cultivar, CAT and SOD activities had no clear trend. Results of regression analysis for antioxidant enzymes in Sangejo cultivar showed a significant correlation between antioxidant enzymes and salt stress levels. By contrast, in Ghaem cultivar there was a weak relationship between both CAT and POD ($R^2$=0.21) and APX ($R^2$=0.33) activities. In conclusion, the results of the current study indicated that Sangejo cultivar was more tolerant to salt stress as compared to newly released cultivar of Ghaem.

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## Introduction

Soil salinity is a major threat to global food security. Up to 20% of the world's irrigated land, which produces one third of the world's food influenced by salt stress (FAO, 2007). Rice (*Oryza sativa* L.) is the primary stable food for over two billion people in Asia, Africa, and Latin America (Salekdeh *et al*., 2002). Salinity affects plant growth and development in two ways. First, it imposes osmotic stress by reducing the soil water potential leading to limiting the water uptake. Second, it causes excessive uptake of ions particularly $Na^+$ and $Cl^-$ that ultimately interferes with various metabolic processes. The different plants have different abilities in saline environments. Nevertheless, differences in salinity stress tolerance not only depend on the genus and species, but also is difference within a species. The degree of plant growth reduction under saline conditions, however, depends on salt composition, salt concentration and plant growth stage (Maas, 1986).

Reactive oxygen species (ROS) are regarded as the main source of damage to cells under biotic and abiotic stresses (Candan and Tarhan, 2003; Gara *et 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 ($O_2^{\cdot-}$), peroxide hydrogen ($H_2O_2$) and hydroxyl radical ($HO^{\cdot}$) (Mittler, 2002). These species of oxygen are highly cytotoxic and can seriously react with vital biomolecules such as lipids (Lipid peroxidation), proteins (Protein denaturing), nucleic acid (DNA mutation) and so on (Quiles and Lopez, 2004).

Increasing respiration induced by salt stress enhance destructive ions production in mitochondria (Ort, 2001). To protect against these toxic oxygen intermediates, plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defense systems. A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against various stresses (Khan and Singh, 2008; Tuteja, 2007). Many researches proved that plants are equipped with a diverse array of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO) against oxidative damages by ROS (Vaidyanathan *et al.,* 2003; (Agarwal and Pandey 2004); Dat *et al*., 2000).

In recent years, salinity is the major problem in decreasing rice growth and yield performance in Iran, where rice has a significant role in food security. This reveals the importance of research on physiological mechanisms of salt stress tolerance especially ROS scavenging systems in rice. The main objective of this study was to investigate the response of two contrast rice cultivars to saline stress by assessment their antioxidant enzymes activities during vegetative growth stage.

## Materials and methods

This research was performed at the Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari Agricultural Sciences and Natural Resources University in 2010**.** The seeds of two contrast cultivars, Ghaem and Sangejo, 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 45-50 seeds were planted into plastic trays covered with Yoshida solution (Yoshida *et al*., 1976). This solution was constantly aerated and renewed 2-3 times a week to minimize pH shift and nutrient depletion. At 16 days after planting (at the 13 stage according to Zadoks (1974) method), salt stress treatment was initiated. Seedlings were treated with Yoshida solution containing 40, 80 and 120 mM NaCl and maintained for 14 days in these conditions. Control seedlings were kept in Yoshida solution without NaCl. After treatment for 14 days, the rice seedling was sampled and transferred to liquid nitrogen and maintained at -70°C.

*Enzyme extraction*

For protein and antioxidant enzyme assays, frozen leaves were ground to a fine powder with liquid nitrogen and were extracted with ice-cold 0.1M Tris-HCl buffer (pH 7.5) containing 5% (w/v) sucrose and 0.1% 2-mercaptoethanol (3:1 buffer volume/FW). The homogenate was centrifuged at 10 000 g for 20 min, at 4°C, and the supernatant was used for enzyme activity and protein determinations. Preparations for enzyme extraction and enzyme assay were carried out at 4°C.

*Protein determination*

The concentration of protein was determined by Bradford (1976) method using bovine serum albumin (BSA) as a standard.

*Assay of antioxidant enzymes*

POD activity was determined spectrophotometrically by measuring the oxidation of o-dianisidine (3, 30-dimethoxybenzidine) at 460 nm. The reaction mixture contained 20 mM phosphate buffer (pH 5.0), 1 mM dianisidine, 3 mM $H_2O_2$ and 50 ml of extract. POD activity was expressed as units (mmol of oxidized dianisidine per min) per mg of protein (Ranieri *et al.*, 2000**)**. CAT activity was determined spectrophotometrically according to Aebi (1984) by monitoring the disappearance of $H_2O_2$ at 240 nm and 25 °C for 2 min (ε = 39.58M−1 cm−1). The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), to which 10.6 mM $H_2O_2$ was added. The reaction was initiated by adding 10_l of the leaf crude extract to this solution for 2 min at 25 °C. SOD activity was assayed by its ability to inhibit photochemical reduction of NBT at 560 nm (Beauchamp and Fridovich, 1971). The assays were carried out at 25°C and reaction mixture (3 ml) contained 0.033 mM nitrotetrazolium blue chloride (NBT), 10 mM l-methionine, 0.66 mM EDTA Na2 and 0.0033 mM riboflavin in 50 mM Na-phosphate buffer (pH 7.8). Riboflavin was added last and the test tubes containing the reaction mixture were incubated for 10 min under 300_molm$^{-2}$s$^{-1}$ irradiance. The reaction mixture with no enzyme developed maximum color because of the maximum rate of reduction of NBT. Non-irradiated reaction mixture was used as the control as it did not develop color. One unit of SOD was defined as the amount of enzyme that inhibits 50% NBT photoreduction. APX activity was measured according to Nakano and Asada (1981). The assay depends on the decrease in absorbance at 290 nm as ascorbate was oxidized. The reaction mixture contained 50mM Na-phosphate buffer (pH 7.0), 50 mM ascorbate, 0.1 mM EDTA Na2, 1.2 mM H2O2 and 0.1 ml of enzyme extract in a final assay volume of 1 ml. The concentration of oxidized ascorbate was calculated by using extinction coefficient of 2.8 mM$^{-1}$cm$^{-1}$. One unit of APX was defined as 1mmolml−1 ascorbate oxidized min$^{-1}$.

*Statistical analysis*

All statistical analysis was performed using the SPSS statistical program. Duncan's multiple range tests were performed when significant differences occurred at 5% level.

**Results and discussion**

*The relationship between antioxidant enzymes*

Regression analysis showed that the relationship between CAT and APX activities was significant in both cultivars (α = 0.01). Regression R-squares were equivalent to 97% (cubic model) and 94% (linear model) in the Sangejo and Ghaem cultivars, respectively (Fig 2). Also, there was a significant relationship between CAT and SOD activities in both cultivars (α=0.01)**.** Regression R-squares were equivalent to 94% (cubic model) and 75% (Sigmoid model) in the Sangejo and Ghaem cultivars, respectively (Fig 3). The relationship between CAT and POD activities in Sangejo cultivar was significant (α = 0.01) according to S model ($R^2$=0.82) while there was no significant ($R^2$=0.21) relationship in Ghaem cultivar (Fig 2). This difference can be caused by decreased POD enzyme activity in Ghaem cultivar**.** Whereas CAT activity directly modulates the amount of ROS (Jiang and Huang, 2001) and also in Sangejo cultivar, CAT activity increased by salinity treatment; so CAT played more active roles than POD in plant cells from oxidative stress. This result is in confirmation with the findings of the

Chookhampaeng (2011). By contrast, Sairam *et al* (2002) and Gosset *et al* (1994) reported that all antioxidant enzymes did not increase in salinity conditions. It seems that response of antioxidants to salinity depending on plant species, growth stage and salt concentration. Furthermore, there was a significant relationship between APX and SOD activities in both cultivars as a cubic model (Fig 5). A power curve fitted ($R^2$=0.86) for APX and POD activities in Sangejo cultivar but there was a weak correlation ($R^2$=0.33) in Ghaem cultivar (Fig 5). In both cultivars, a sigmoid and cubic models was observed between SOD and POD activities (Fig 7).

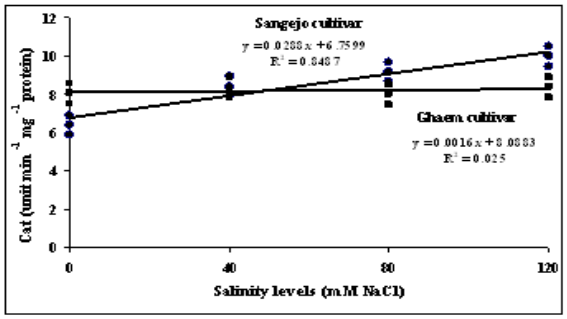


**Fig. 1.** CAT enzyme activity under different salinity levels.

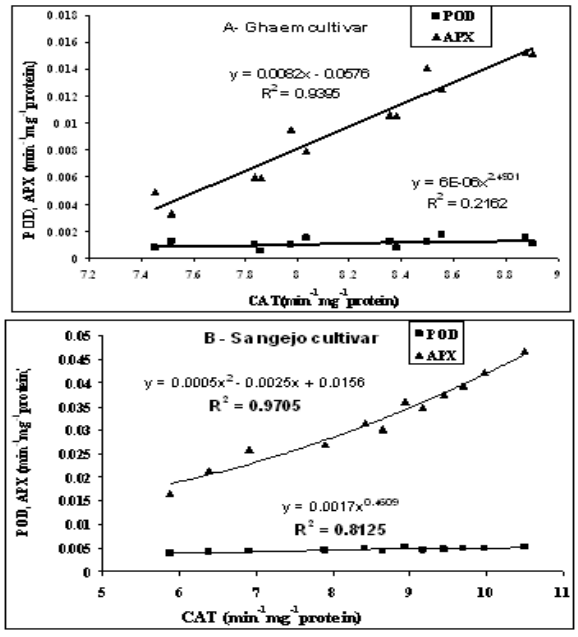


**Fig. 2.** The relationship between CAT with POD and APX activity (A- Ghaem cultivar and B- Sangejo cultivar).

*Catalase (CAT)*

CAT enzyme activity in Sangejo cultivar increased just after salinity stress starting. The velocity enhancement of enzyme activity with 120 mM NaCl treatment was higher than other treatments while there was a weak linear regression for CAT activity in Ghaem cultivar (Fig 1). Vaidyanathan *et al* (2003) reported that an increase of CAT activity is a strategy for improving the salt tolerance of rice. In plants, a number of enzymes regulate $H_2O_2$ intracellular levels. APX and CAT are considered to be the most important ones (Deneto *et al*., 2006). $H_2O_2$ is a constituent of oxidative metabolism. It is a product of peroxisomal and chloroplastic oxidative reactions (Del Rio *et al*., 1992). CAT is used to break down the $H_2O_2$ (Mittler, 2002). So CAT is responsible for detoxification of $H_2O_2$, is probably equally important in the detoxification step in plants (Vaidyanathan *et al*., 2003). In the present study, CAT activity did not increase in Ghaem cultivar; so the toxic metabolite accumulates in the cell. Its cause may be no increases in SOD activity. Since SOD enzyme can be converts two superoxide anions into a molecule of hydrogen peroxide and one of oxygen (Polle, 2001), therefore the plant does not need to increase CAT activity for detoxification. Demiral and Türkan (2005) also reported that increased CAT activity in salt tolerant cultivar "Pokkali" under different salinity levels whereas no changes were observed in the salt-susceptible cultivar "IR28" and there *was* a little change in SOD Activity. Also Abbaspour (2012) stated that CAT activity increased with increasing salt stress (Abbaspour 2012).

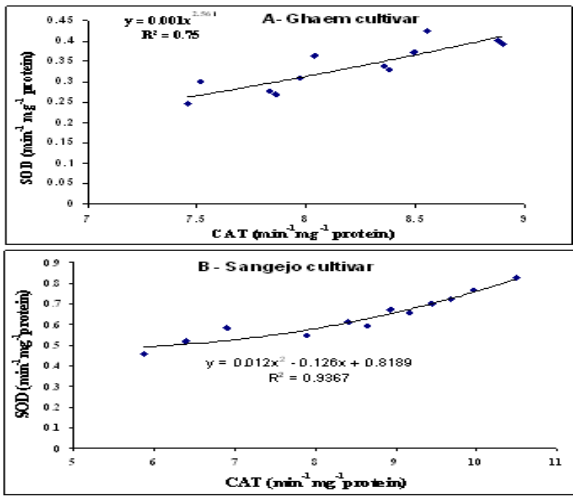


**Fig. 3.** The relationship between CAT and SOD activity (A- Ghaem cultivar and B- Sangejo cultivar).

*Superoxide dismutase (SOD)*

SOD activity in Sangejo cultivar increased after the salt treatment as linear regression (Fig 4). The maximum activity of SOD was observed at 120 mM of NaCl treatment while it did not increased in Ghaem cultivar.

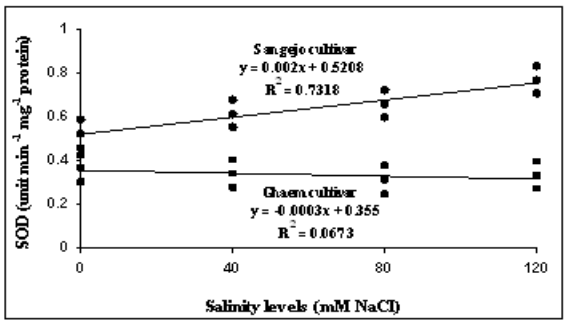


**Fig. 4.** SOD enzyme activity under different salinity levels.

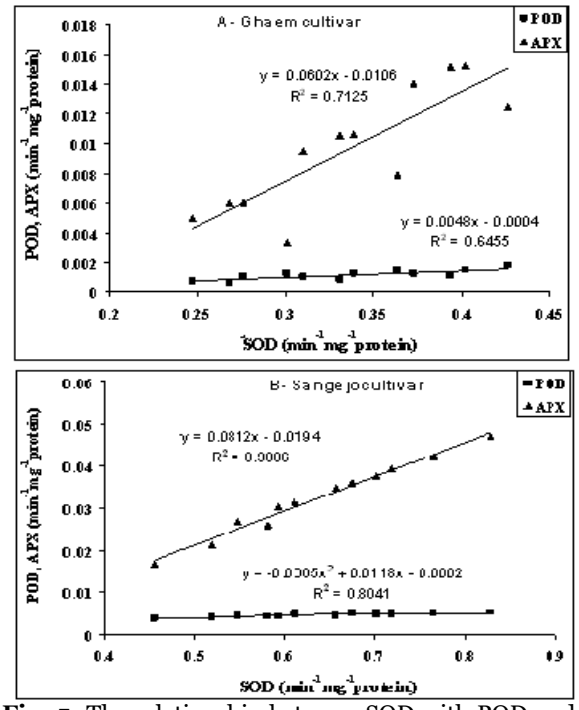


**Fig. 5.** The relationship between SOD with POD and APX activity (A- Ghaem cultivar and B- Sangejo cultivar).

The enzymatic antioxidant system is one of the protective mechanisms including SOD, which can be found in various cell compartments and it catalyses the disproportion of two $O^{2\cdot-}$ radicals to $H_2O_2$ and $O_2$ (Mittler *et al.*, 2004; (Alscher and Heath 2002)). Superoxide anion toxicity produced under oxidatively salt stress circumstances. If this radical is not scavenged by SOD, it disturbs vital biomolecules (Mittler, 2002). So increases SOD enzyme activity destroys the superoxide ion (Ort, 2001). The same process was reported by Doulatabadian *et al* (2008) in wheat under salinity stress. Therefore 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 (Mittler, 2002). In Ghaem cultivar, superoxide radical production increases with the increase of salt stress. SOD activity, however, was constant at all salt stress levels so it seems that scavenging of this dangerous radical was not done perfectly. Consequently, this radical attacks vital biomolecules (Esfandiari *et al.*, 2007).

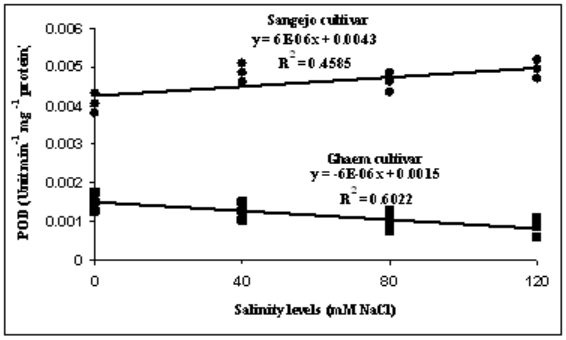


**Fig. 6.** POD enzyme activity under different salinity levels.

Elevated SOD activity is accompanied with an increase in the activity of major $H_2O_2$ scavenging enzymes like APX and CAT in Sangejo cultivar which may be attributed to its salt tolerance as compared to Ghaem cultivar. The increase in SOD activity was reported previously in tolerance Basmati rice variety (Singh *et al.* 2007) and Pokkali while a significant decrease of this enzyme was reported in the salt-sensitive variety (Dionisio-Sese and Tobita, 1998). Similar observations have been made for other plants (Reddy and Srivastava, 2003; Zamani *et al.*, 2011).

*Peroxidase (POD)*

The Sangejo and Ghaem cultivars had different patterns in POD activity. As POD activity increased in Sangejo cultivar but decreased in Ghaem cultivar. The lowest POD activity was observed at the 120mM NaCl salinity treatment (Fig 6).

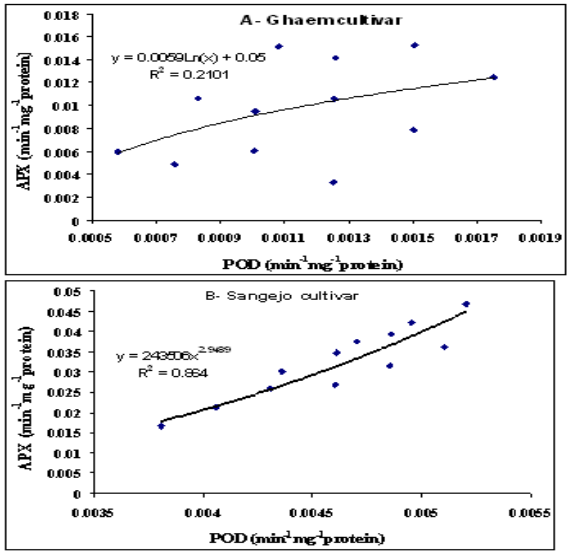


**Fig. 7.** The relationship between POD and APX activity (A- Ghaem cultivar and B- Sangejo cultivar.

PODs are involved not only in scavenging $H_2O_2$ but also in plant growth, development, lignification, suberization, and cross-linking of cell wall compounds (Passardi *et al*., 2005). Salt- tolerant plants often have higher POD activity than sensitive plants under stress conditions; this is true for salt-tolerant tomato (Shalata and Tal, 1998). Various researchers dealing with rice (Mittal and Dubey, 1991), sea purslane (Kalir *et al*., 1984) and mung bean plant (Sheoran and Garg, 1979) have also indicated an increase in POD activity in salt-tolerant cultivars under salt stress. It is not clear whether the observed increase in peroxidase activity under salt stress was due to increased activity of peroxidase encoding genes or increased activation of already existing enzymes (Dionisio-Sese and Satoshi, 1998).

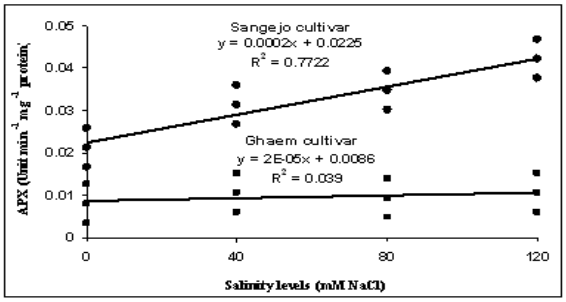


**Fig. 8.** APX enzyme activity under different salinity levels.

*Ascorbate peroxidase (APX)*

APX activity significantly increased in both cultivars after the onset of salinity stress as compared to the untreated plants (Fig 8). This is in agreement with the reports of Abbaspour (2012) in pistachio (*Pistacia vera* L.). It can be assumed that both CAT and APX which are responsible for detoxification of $H_2O_2$, have probably equally important in the detoxification process in plants. These results are consistent with observation of some researchers which revealed that APX activity coordinated with CAT activity and plays a key protective role during salt stress (e.g. Vaidyanathan *et al*., 2003). Lopez *et al* (1996) also has shown that the salt-induced increase in APX activity in radish plants was not accompanied by a corresponding increase in mRNA level, suggesting that the salt-induced ascorbate peroxidase expression is probably the consequence of post-transcriptional events. Lee and colleagues (2001) also noted an increase in enzyme activity under salt stress, and reported that there were positive correlation between these enzymes and salinity stress. Noctor and Foyer (1998) stated that plant responds to ROS by increasing the synthesis of antioxidant enzymes such as SOD, APX and CAT. Similar results were recorded for Sangejo cultivar while sensitive cultivar of Ghaem showed different reaction as APX synthesis increased. Enhancement of APX activity is an important sign of higher salinity tolerance (Koca *et al*., 2007). Bor *et al* (2003) in wild beet and Mittova *et al* (2002) in tomato also found that induced APX activity in salt tolerant plants. Several studies have pointed out that salt-tolerant species increased both their antioxidant enzyme activities and contents in response to salt treatments, whereas salt-sensitive species failed to do so (Shalata *et al*., 2001; Demiral and Türkan, 2005).

## Conclusion

To date considerable efforts have been exerted in the selection and development of rice varieties resistant to salinity stress. Progress, however, seems slow primarily due to inadequate understanding of the mechanism of salt tolerance. The results of the present study showed that some antioxidant enzymes must cooperate with each other to form a scavenging system and removing ROS. Between two studied cultivars, Sangejo as a traditional cultivar could be recommended as a salt-tolerant genotype for further

evaluation and plant breeding programs.

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