

# Effects of exogenous proline and trehalose on physiological responses in rice seedlings during salt-stress and after recovery

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## ABSTRACT

Two osmoprotectants (proline – Pro; trehalose – Tre) were exogenously supplied to seedlings of rice cvs. Pokkali (PK – salt-tolerant) and Khao Dawk Mali 105 (KDML105 – salt-sensitive) to investigate their effects on plants exposed to 200 mmol/L NaCl for 6 days and 5 days after recovery from stress. The reduction of growth, increase in  $\text{Na}^+$  to  $\text{K}^+$  ratio, high level of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content, enhanced activity of antioxidant enzymes (superoxide dismutase – SOD, peroxidase – POX, catalase – CAT and ascorbate peroxidase – APX) were observed in both rice cultivars under NaCl treatments. Exogenous Pro and Tre supplement to NaCl-stressed plants did not mitigate the reduction of growth during salt-stress. Nevertheless, during recovery plants previously supplied with Tre showed markedly higher percentage of growth recovery than those treated with NaCl alone or supplied with Pro. The beneficial effect of Tre on growth recovery was clearly demonstrated in KDML105 in which growth enhancement was related to reduction in  $\text{Na}^+$  to  $\text{K}^+$  ratio. Exogenous Pro was able to reduce  $\text{H}_2\text{O}_2$  in both cultivars during salt stress whereas Tre could reduce it only in KDML105. Exogenous Tre did not enhance any antioxidant enzymes during stress but enhanced APX activity in KDML105 during recovery. Exogenous Pro enhanced the activity of APX in PK, and POX, CAT and APX in KDML105 during both stress and recovery period.

**Keywords:** antioxidant enzymes; hydrogen peroxide; osmoprotectants; rice; salinity

Saline soil is an important factor causing relatively low production of rice in the arid, semi-arid and coastal regions. Salinity induces osmotic stress from low soil water potential, ion toxicity mainly from  $\text{Na}^+$  and  $\text{Cl}^-$  accumulation and oxidative stress from reactive oxygen species (ROS). The overproduction of ROS such as superoxide anion ( $\text{O}_2^{\bullet-}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) during salt stress causes oxidative damage to biomolecules. To counteract the ROS, plants generate non-enzymatic antioxidants as well as antioxidant enzymes i.e. peroxidase (POX), superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Sairam and Tyagi 2004). Accumulation of compatible solutes in high levels is needed to adjust the osmotic imbalance when plants confront several abiotic stresses including drought, salinity and chilling. A major category of organic compatible solutes consists of sugars such as trehalose (Tre) and fructans. Others include amino acid deriva-

tives such as proline (Pro), glycine betaine and choline *o*-sulphate (Parvaiz and Satyawati 2008).

Pro offers a wide range of protective roles including osmotic adjustment, stabilizer for cellular structure and reducing damage to photosynthetic apparatus. Tre is a non-reducing disaccharide consisting of two units of glucose. Tre can accumulate in response to abiotic stresses in several plants. Tre can act as a stabilizer, serves as an energy source, an osmolyte or a protector for protein and membrane structure (Iordachescu and Imai 2008). Application of Pro and Tre for improvement of environmental stress tolerance was reported in several plants. Hossain and Fujita (2010) demonstrated that exogenous Pro provided a protective action against salt-induced oxidative damage by reducing  $\text{H}_2\text{O}_2$  and lipid peroxidation level and by enhancing antioxidant defense and methylglyoxal detoxification systems. Exogenous Tre played a beneficial role to maize under salt

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stress by reducing the rate of ion leakage and lipid peroxidation of root cells (Zeid 2009).

Several types of chemicals including compatible solutes such as Pro, glycinebetaine and Tre were successfully used in laboratory and greenhouse experiments to enhance the plants' ability to counteract abiotic stresses (Djilianov et al. 2005). Rice is a salt-sensitive crop and salinity affects all aspects of rice growth to varying degrees at all stages of growth from germination to maturity. Increased salt tolerance of elite rice cultivars is needed to sustain food production in many drier regions of the world especially where the area of salt-affected soils are ever expanding due to increasing temperature and land clearing.

In the present study, we investigated the role of exogenous Pro and Tre on physiological parameters and antioxidant enzymes activities in seedlings of two rice cvs. Pokkali (PK – salt-tolerant) and Khao Dawk Mali 105 (KDML105 – salt-sensitive) during salt stress and after recovery period under high concentration of NaCl (200 mmol/L).

## MATERIAL AND METHODS

**Plant materials and treatments.** Rice seeds (*Oryza sativa* L. cvs. PK and KDML105) were germinated in water for 5 days at room temperature. The seedlings were then transferred to plastic chambers containing 1 L of nutrient solution pH 5.0 (Yoshida et al. 1976) and were allowed to grow for 28 days in a greenhouse under natural conditions. The average day/night temperature was 26.5/19.4°C and the relative humidity was 62–69%. The plants were exposed to 0 mmol/L NaCl (C), 200 mmol/L NaCl (NaCl), 10 mmol/L Pro plus 200 mmol/L NaCl (P + NaCl) and 10 mmol/L Tre plus 200 mmol/L NaCl (T + NaCl) for 6 days. After six-day treatment the plants were then allowed to recover for 5 days by replacing the treatment solutions with fresh nutrient solutions. The plants were harvested twice, the first after 6-day stressed period and the second after 5-day recovery for the determination of fresh (FW) and dry (DW) weights, and physiological parameters.

**Determination of ions and H<sub>2</sub>O<sub>2</sub> content.** For determination of the concentration of Na and K ions, dried shoot samples (0.1 g) were digested with 10 mL of nitric acid (300°C), 5 mL perchloric acid (200°C) and 20 mL of 6 mol/L hydrochloric acid. The concentration of Na and K ions were analyzed using an atomic absorption spectrometer (Model GBC 932 AAA, Cambridge, UK). For H<sub>2</sub>O<sub>2</sub> content, the method described by Velikova et al. (2000) was applied.

**Enzyme extraction and assay for antioxidant enzyme activities.** The leaves of rice (0.5 g) were homogenized in 10 mmol/L potassium phosphate buffer (pH 7.0) containing 4% polyvinyl pyrrolidone (PVP), the homogenates were centrifuged at 12 000 × g at 4°C for 15 min, and the supernatants were immediately used for determination of enzyme activity. Total protein was determined based on the Bradford method (Bradford 1976). The supernatant (20 µL) was mixed with 980 µL of Bradford reagent (Biorad, CA, USA) and the absorbance was measured at 595 nm. Protein concentration was determined from the standard curve using bovine serum albumin.

The methods from Beauchamp and Fridovich (1971) and Dhindsa et al. (1981) were followed for determination of SOD activity. The reaction mixture (3 mL) contained 50 mmol/L potassium phosphate buffer (pH 7.8), 13 mmol methionine, 75 µmol/L nitroblue tetrazolium chloride (NBT), 0.1 mmol/L EDTA and 0.05 mL of enzyme extract. The reaction started when adding 2 µmol/L riboflavin, the mixture was incubated under fluorescent lamps for 10 min then kept in the dark to stop the reaction. The absorbance of the mixture was measured at 560 nm. The reaction mixture with no enzyme developed maximum color due to the maximum rate of reduction of NBT. One unit of SOD was determined as the amount of enzyme that inhibits 50% NBT photoreduction. The activity was expressed as unit/min/mg protein.

The method described by Velikova et al. (2000) was applied for the detection of POX activity. The 3 mL reaction mixture contained 10 mmol/L potassium phosphate buffer (pH 7.0), 0.2% of guaiacol (w/v) and 0.04 mL of enzymes extract. The reaction mixture was incubated at room temperature for 5 min after the addition of 3 mmol/L H<sub>2</sub>O<sub>2</sub>. The absorbance was then measured at 470 nm. The activity of POX was calculated from the rate of formation of guaiacol dehydrogenation product (GDHP) using the extinction coefficient of 26.6 mmol/L/cm, and the activity was expressed as µmol GDHP min/mg protein.

For CAT activity, three milliliters of reaction mixture contained 10 mmol/L potassium phosphate buffer (pH 7.0), 0.1 mL of enzyme extract and 0.035% H<sub>2</sub>O<sub>2</sub>. The activity of CAT was calculated based on the rate of disappearance of H<sub>2</sub>O<sub>2</sub> measured as a decline in the absorbance at 240 nm. The activity was calculated using the extinction coefficient of 40 mmol/L/cm, and expressed as H<sub>2</sub>O<sub>2</sub> reduced min/mg protein (Velikova et al. 2000).

For APX assay, the reaction mixture (3 mL) contained 0.5 mmol/L ascorbic acid, 0.1 mmol/L EDTA and 0.1 mL of enzyme extract. The reaction was

started when adding 1.5 mmol/L  $H_2O_2$ . The absorbance of the reaction mixture was measured at 290 nm 2 min after  $H_2O_2$  was added. The activity of APX was calculated using the extinction coefficient of 2.8 mmol/L/cm and the activity was expressed as  $\mu\text{mol}$  ascorbate oxidized min/mg protein (Nakano and Asada 1980).

**Statistical analysis.** All the experiments were replicated four times with 12 plants per replication. All results were presented as means  $\pm$  SD. The significance of differences among the treatment means was determined by ANOVA ( $P \leq 0.05$  was considered significantly different) using the Duncan's multiple range test (DMRT). Student's *t*-test was used for comparison between the means obtained after 6-day treatment and after 5-day recovery.

## RESULTS AND DISCUSSION

**Growth parameters.** Growth of both rice cultivars during the treatment with high concentration of NaCl (200 mmol/L) was severely inhibited. Plants treated with Pro or Tre neither showed an improvement nor a reduction in growth compared with plants treated with NaCl alone (Figure 1). Either positive or negative effects of exogenous Pro or Tre combined with abiotic stresses were demonstrated depending on plant species, types

of stress, concentrations and methods of application. An experiment carried out by Heuer (2003) showed that the growth of salt-stressed tomato was exacerbated when Pro was supplemented in the growth medium. Mani et al. (2002) suggested that the toxicity from exogenous Pro was caused by an over accumulation of pyrroline-5-carboxylate (P5C) in the Pro degradation pathway. In *Arabidopsis* seedlings, Tre caused a strongly reduced root growth, failure to develop primary leaves and primary roots (Aghdasi et al. 2010). The toxicity of Tre was found to relate to the accumulation of trehalose-6-phosphate (T6P), an intermediate in Tre synthesis pathway. It was suggested that growth inhibition by T6P was associated with an interference with carbon utilization in the source tissues and starvation of carbon in the sink tissues important for growth (Schluepmann et al. 2004, Aghdasi et al. 2010). Although exogenous Pro and Tre did not improve plant growth during the salt-stress period from this study, Tre clearly enhanced plant growth (based on DW measurement) during the recovery in both cultivars. PK plants previously supplied with Tre plus NaCl showed the highest potential of growth recovery as indicated by the highest percentage (87.14%) of DW increase compared with plants supplied with NaCl alone (33.69%) and with NaCl plus Pro (8.23%) (Figure 1C). The positive effects of Tre

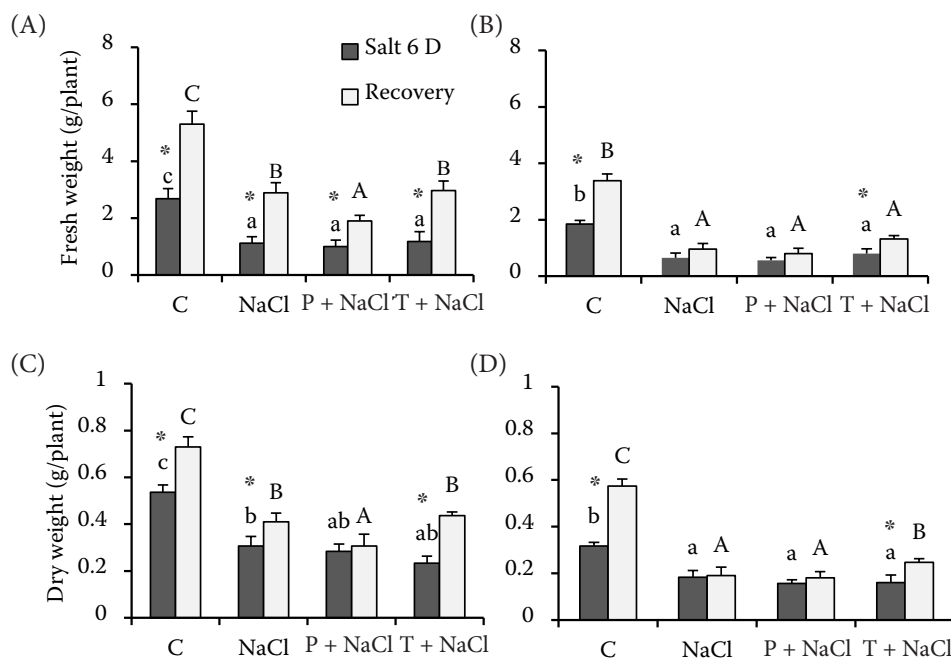


Figure 1. The effect of NaCl and exogenous proline (Pro) or trehalose (Tre) combined with NaCl on (A) FW of Pokkali (PK); (B) FW of Khao Dawk Mali (KDML105); (C) DW of PK, and (D) DW of KDML105, 6 days after stress (dark bars) and 5 days after recovery (grey bars). The values showed means  $\pm$  SD. Different small letters for the dark bars and capital letters for the grey bars indicated that the means are significantly different. \*indicates the significant difference in the mean values between 6 days after salt stress (dark bars) and 5 days after recovery (grey bars)

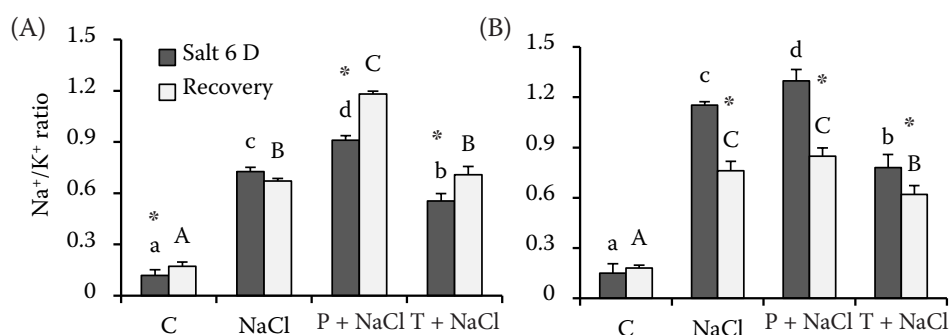


Figure 2. The effect of NaCl and exogenous proline (Pro) or trehalose (Tre) combined with NaCl on Na<sup>+</sup> to K<sup>+</sup> ratio of (A) Pokkali (PK), and (B) Khao Dawk Mali (KDML105) seedlings 6 days after stress (dark bars) and 5 days after recovery (grey bars). For details of statistical symbols see Figure 1

during recovery were even more pronounced in salt-sensitive KDML105. In the absence of Tre, stressed KDML105 plants were unable to recover (both FW and DW remained unchanged after 5-day recovery), whereas 54.17% DW increase was observed in KDML105 previously supplied with Tre (Figure 1D). It is possible that T6P which accumulated during stress was metabolized to usable sugars resulting in improved growth during recovery in the Tre-treated plants of both cultivars.

**Ratio of Na<sup>+</sup> to K<sup>+</sup>.** As shown in Figure 2. The ratio of Na<sup>+</sup> to K<sup>+</sup> increased appreciably when plants of both cultivars were under salt stress for 6 days. Exogenous Pro exacerbated the ion balance by further increasing Na<sup>+</sup> to K<sup>+</sup> ratio in both PK and

KDML105. In contrast, Tre showed the beneficial effect by significantly reducing the Na<sup>+</sup> to K<sup>+</sup> ratio in both rice cultivars. These results corresponded with the findings of Garcia et al. (1997) in two rice cultivars PK and IR 29 (salt-sensitive) that Pro either had no effects or in some cases exacerbated the effects of NaCl on ion balance while Tre reduced Na<sup>+</sup> accumulation. Similar results were observed in salt-stressed maize pre-treated with Tre (Zeid 2009). Furthermore, in transgenic rice which received *otsA* and *otsB* gene (TPS and TPP in higher plants) from *Escherichia coli*, was able to maintain a higher level of selectivity for K<sup>+</sup> over Na<sup>+</sup> uptake, resulting in lower ratio of Na<sup>+</sup> to K<sup>+</sup> (Garg et al. 2002). Five days after recovery from

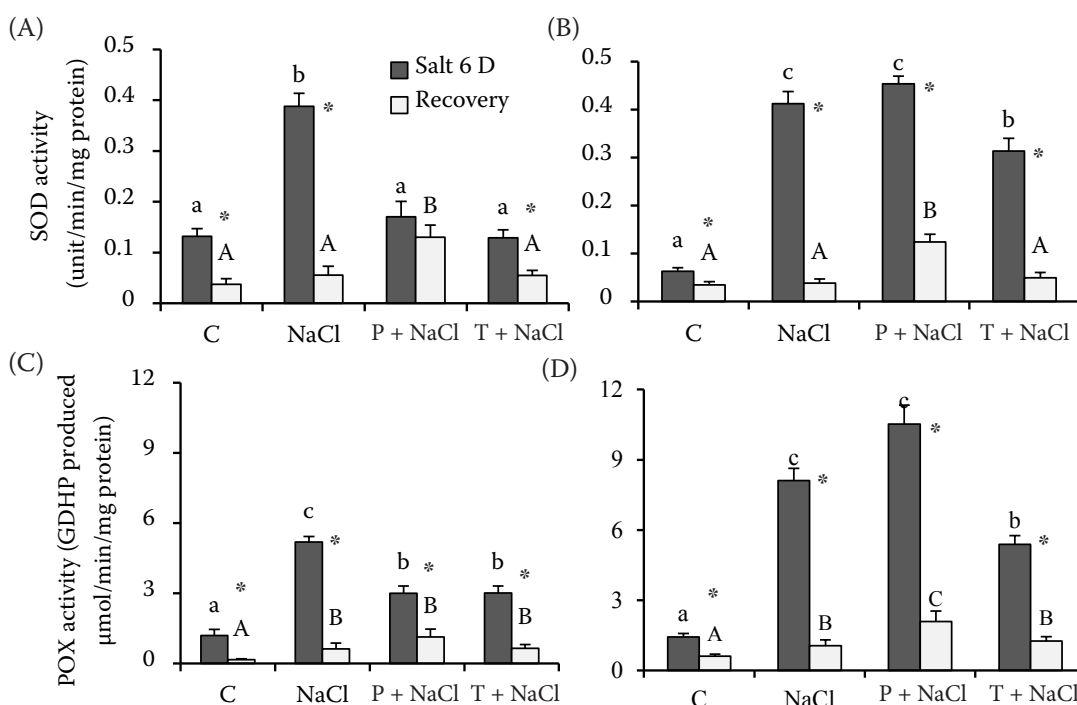


Figure 3. The effect of NaCl and exogenous proline (Pro) or trehalose (Tre) combined with NaCl on superoxide dismutase (SOD) and peroxidase (POX) activity in rice seedlings 6 days after stress (dark bars) and 5 days after recovery (grey bars). (A) SOD in Pokkali (PK); (B) SOD in Khao Dawk Mali (KDML105); (C) POX in PK, and (D) POX in KDML105. For details of statistical symbols see Figure 1

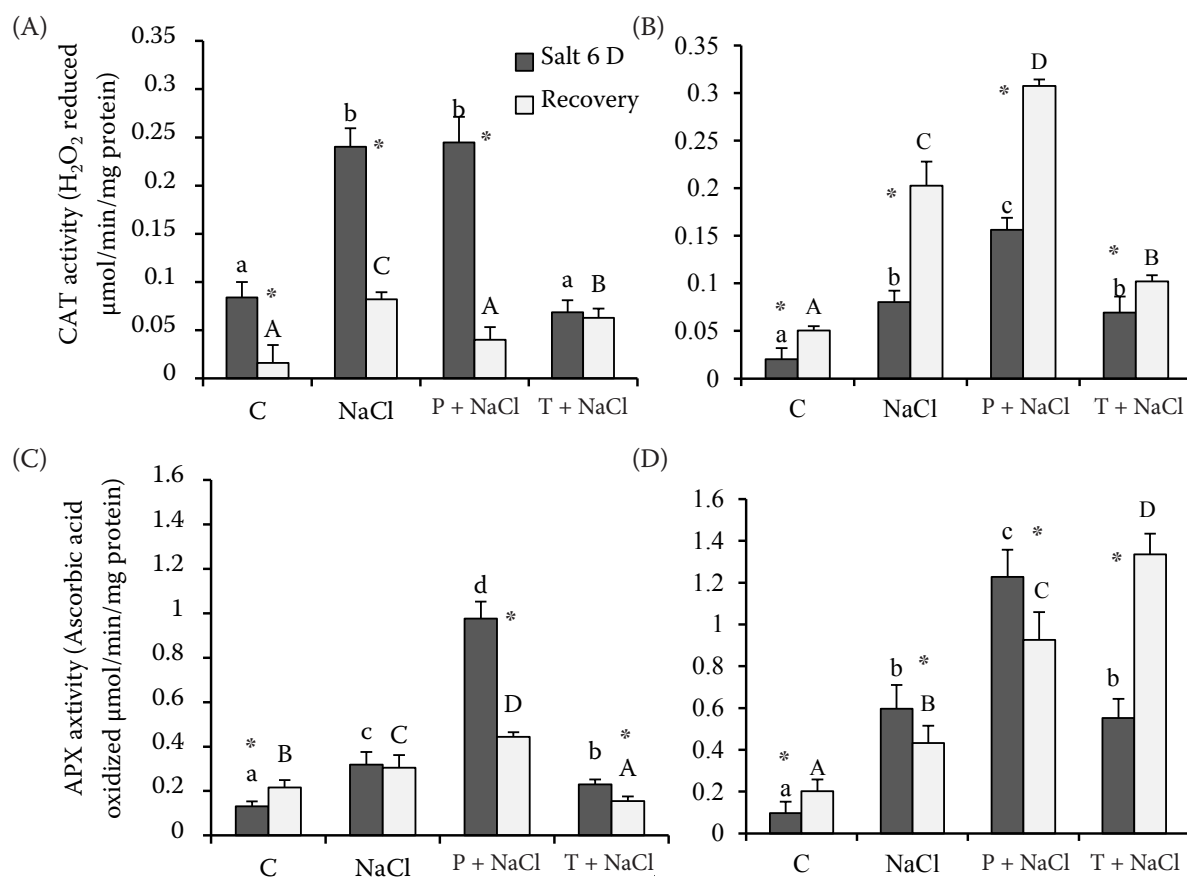


Figure 4. The effect of NaCl and exogenous proline (Pro) or trehalose (Tre) combined with NaCl on catalase (CAT) and ascorbate peroxidase (APX) activity in rice seedlings 6 days after stress (dark bars) and 5 days after recovery (grey bars). (A) CAT in Pokkali (PK); (B) CAT in Khao Dawk Mali (KDML105); (C) APX in PK, and (D) APX in KDML105. For details of statistical symbols see Figure 1

stress, in PK, the Na<sup>+</sup> to K<sup>+</sup> ratios in the NaCl-stress group decreased after the salt stress was removed. Interestingly, in salt-tolerant PK the presence of Pro or Tre during salt stress resulted in a marked and significant increase in Na<sup>+</sup> to K<sup>+</sup> ratios during the recovery period. For KDML105, the Na<sup>+</sup> to K<sup>+</sup> ratios in KDML105 plants previously treated with NaCl (NaCl, P + NaCl and T + NaCl) dramatically decreased from the values on day 6 after stress.

**Activity of antioxidant enzymes (SOD, APX, POX and CAT) and H<sub>2</sub>O<sub>2</sub> content.** It was noted that, in the normal non-stressed conditions, PK had higher activities of SOD, CAT and APX than KDML105. Under salt stress PK had much higher activity of CAT. On the other hand, salt-stressed KDML105 had greater activities of POX and APX. The activity of SOD during salt stress was raised to a similar level for both cultivars.

The data obtained in this work are in good agreement with the results of Vaidyanathan et al. (2003) that the salt-tolerant (PK) rice showed higher activity of CAT than the salt-sensitive (cv. PB) rice. Demiral and Türkan (2005) also found

that CAT activity in PK roots greatly increased in response to salt stress but the activity in IR-28 (salt-sensitive) remained unchanged. Our observation that salt-induced POX activity was higher in the salt-sensitive rice was consistent with that reported by Dionisio-Sese and Tobita (1998) which compared rice cv. Hitomebore (salt-sensitive) with PK. Similarly, Demiral and Türkan (2005) also observed higher activity of POX during salt-stress in salt-sensitive rice (cv. IR28) than in PK. The APX activity under salt stress (200 mmol/L) in this investigation was higher in KDML105 than PK.

For PK plants supplied with Pro and NaCl, the high activities of CAT and APX (Figure 4A and 4C) were coinciding with lower concentration of H<sub>2</sub>O<sub>2</sub> (Figure 5A). Feeding Pro to salt-stressed KDML105 enhanced POX, CAT and APX activities (Figure 3D, Figure 4B and 4D) which related to lower H<sub>2</sub>O<sub>2</sub> accumulation (Figure 5B). Similar effects of exogenous Pro on H<sub>2</sub>O<sub>2</sub> reduction were also observed in mung bean seedlings (Hossain and Fujita 2010) and olive plants under salt stress (Ahmed et al. 2010).



Exogenous Pro added in combination with NaCl tended to have the opposite effects on PK and KDML105 in relation to SOD and POX activities (Figure 3). The activity of CAT remained unchanged for PK but increased in KDML105 (Figure 4A and 4B). The activity of APX activity increased to a similar extent in PK and KDML105 (Figure 4C and 4D). Similar to our observation, external supply of Pro to plants under stress conditions was reported to suppress SOD activity in common ice plant under paraquat-induced stress (Shevyakova et al. 2009). Huang et al. (2009) demonstrated that exogenous Pro also enhanced POX activity in salt-stressed cucumber (a salt-sensitive cultivar). The elevated CAT activity under salt stress in response to exogenous Pro was also found in olive plants (Ahmed et al. 2010). In grapes exogenous Pro enhanced APX activity under oxidative stress (Ozden et al. 2009).

Exogenous Tre supplied to NaCl-stressed PK plants caused a significant reduction in all antioxidant enzymes investigated compared with plants treated with NaCl alone (Figure 3A and 3C, Figure 4A and 4C) leading to higher accumulation of  $H_2O_2$  (Figure 5A). For KDML105, although exogenous Tre suppressed SOD and POX activities (Figure 3B and 3D) it did not affect CAT and APX (Figure 4B and 4D). Therefore CAT and APX presumably acted in concert with other ROS-scavenging systems to control  $H_2O_2$  level in KDML105 (Figure 5B). The data obtained in this work were different from the findings of Ali and Ashraf (2011) that exogenous Tre significantly increased POX and CAT activities in maize under drought stress.

For the salt-stress groups (without Pro or Tre) after recovery, in most cases, both antioxidant enzyme activities (except CAT activity in KDML105) and the  $H_2O_2$  content markedly reduced presumably because ROS-generating activities associated with NaCl-stress were much reduced and hence the reduction in antioxidant enzyme activities.

Lee et al. (2001) reported in rice during recovery from salt stress that CAT activity increased and  $H_2O_2$  decreased whereas SOD, POX and APX activities decreased. Khan and Panda (2008) recently reported that the activities of POX and CAT increased and  $H_2O_2$  decreased in roots of rice after relieving from NaCl stress.

A significant decrease in  $H_2O_2$  level was also observed in PK plants previously supplied with Pro. Low level of  $H_2O_2$  was most related to APX whose activity in P + NaCl plants during recovery was higher than those found in the NaCl-stress and the T + NaCl treatments. The effect of Tre was different from Pro; T + NaCl plants during recovery showed lower activities of all antioxidant enzymes, hence relatively high  $H_2O_2$  content.

For KDML105 after recovery,  $H_2O_2$  content was markedly increased in plants previously supplied with Pro or Tre and the concentration was higher in Pro- than Tre-treated plants (Figure 5B). The activities of POX, CAT and APX in the P + NaCl plants were significantly higher than those of the plants treated with NaCl without Pro. However, the enhanced levels of these antioxidant enzymes could not efficiently detoxify  $H_2O_2$ . In case of exogenous Tre, substantial enhancement of APX during recovery presumably led to lower  $H_2O_2$  level in T + NaCl than P + NaCl plants.

In conclusion, the mitigating effect of exogenous Tre was evident during the recovery period by increasing the potential for growth recovery and the effect was more pronounced in the salt-sensitive cultivar which was related to the reduction in  $Na^+$  to  $K^+$  ratio. During salt stress and recovery, Pro supplement promoted APX activity in the salt-tolerant rice and enhanced APX as well as CAT and POX in the salt-sensitive rice. The beneficial effect of exogenous Tre on antioxidant enzymes was the most pronounced in salt-sensitive rice during recovery period by greatly stimulating APX activity.

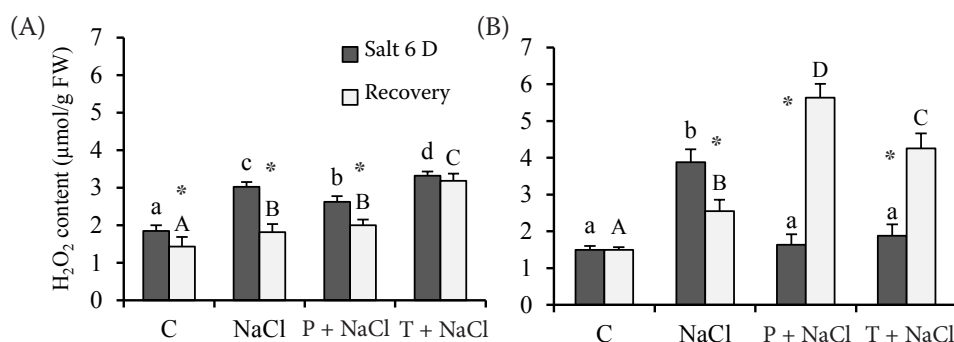


Figure 5. The effect of NaCl and exogenous proline (Pro) or trehalose (Tre) combined with NaCl on  $H_2O_2$  content in (A) Pokkali (PK), and (B) Khao Dawk Mali (KDML105) seedlings 6 days after stress (dark bars) and 5 days after recovery (grey bars). For details of statistical symbols see Figure 1

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