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Research Article

Role of antioxidative enzymes activity in salt stress and salinity screening in rice grown under *in vitro* condition

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Abstract
Role of antioxidative enzyme activity in salt stress and salinity screening was studied in the callus of two rice cultivars (White ponni and BPT-5204). The antioxidant activities of the rice callus were determined by analyzing three enzymes activity namely, Superoxide Dismutase (SOD), Catalase (CAT) and Ascorbate Peroxidase (APX) grown under saline condition. Enzymes were analysed in 15 days old rice callus culture grown under salt stress and non-saline conditions. All the three enzyme activities were varied according to salt concentrations in the medium. SOD and CAT activities were higher in BPT-5204 but APX activities were higher in White ponni. Among the NaCl treatment, medium containing 40 mM NaCl observed higher enzyme activity than 20 and 30 mM NaCl. Under non saline condition there is no significant difference was noticed in the enzymatic activities and callus growth parameter in both the cultivars. Observation was carried out on the change in callus growth parameter like weight and color of the callus. There was a significant reduction in weight and also change in colour of the callus was noticed with respect to higher salt concentrations (30 and 40 mM NaCl) in the medium for both the cultivars. The observed data indicated that rice plant responds well to salt-induced oxidative stress by increasing their enzymatic antioxidant defense systems. The antioxidant enzyme activity play vital role in defense against salt stress and this may help to screen the salt tolerant line grown under *in vitro* condition in early callus stage itself.

Keywords:
Rice, antioxidative enzyme, salinity screening, callus culture.

Introduction:

Salinity is one of the important abiotic stress limiting plant productivity (Munns *et al*., 2002). Worldwide 19.5% of the irrigated agricultural land is considered as saline (Flowers, 2004). Rice is considered as stable food over half of the world population. Rice is characterized as the salt sensitive crop. Plants exposed to saline environment suffer from ion excess or water deficit and oxidative stress linked to the production of reactive oxygen species (ROS), which cause damage to lipids, proteins and nucleic acids (Hernandez *et al*., 2000).

Oxidative stress is considered to be one of the major damaging factors in plant cells exposed to salinity (Gossette *et al*., 1994; Hernandez *et al*., 1995; Khan and Panda, 2008; Queiros *et al*., 2007). Plants possess a number of antioxidant enzymes that protects them from these potential cytotoxic effects (Gossette *et al*., 1994 ; Kusvuran *et al*., 2012; Li, 2009; Chookhampaeng, 2011).

When plants are subjected to environmental stress such as temperature extremes, drought, herbicide treatment and mineral deficiency, the balance between the production of ROS and the quenching activity of antioxidants is upset, often resulting in oxidative damage. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage (Yasar, 2007; Dolatabadian *et al*., 2008; Amirjani, 2010; Siringam *et al*., 2011).

In recent years tissue culture techniques are being used as a useful tool to elucidate the mechanism involved in salt tolerance by using *in vitro* selected salt tolerant cell lines (Lutts *et al*., 2004; Venkataiah *et al*., 2004).

Research on squash and eggplant indicated that superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) enzyme activities in salt-tolerant genotypes are higher compared to salt-susceptible genotypes in both seedling and callus tissues (Yasar, 2003; Sevengor, 2010). The aim of this study was to elucidate the activities of three antioxidative stress enzymes (SOD, APX and CAT) in two rice cultivars grown under salt-stress conditions and screening saline tolerant line grown under *in vitro* condition.

Material and methods

Explants preparation: Present study was carried out at Agricultural College and Research Institute, Killikulam during 2012, under *in vitro* condition. Healthy seeds rice varieties viz., White Ponni and BPT 5204 were manually dehusked and

surface sterilized with 0.1 % (w/v) $HgCl_2$ for 15 min followed by 70 % ethanol for 30 sec under aseptic conditions in a laminar air flow chamber. Seeds were thoroughly rinsed four or five times with sterile distilled water and blotted in 90 mm Petri dishes containing a layer of filter paper.

Growth media and culture condition**:** Seeds were inoculated for callus induction on MS (Murashige and Skoog, 1962) salts and vitamins supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kinetin. Media were treated with various concentration of NaCl (20, 30 and 40 mM) along with control. Inoculated seeds were kept under dark condition with temperature range of 23 ± 2ºC for 15 days. Embryogenic callus were transferred to fresh media for callus proliferation, then callus was studied to determine the various antioxidative enzyme activities.

Salt treatment: Callus induction media was treated with different concentration of NaCl (20, 30 and 40 mM). Media were disbursed in petridishes and incubated under aseptic controlled conditions. Seeds were inoculated on salt treated media for callus induction. Each NaCl treatment was maintained with 15 plates.

Enzyme assays: All the enzyme assays such as SOD, APX and CAT were performed at 30º C under controlled condition using spectrophotometer (model: UV – 600 (PC), Manufacturer: Tomos Life Science Group Pvt Ltd).

Enzyme extraction: Frozen control and salt treated callus samples (0.3 g each) were ground into a fine powder using liquid nitrogen in a chilled mortar and pestle. The powder was then suspended in 3 ml of sodium phosphate buffer (0.1 M, pH 6.2) and then centrifuged at 8000 X g for 15 min at 4º C. The supernatant was used to estimate the activities of antioxidative enzymes namely superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). To prevent denaturation of enzymes, all steps during extraction were carried out at 4ºC. SOD was assayed according to Cakmak and Marschner (1992), by monitoring the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm. It was defined that 1 unit of SOD activity was the amount of enzyme which causes 50% inhibition of the photochemical reduction of NBT. CAT activity was determined by monitoring the disappearance of hydrogen peroxide (H_2O_2) according to the method of Cakmak and Marschner (1992).

Super oxide dismutase (SOD) assay: SOD was assayed according to Cakmak and Marschner (1992). Assay mixture for SOD activity contained 1.15 ml distilled water 1.2 ml of pyrophosphate buffer (0.05 M, pH8.3), 0.1 ml Phenazine meta sulfate (PMS - 186 µM), 0.3 ml Nitroblue tetrazolium (NBT-300 µM) and 0.05 ml of enzyme extract. At the end 0.2 ml NADH was added to start the reaction. It was mixed well to ensure the reaction proceeded properly. The test tubes were incubated at 30º for 90 sec, after which 1 ml of glacial acetic acid was added to stop the reaction. The optical density (OD) was read at 560 nm.

Catalase (CAT) assay**:**The reaction mixture was contained 0.6 ml of phosphate buffer (0.1M. pH 7) in 0.1 ml of enzyme extract. 0.3 ml of hydrogen peroxide H_2O_2, 75 mM was added in the reaction mixture at the end with following immediate observation for decrease in OD at 240 nm for 3 min. H_2O_2 concentration is taken so as to get a starting OD ~0.7-0.8.

Ascorbate Peroxidase (APX) assay: Assay mixture for APX activity was contained 0.15 ml of distilled water, 0.5 ml of sodium phosphate buffer (0.1M, pH7), 0.1 ml of sodium ascorbate (5 mM) and 0.1 ml of EDTA (10 mM) in 0.05 ml of enzyme extract. In this mixture 0.1 ml of 1 mM, H_2O_2 was added and immediately observed for decrease in OD at 290 nm for 2 min.

Statistical analysis: The experiment was laid out in a Completely Randomized Block design, each NaCl treatment with 15 plates and each plate with three calli under *in vitro* conditions. Data was recorded on callus growth parameter like weight of the callus and antioxidative enzymes namely APX, CAT and SOD activites. Experimental data was analysed with AGRES software, detailed results and discussion were furnished below.

Results and discussion

Effect of NaCl on callus growth parameters: Callus induction on control and salinized medium with different concentration of NaCl is presented in Fig. 1. from which all enzyme analysis was carried out. Results on the effect of NaCl on rice callus were presented in Table 1. The callus inductions of both the cultivars were noticed considerably slow on media containing NaCl. White ponni has observed the lowest callus induction, proliferation and callus weight on salt treatment. Decreasing ratio of callus weight in White ponni was 37.66 mg, 33 mg, 27.67 mg and 20 mg respectively from control to 40 mM NaCl treatment, while it was 43 mg, 37.33 mg, 28.67 mg and 20.83 mg in BPT-5204. Results clearly revealed that salt concentration above the permissible level should detrimental to callus and ultimately reduce the callus proliferation rate. In the callus culture, all of the conditions were optimized such as growth media and continued darkness. In White ponni, on the 8th day of the salt application, a significant difference was observed in color compared to the controls (Fig 1.).

Effect of salinity on Superoxide dismutase (SOD) activity**:** SOD activities in callus grown under non saline condition were found to be significantly lower than on saline conditions in both the cultivars. Salt stress caused an increase in the SOD activity in both the genotypes. An increase in SOD activities of BPT-5204 was higher than White ponni. The SOD activity in BPT-5204 treated with 40 mM NaCl was (759.11 unit / g FW) higher than those measured in the control (564.40 unit/ g FW) (Table 1.). White ponni showed lower SOD activity (617.70 unit/g FW) with 20 mM NaCl treatment. The SOD activity of White ponni was increased slightly with reference to different salt concentration in the medium. On the other hand, SOD activity in BPT-5204 was found differed among various salt concentrations was coincide with earlier report. Increases in SOD activity and differential varietal salt susceptibility have also been reported in salt-treated wheat (Sairam *et al*., 2002), rice (Khan and Panda, 2008), and cucumber (Baysal and Tipirdamaz, 2010). Zaefyzadeh *et al*. (2009) reported that SOD production is one of the stress confrontation systems under oxidative stress that is activated in drought and salinity conditions.

Effect of salinity on Catalase (CAT) activity**:**The Catalase (CAT**)** activity were analysed on the 8th day old rice callus culture grown on various NaCl medium showed variation in CAT activities in both the genotypes compared with the control. The highest CAT activity was observed in BPT-5204 with a value of 0.0426 unit/ g FW in callus grown on media treated with 40 mM NaCl. The variety BPT-5204 showed higher CAT enzyme activity under salt stress condition. It was followed by White ponni with a value of 0.0353 unit/g FW at 40 mM NaCl. (Table 1). This findings coincided with previous studies reported that CAT activities were higher in varieties of pumpkin (Sevengor, 2010) and in barley (Perez-Lopez *et al*., 2009). They concluded that CAT activity is important for the elimination of H_2O_2 under salinity.

Effect of salinity on Ascorbate Peroxidase (APX) activity**:**White ponni showed two times increase in APX activity (0.9461 unit / g FW) at 40 mM NaCl and it was higher than BPT-5204 (0.4834 unit /g FW). Both these genotypes showed greater APX activity in NaCl condition than under control (Table 1). The APX activity was increased with increased concentration of salt stress in the medium. Treatment with NaCl caused an increase in the activities of APX in rice roots (Tsai *et al*., 2005). The enhanced activities of APX were found to be higher in White ponni. The induced activity of APX was increased with increase concentration of salt in the medium. Higher antioxidative capacity conferring salt tolerant in rice under salt-stress has been reported earlier (Nguyen *et al*., 2005; Pal *et al*., 2004).

Effect of antioxidative enzymes on reactive oxygen species (ROS) under salt stress: In the plant cells certain reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals can be responsible for cellular damage under stress conditions was reported by Foyer et al., 1994 and Mitler, 2002. Under salt stress, cellular homeostasis is disrupted and leads to the production of relatively high levels of ROS (Huang et al., 2009). These radicals can damage vital cellular macromolecules (example via denaturation of proteins, peroxidation of lipids). Plants have evolved both enzymatic and nonenzymatic mechanisms to scavenge ROS (Asada, 1999; Kusvuran et al., 2012). Several reports have clearly demonstrated enhanced activity of various antioxidant enzymes under salt stress conditions (Huang et al., 2009). The SOD enzyme destroys the superoxide radical. It was stated by other researchers that SOD activity increases with salt application (Gossett et al., 1994; Lin and Kao, 2000). This report is concordance with our result. The SOD enzyme activity under salt stress condition was showed increased in concentration in both the cultivars with reference to concentration of salt in the medium. Under controlled condition there is no variation is observed in SOD activity but BPT-5204 exhibited higher SOD enzyme activity than White ponni. CAT eliminates H_2O_2 by decomposing it directly to water and oxygen (Yasar et al., 2007; Amirjani, 2010). Salt treatment increased CAT activities in both the varieties compared with the control plants. Similar results have been reported by Perez-Lopez et al. (2009). They concluded that CAT activity is important for the elimination of H_2O_2 under salinity.

In conclusion, the antioxidative enzyme activities play a protective role against salt stress. Antioxidative defense mechanism was effective in providing tolerance to salt stress in rice plantlets grown on salinized media. These results suggests that BPT-5204 performed well in all aspects like by increased activity of SOD, CAT and lower reduction in callus fresh weight except APX activity was higher in White ponni under salt stress conditions. Callus culture could be a useful screening method for determining the salt tolerance level of rice genotypes. The change in callus fresh weight under salt stress was not found to be indicative for detecting the salt tolerant rice variety but enzyme activities provided relevance to saline tolerant line screening. The fact that the callus culture can be used very easily in experiments made for enzyme analyses, because it provides a homogeneous samples without residues and also easier to enzyme analysis. The increasing ratio in CAT activity

and moreover, SOD activity in the rice culture after salt treatment under *in vitro* condition could be useful to screen the plant for salt-tolerance. This could suggests that antioxidative enzymes behave as an important enzyme marker for screening salt tolerant genotypes from susceptible one in callus stage itself.

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Table 1. Effect of NaCl on enzyme activities and on fresh weight of the callus in two rice cultivars

Cultivars	(Callus fresh weight (mg))				CD 0.05	CD 0.01
	Control	20 mM	30 mM	40 mM		
White Ponni	37.66 ± 1.25	33.00 ± 0.82	27.67 ± 1.25	20.00 ± 1.63	0.2089	0.2878
BPT-5204	43.00 ± 2.16	37.33 ± 0.47	29.67 ± 1.25	21.67 ± 1.25		
	Superoxide dismutase (SOD (unit / g FW))					
White Ponni	520.81 ± 19.3	617.70 ± 20.1	683.00 ± 20.5	754.07 ± 30.40	28.30	39.00
BPT-5204	564.40 ± 16.04	648.82 ± 20.68	711.14 ± 9.50	759.11 ± 9.05		
	Ascorbate Peroxidase (APX (unit / g FW))					
White Ponni	0.3248 ± 0.0327	0.2753 ± 0.0154	0.7072 ± 0.0157	0.9461 ± 0.0282	0.0259	0.0357
BPT-5204	0.3712 ± 0.0026	0.3756 ± 0.0024	0.4190 ± 0.0048	0.4834 ± 0.0012		
	Catalase (CAT (unit / g FW))					
White Ponni	0.0179 ± 0.0006	0.0210 ± 0.0009	0.0238 ± 0.0002	0.0353 ± 0.0004	0.0009	0.00132
BPT-5204	0.0230 ± 0.0009	0.0323 ± 0.0006	0.0370 ± 0.0001	0.0426 ± 0.0006		

± Denote Standard deviation
Figures in parentheses are percentage increase\ decrease over control.

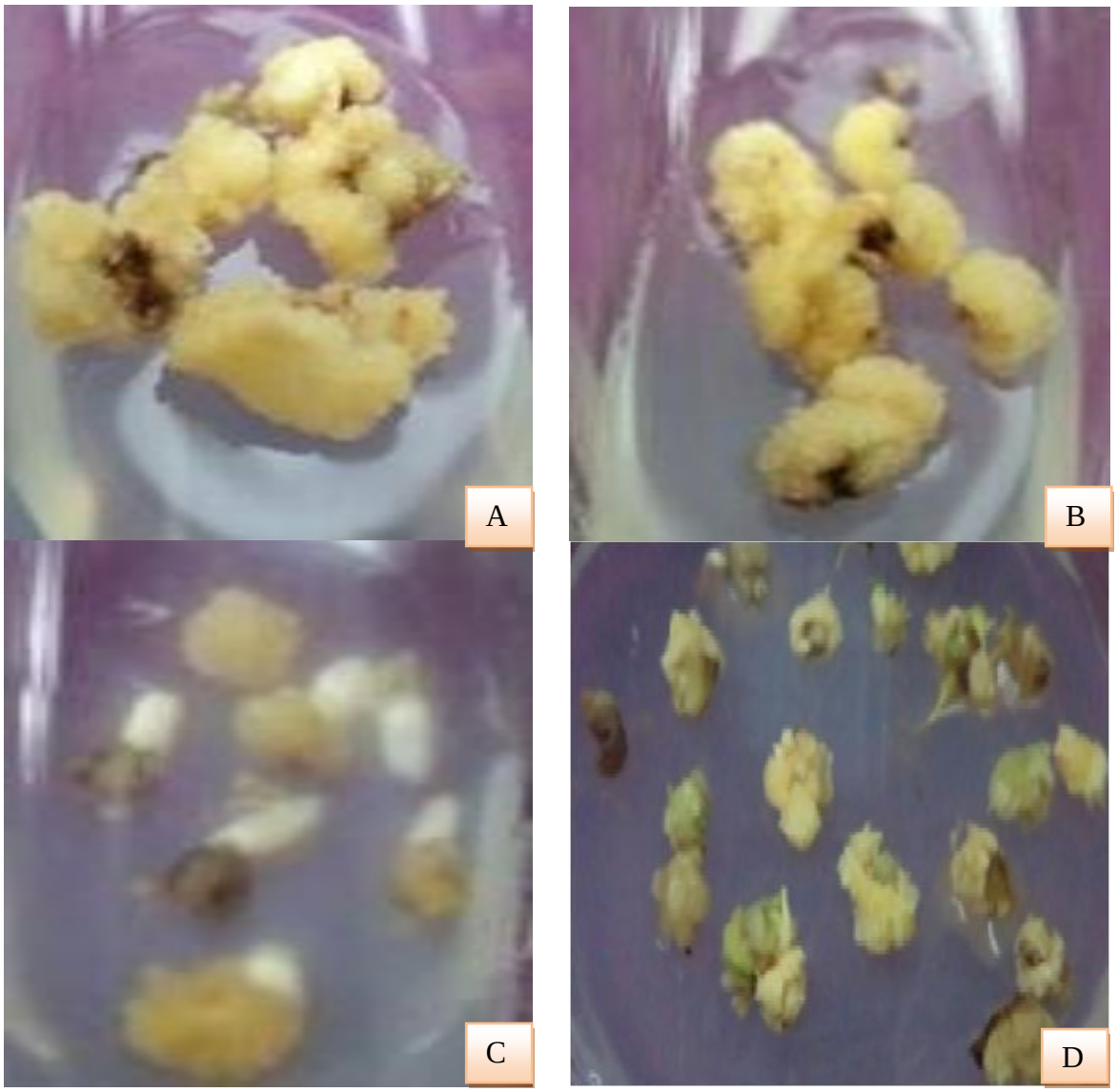

Figure 1. Callus induction on salinized medium, A: control, B: 10 mM, C: 20 mM, D: 30 mM NaCl,