ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 10, pp. 13964-13969, October, 2016

International Journal of Recent Scientific Research

Research Article

COMPARATIVE STUDY OF ANTIOXIDANT ENZYMES IN TOLERANT AND SENSITIVE RICE VARIETIES SUBJECTED TO SALINITY STRESS

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ARTICLE INFO

Article History:

Received 17th July, 2016
Received in revised form 12th August, 2016
Accepted 04th September, 2016
Published online 28th October, 2016

Key Words:

Antioxidant enzymes, Ascorbate, Glutathione, khazan, Salinity, Superoxide dismutase

ABSTRACT

Comparative study of antioxidant enzymes in salinity tolerant and sensitive rice varieties commonly grown in khazan fields of Goa was undertaken, with an objective to understand the possible role of antioxidant enzymes . Jaya, Jyoti and Korgut (salinity tolerant, sensitive hybrid and tolerant non-hybrid rice varieties resp.) were grown in laboratory conditions with relative humidity (RH) of 70% at 25^0C, 200µmol m^{-2} s^{-1} light intensity for 2 weeks and were further subjected to salinity stress (50, 100 and 150 mM NaCl) for 12 days. Spectrophotometric analysis of enzymatic & non enzymatic antioxidants such as, Superoxide dismutase (SOD), Peroxidase (POD), Ascorbate peroxidase (APX), Glutathione reductase (GR), Total ascorbate (ASC+DHA) and reduced glutathione (GSH) were studied. The present study showed an increase in enzymatic and non enzymatic antioxidants like SOD, GR and GSH in all the three rice varieties. However, the APX activity declined in Jaya and Jyoti in comparison to Korgut. The data thus indicate, the adaptability of these rice varieties to given salinity stress with Korgut being the most tolerant rice variety.

INTRODUCTION

In nature, plants are exposed to variety of biotic and abiotic stresses such as pathogens, adverse temperature, excess of light, salinity, chilling and heavy metals (Andras *et al.*2012). Abiotic stresses like salt stress is one of the major stresses affecting plant growth and development resulting in significant crop loss with overall reduction in agricultural productivity (Todaka *et al.* 2012, Zhang *et al.* 2011a, Joseph and Jini 2011, Munns and Tester 2008). Salt stress leads to ionic and osmotic imbalance followed by alteration in metabolic rate in plants (Quadir *et al.* 2008, Chen *et al.* 2007). Hyper-ionic stress increases the production of ROS like superoxide ions ($O2^{\bullet}$), hydroxyl radicals ($^{\bullet}OH$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) (Kundan *et al.* 2013). Enhanced production of ROS thus, pose a threat to the cellular component with lipid peroxidation, protein oxidation, enzyme inhibition and activation of programmed cell death (PCD) (El-Shabrawi *et al.* 2010). Plants, however maintain an equilibrium between ROS production and its scavenging by efficient enzymatic and non enzymatic anti-oxidative system (Miller *et al.* 2010, Noctor and Foyer 2011).

Amongst the non enzymatic antioxidative defense system, Ascorbate–glutathione (ASC-GSH) cycle has been regarded as the most effective detoxifying mechanism of antioxidant pathway (Anjum *et al.* 2011). Role of enzymatic antioxidants like Catalase (CAT), Ascorbate peroxidase (APX), Monodehydroascorbate Reductase (MDHAR), Dehydroascorbate Reductase (DHAR) & Glutathione reductase (GR) in detoxification of H_2O_2 through Foyer-Halliwell-Asada pathway is well understood (Foyer and Halliwell 1976, Halliwell 2006).

Rice is the staple food of Goa, and is largely grown in low lying areas called Khazan fields that are subjected to salinity stress. Korgut is a traditional non-hybrid rice variety grown in these fields over the years. However, in recent years it has been replaced by high yielding salinity tolerant & sensitive hybrid varieties like Jaya and Jyoti. Although rice is a glycophyte, yet is highly sensitive to salinity stress (Kumar *et al.* 2008). Understanding the importance of rice cultivation in the khazan fields, the present work was undertaken with an objective to compare the role of antioxidants in tolerant and sensitive rice varieties subjected to salinity stress.

MATERIALS AND METHODS

***Plant material*:** Hybrid seeds of salinity tolerant Jaya and sensitive Jyoti rice varieties were obtained from ICAR old Goa, while, traditional non–hybrid Korgut rice seeds were obtained from local farmers.

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Growth condition and salinity treatment: The seeds were surface sterilized with 0.1 % Mercuric chloride ($HgCl_2$) solution for about 5 minutes and were then thoroughly washed 3-4 times with distilled water. Seeds were soaked in distilled water overnight, drained, for tied to muslin cloth for sprouting. Sprouted seeds were then potted in plastic pots containing soil: sand: peat (1:1:2 v/v) and grown in the laboratory condition under illumination at 25^0C, Relative Humidity (RH) of 70% and 200µmol m^{-2} s^{-1} light intensity for 2 weeks. Two weeks old rice seedlings were then subjected to salinity stress (50, 100, 150mM NaCl) by regular watering with respective salt solution. The plants were grown for next 12 days. Control plants were grown in distilled water alone. After 12 days of salinity treatment, the fully expanded leaf tissue was harvested, frozen in liquid Nitrogen (Liq. N_2) and used for various enzymatic assays.

Enzymatic antioxidants

Superoxide dismutase enzyme activity: Spectrophotometric analysis of superoxide dismutase (SOD; EC 1.15.1.1) enzyme was performed according to the method of Beauchamp & Fridovich (1971). Leaf samples (0.5gm) were homogenized in 3 mL of 25 mM ice cold phosphate buffer (pH 7.8). The homogenate was then centrifuged at 18,000 rpm at 4 C for 30 min and supernatant was used for the assay. The reaction mixture (3 mL) contained 1mM Riboflavin, 12mM L-Methionine, 0.1mM EDTA, 50mM $NaCO_2$, 75 µM NBT and 200 µl of plant enzyme extract. The test sample was shaken and illuminated with 120W lamp for 15 mins and absorbance was read at 560nm. An unilluminated tube without enzyme was taken as a blank and the activity was expressed in terms of UmL^{-1}.

Peroxidase enzyme activity: Peroxidase assay (POD; EC 1.11.1.7) was performed according to the method of Motonaka Kuroda *et al*. (1996). Leaf tissue (0.2gm) was homogenized in 50mM Phosphate buffer (pH 7.0). The homogenate was then centrifuged at 15,000 rpm at 4 C for 15 min and the supernatant was used as enzyme source. The reaction mixture (3mL) contained 50mM phosphate buffer (pH 7.0), 3.4 mM Guaicol, 0.9 mM H_2O_2 and 100µL enzyme extract. Activity of Peroxidase was monitored at 436 nm.

Ascorbate Peroxidase (APX) enzyme activity: Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to the method of Rama-devi & Prasad (1998). Leaf sample (0.1 gm) was homogenized in 5mL of 50mM Tris HCL buffer (pH 7.8). The homogenate was centrifuged at 12000 rpm at 4 C for 20 min and supernatant was used as enzyme source. The reaction mixture in a final volume of 3ml contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM sodium ascorbate, 0.1 mM H_2O_2, 2 mM ascorbate and 100µL enzyme. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of ascorbate oxidized was calculated using extinction coefficient (2.8 mM^{-1}).

Glutathione Reductase (GR) enzyme activity: Glutathione Reductase (GR; EC 1.6.4.2) activity was performed according to the method of Smirnoff & Colombe (1988). Leaf tissue was homogenized in ice cold 0.1M HCL in 1mM EDTA buffer (pH 7.0). The homogenate was then centrifuged at 12000 rpm at 4 C for 10 min and supernatant was used as enzyme source. The reaction mixture in a final volume of 3ml contained 100mM Tricine KOH (pH 7.6), 3.24 mM NADPH, 16 mM oxidized glutathione and 300 µL of plant extract. Rate of NADPH oxidation was then monitored at 340nm.

Ascorbate and Dehydroxyascorbate Assay (ASC+DHA): Ascorbate and Dehydroascorbate was measured using 2,2-bipyridal based calorimetric assay Nakano & Asada (1981). Leaf tissue (0.2g) was homogenized in liquid nitrogen (Liq.N_2) and extracted with 0.1 M HCL in 1mM EDTA (pH 7.0). After centrifugation for 2 min at 5000 rpm, 20 µL of supernatant was taken for the assay with addition of 0.4 M phosphate buffer and 10mM DTT and incubated for 10 min at room temperature. Excess DTT was removed by addition of 0.5 % N-ethylmaleimide (NEM). Color reagent (80 µL) comprising of 4.6 % TCA, 15.3 % ortho-phosphoric acid, 4 % 2, 2- bipyridyl in 70 % ethanol and 0.6 % $FeCl_3$ were added to all the reaction tubes. Blanks and ascorbate standards were prepared using 6% TCA alone. All the assay tubes were again incubated at 42 C for 45min and the absorbance was read at 520nm. Concentration of total and reduced ascorbate was calculated from the standard curve maintained with ascorbate. Concentration of dehydroascorbate (DHA) was calculated after subtracting the values of reduced ascorbate from those of total ascorbate.

Reduced Glutathione (GSH) activity: Reduced glutathione activity was assayed according to the method of Doulis *et al.* (1997). Plant tissue (0.2 gm) was ground in liquid nitrogen and homogenized in 2mL of 0.1M HCL in 1mM EDTA (pH 7.0). The reaction mixture in a final volume of 4mL consisting of 50mM KH_2PO_4, 125 mM GSH, 10 mM DHA and 100 µL plant extract was monitored spectrophotometrically at 280nm.

Statistical Analysis: Statistical analysis was performed by using 1 way analysis of variance (ANOVA). All the experiments were repeated thrice and data presented were mean of three experiments. All statistical analysis was performed by using Microsoft excel version -7.

RESULTS AND DISCUSSION

Salinity stress is known to accelerate generation of reactive oxygen species (ROS) (Mihaela *et al.* 2012). Reactive oxygen species comprises of both, free radicals such as superoxide, hydroxyl, peroxyl, alkoxyl, hydroperoxyl radicals and non radical oxygen derivatives like hydrogen peroxide, ozone, singlet oxygen & peroxynitrite (Kotchoni 2004). The life span of ROS in a cell depend on the activity of antioxidant molecules that quench thereby providing protection (Khattab *et al.* 2007).

Reactive oxygen species (ROS) generated under salinity stress, attacks proteins and other biomolecules directly or indirectly through carbonylation and nitrosylation (Pallavi Sharma *et al.* 2012, S.Sankhalkar and V. Vernekar, 2016).

Effect of salinity stress alone on superoxide dismutase (SOD) activity

SOD is known to catalyze dismutation of superoxide radicals to H_2O_2. Our results on effect of salinity stress on SOD activity are shown in Figure 1. Results obtained showed highly significant increase ($^{\#}P<0.001$) in SOD activity with increasing

salinity stress in all the three rice varieties studied in comparison to its respective control. An increase of 131.96 % & 205% in SOD activity was observed in Jaya & Korgut rice varieties at 150mM NaCl stress. Amongst the studied rice varieties salinity tolerant Jaya rice showed better protection over Korgut and Jyoti rice varieties.

Superoxide dismutase (SOD) is a major enzyme involved in regulation of intracellular ROS level thereby helping to cope against oxidative stress and maintain normal physiological condition against the oxidative stress (Mitler 2002). There are reports of increase in SOD being correlated to better tolerance under salt stress (Anjaneyulu *et al.* 2013). Similar increase in SOD activity under salinity stress is reported in *solanum tuberosum* L. by Rehnama *et al.* (2006) and in salinity tolerant Sardari wheat variety by (Shekari *et al.* 2007). Our results are also in correlation to the above findings and indicate more scavenging of superoxide radicals thereby reducing further damage to membranes and other cellular organelles.

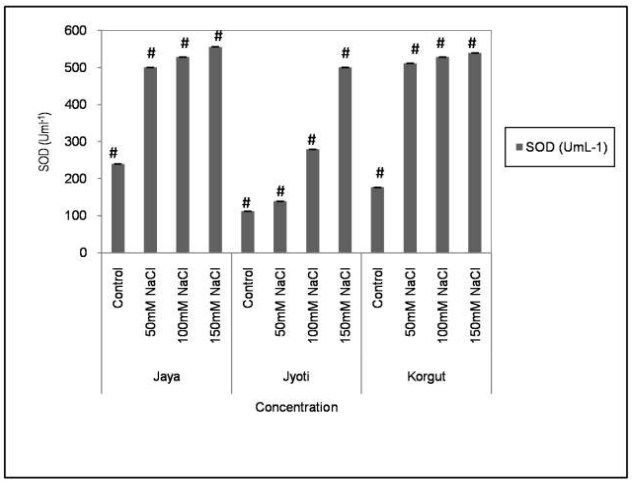

Fig 1 Effect of salinity stress alone on Superoxide dismutase (SOD). The values are mean of 3 experiments, ± S.D (n = 3). Statistical data shows highly significant difference at $^{\#}P<0.001$ by one way ANOVA.

Effect of Salinity stress alone on peroxidase (POD) activity

Our results on peroxidase activity are shown in Figure 2. The study showed 134.46 % increase in POD activity in Jyoti rice variety at 150 mM NaCl stress in comparison to its control. Although there was a linear increase in POD activity from 50-150mM NaCl stress, the increase was non-significant (^{ns} P>0.05). However, with increasing salinity stress, in comparison to their respective controls the activity of POD declined in Jaya (43.23%) and Korgut (32.38%) rice varieties. When these varieties were compared with each other, salinity sensitive Jyoti rice variety showed 95.06 % & 75.44% increase in POD at 150mM NaCl stress over Jaya & Korgut. Peroxidases are involved in decomposition of H_2O_2 by oxidation of phenolics or antioxidants. The decline in POD activity in salt tolerant Jaya and Korgut rice varieties indicate that this enzyme may not be directly involved in protection against oxidative stress and there may be activated coordination between various antioxidant enzymes for establishing proper H_2O_2 homeostasis as reported earlier by Mittova *et al.* 2002, Neil *et al.* 2002. Similar increase in POD activity is reported by Ashrafuzzaman *et al.* (2013) in BRRI dhan 29 (salt sensitive) rice variety over tolerant Pokkali rice variety.

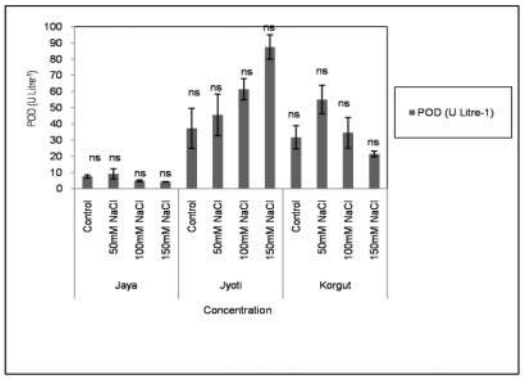

Fig 2 Effect of salinity stress alone on Peroxidase (POD). The values are mean of 3 experiments, ± S.D (n = 3). Statistical data shows non-significant difference at ^{ns} P>0.05 by one way ANOVA.

Effect of salinity stress alone on glutathione reductase (GR) activity

Our results on salinity stress induced glutathione reductase (GR) activity are shown in Figure 3. Glutathione reductase (GR) is an oxidoreductase type of enzyme involved in defense against oxidative stress (Vidyanathan *et al.* 2003). It is an important enzyme of ascorbate glutathione cycle catalyzing NADPH dependent reduction of oxidized glutathione (Satpal and Tripathy 2011). In the present study, total GR activity was measured by monitoring the decline in absorbance at 340 nm due to oxidation of NADPH. Our study showed linear increase in GR activity with increasing salinity stress in all the rice varieties. In comparison to their respective controls highly significant ($^{\#}$ P<0.001) increase was observed in Jaya (163.6 %), and Jyoti (267.6 %). This increase in GR activity indicates activated rapid tolerance capacity of Jaya over Jyoti & Korgut. Our results are thus in correlation to the existing report by Vaidyanathan *et al.* (2003) that showed increase in GR in salt tolerant Pokkali (PK) rice variety. As reported by (Ben Amor *et. al.* 2006) this possible increase in GR activity may be due to increased NADP/NADPH ratio favoring availability of NADP to accept electrons from the ETC thereby reducing down the production of ROS in the chloroplasts.

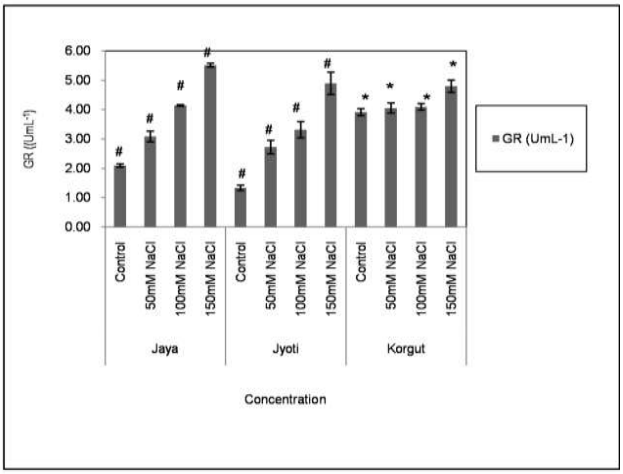

Fig 3 Effect of salinity stress alone on Glutathione reductase (GR). The values are mean of 3 experiments, ± S.D (n =3). Statistical data shows highly significant difference at $^{\#}P<0.001$ and Significant difference at $^{*}P<0.05$ by one way ANOVA.

Effect of salinity stress alone on ascorbate peroxidase (APX) activity

Our results on APX activity are shown in Figure 4. The results showed non significant ($^{ns}p>0.05$) increase in APX activity with increasing salinity stress (50-150mM NaCl) in tolerant Korgut rice variety while, it declined in Jaya (3.84 %) and Jyoti (67.74 %) rice varieties. An increase of 44% was observed in Korgut rice at 150 mM NaCl stress in comparison to its control. Similar results are shown by Hossain *et al.* (2013) on salt tolerant Pokkali and sensitive BRRI dhan 29 rice varieties. Their results indicated detoxification of H_2O_2 with increasing APX activity in presence of ascorbate serving as electron donor. APX thus play an important role in lowering of H_2O_2 and lipid peroxidation level thereby protection. According to our study an increase in APX activity in Korgut indicates that salinity stress influences production of more H_2O_2 and is detoxified to less harmful radicals hence showing better protection over other two varieties.

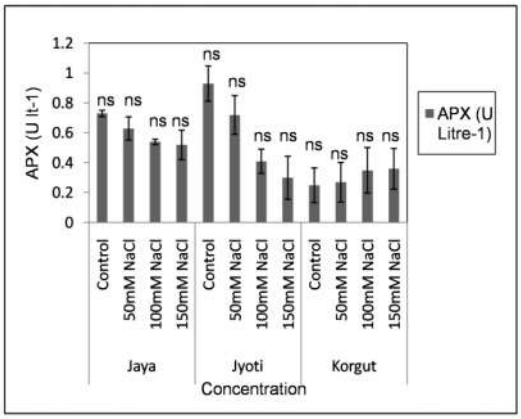

Fig 4 Effect of salinity stress alone on ascorbate peroxidase (APX). The values are mean of 3 experiments, ± S.D (n = 3). Statistical data shows non- significant difference at ^{ns} P>0.05 by one way ANOVA.

Effect of salinity stress on total ascorbate (ASC+DASC) and reduced glutathione (GSH) activity

We tried to correlate the enzymatic antioxidant activity with non-enzymatic antioxidants such as total ascorbate and GSH. ASC and GSH seem to play an important role in cellular compartments disposing H_2O_2 (Hossain *et al.* 2012, Chao *et al.* 2010, Ashraf 2009). Increased level of these non enzymatic antioxidants under stress is directly correlated to reduced ROS. Results of total ascorbate (ASC+DASC) and GSH are shown in Figure 5 and 6.

With increasing salinity stress, non-significant ($^{ns}P>0.05$) decline in total ascorbate content was observed in Jaya, Jyoti & Korgut. Comparing this to their respective controls the decline in total ascorbate content at 150mM NaCl stress was 27.90 %, 13.88 % and 21.73 % resp. Activity of GSH showed highly significant ($^{\#}P<0.001$) increase in Jaya and Jyoti having 1.67 and 1.57µmoles gm^{-1} F.w respt. Niyogi *et al* 2004 have shown that increase in glutathione overlaps with antioxidant functions of Ascorbate. Based on the increasing GSH content in our findings, we also hypothesize the networking of ascorbate-glutathione cycle with other antioxidant enzymes that may possibly be involved in protection against oxidative stress.

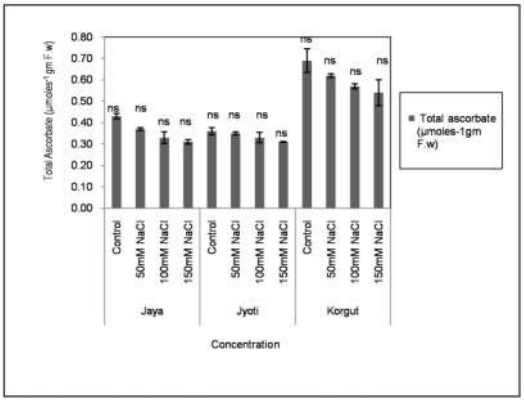

Fig 5 Effect of salinity stress alone on Total Ascorbate (ASC+ DASC).The values are mean of 3 experiments, ± S.D (n=3). Statistical data shows non- significant difference at ^{ns}P>0.05 by one way ANOVA.

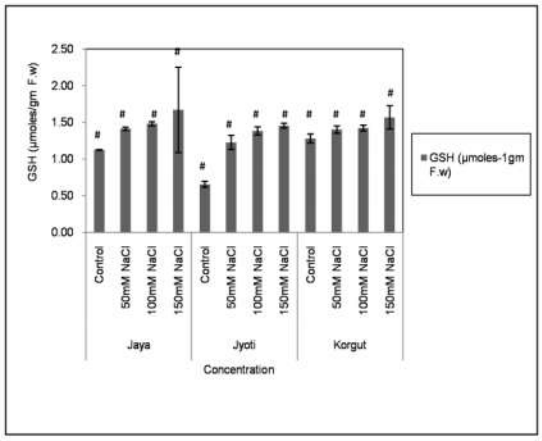

Fig 6 Effect of salinity stress alone on Reduced glutathione (GSH).The values are mean of 3 experiments, ± S.D (n=3). Statistical data shows highly significant difference at $^{\#}$P<0.001 by one way ANOVA.

CONCLUSION

From the above study it is concluded that increasing salinity stress resulted in varied responses of antioxidant enzymes in studied rice varieties. The increase in SOD, GR, and GSH content in Jaya & Korgut rice observed in our study indicated their tolerance to higher levels of NaCl stress. However, further study is necessary to understand the regulatory mechanism of these antioxidant enzymes at gene level that would give a possible insight in inter and intracellular molecular interaction to salinity stress. Isolation and identification of salt tolerant genes from traditional non hybrid rice varieties can thus be a better tool for plant breeding and genetic engineering program.

Acknowledgement

The authors would like to highly acknowledge Department of Science and Technology (SERB), Govt. of India, New Delhi for financial assistance to undertake the above work.

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How to cite this article:

Sankhalkar Sangeeta and Vernekar Vrunda.2016, Comparative Study of Antioxidant Enzymes in Tolerant and Sensitive Rice Varieties Subjected To Salinity Stress . *Int J Recent Sci Res.* 7(10), pp. 13964-13969.