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Effect of Exogenous Proline and Glycinebetaine on Antioxidant Enzymes Activity in Rice Seedlings under Salt Stress

MUHAMMAD ABDUS SOBAHAN
School of Agriculture and Rural Development, Bangladesh Open University, Gazipur-1705, Bangladesh.

Abstract

Exogenous application of proline and glycinebetaine (betaine) counteracted the adverse effects of salinity. The effect of exogenous proline and betaine on the activity of antioxidant enzymes in the leaves of rice plants (*Oryza sativa* L., cv. Nipponbare) was studied under salt stress conditions. Salt stress increased the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR). Exogenous application of proline and betaine increased CAT activity and GR activity, whereas SOD, APX, POX and DHAR activities decreased under salt stress conditions. It is suggest that exogenous proline and betaine mitigate the detrimental effects of salt stress in rice plants via increasing CAT and GR activities rather than SOD, APX, POX and DHAR activities.

Keywords: Proline, Betaine, Antioxidant enzymes, Rice, Salt stress.

1. Introduction

Salinity is one of the major abiotic stresses limiting plant growth and yield of most crops all over the world [33] [5]. Rice is one of the most important crops and suitable for saline soil [53].

Salt stress causes both ionic and osmotic effects in plants, leading to membrane disorganization, metabolic toxicity, and genesis of reactive oxygen species (ROS), which may cause oxidative damage [13]. ROS such as singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (.0H) which are inevitably generated naturally via a number of cell metabolic pathways [21]. ROS have the potential to interact with all types of bio-molecules, such as DNA, proteins and lipids, leading to radical chain processes, membrane leakage [22].

Plants cope with oxidative stress by using enzymatic and non-enzymatic antioxidant systems. The enzymatic system includes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) [21] [2]. They act together in scavenging ROS and subsequent protection of plant cells from oxidative damage [25] [26]. As a major scavenger, SOD catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen. However, H_2O_2 is also toxic to the cells and has to be further scavenged by CAT or peroxidase, or both, to water and oxygen [54] [44]. The first step of the ascorbate-glutathione cycle (ASC-GSH), which removes H_2O_2 is catalyzed by APX [3]. In these cycle, reduced glutathione (GSH) functions as an electron donor for dehydroascorbate reductase (DHAR) to regenerate ASC through reduction of dehydroascorbate (DHA) via the Halliwell-Asada pathway [37] [4].

Environmental stresses including salinity can induce a significant accumulation of compatible solutes [9]. Proline and betaine are regarded as one of the most effective compatible solutes, plays an important role in plant salt tolerance by osmotic adjustment, protecting the enzymes by stabilizing the structure of proteins such as RuBisCO, protecting membrane structures, up-regulate stress protective proteins and by functioning as oxygen radical scavengers [41] [34] [24] [35] [30] [39] [38]. In addition, exogenous proline and betaine may have also contributed to the improvement of plant salt tolerance through its role in ion homeostasis and Na^+/K^+ discrimination under salinity conditions [51][50][23]. Although exogenous application of proline or betaine mitigates detrimental effect of salt stress by increasing antioxidant enzymes activity in many plant species [40][43], there is little information available on proline and betaine-induced modulation of antioxidant enzymes in rice plants. The present work was conducted to study the effect of exogenous proline and betaine on the

*Corresponding author: Muhammad Abdus Sobahan; E-mail: sobahan_74@yahoo.com
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antioxidant enzymes activity in rice plants under saline conditions.

2. Materials and Methods

Plant material and growth: Seeds of rice (*Oryza sativa* L. cv. Nipponbare) were collected from Field Science Center, Faculty of Agriculture, Okayama University, Japan. Seeds of rice were washed with 10% H_2O_2 and thoroughly rinsed with distilled water. The washed seeds were placed on water-soaked filter paper in a Petri dish. The Petri dishes were sealed with a strip of Parafilm to prevent water vaporizations, and were incubated in a growth room (a 12-h-light/30°C and 12-h-dark/25°C regime and a photon influence rate of 80 µmol m^{-2} s^{-1}) for germination. After 7d of incubation, the seedlings were transferred from the dish to a floating net so that only the roots of the rice plants could be dipped in Kimura B solution, supplemented with 2.86 mg/l of H_3BO_3, 1.80 mg/l of $MnCl_2.4H_2O$, 0.079 mg/l of $CuSO_4.5H_2O$, 0.126 mg/l of $Na_2MoO_4.2H_2O$, and 0.220 mg/l of $ZnSO_4.H_2O$ [19], in a Wagner pot (r = 1/5,000). The plants were grown in growth room under the same conditions as for germination, the nutrient solution was changed every other day, and the pH was adjusted to 5.5 with 1N NaOH or 1N HCl. The rice plants were grown for 14d followed by treatment with or without 25 mM NaCl in the presence and the absence of 1 mM proline or betaine for 12 h.

Extraction of antioxidant enzymes: Leaf sample (50 mg) was frozen in liquid nitrogen immediately after harvesting and stored at -80˚C until enzyme assays. Leaf sample was homogenized in 3 ml of 50 mM NaH_2PO_4 buffer (pH 7.8) including 1 mM of EDTA and 2% (w/v) of PVP with a chilled mortar and pestle. The homogenate were centrifuged at 11,000 x g for 20 min at 4˚C. All spectrophotometric analyses were conducted on a Shimadzu (model UV-2400 PC, Japan) spectrophotometer.

Superoxide dismutase: SOD activity was measured by using an SOD Assay Kit-WST (Dojindo Molecular Technologies, Kumamoto, Japan). For each SOD activity measurement, 20 µl of the sample solution was placed in the wells for sample and blank 2. Twenty µl of distilled water was placed in the wells for blanks 1 and 3. Two hundred µl of WST working solution was added to each well, and 20 µl of dilution buffer was added to the wells for blanks 2 and 3. Twenty µl of enzyme working solution was added to the wells for sample and blank 1 and then mixed thoroughly. The absorbance was measured at 450 nm using a microplate reader (model 680, Nippon Bio-Red, Tokyo) after incubation at 37˚C for 20 min. The activity was calculated (inhibition rate %) using the following equation: SOD activity (inhibition rate %) = {[(A blank 1-A blank 3)-(A sample-A blank 2)] / (A blank 1-A blank 3)} x 100. SOD activity was expressed as a percentage, where the activity of non-stressed plants was 100%.

Catalase: Catalase (CAT) activity was assayed as previously described [8]. The reaction mixture contained 50 mM of NaH_2PO_4 buffer (pH 7), 0.1 mM of EDTA, 3% of H_2O_2 and 0.1 ml of enzyme extract. The decrease in H_2O_2 was measured as a decline in optical density at 240 nm for 2 min when the extinction coefficient was 39.4 mM^{-1} cm^{-1}.

Peroxidase: Peroxidase (POX) activity was determined by the procedure previously reported [36]. The reaction buffer solution contained 50 mM of NaH_2PO_4 buffer (pH 7), 0.1 mM of EDTA, 0.1 mM of H_2O_2 and 10 mM of guaiacol. The reaction was started by adding sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 470 nm for 1 min when the extinction coefficient was 26.6 mM^{-1} cm^{-1}.

Ascorbate peroxidase: Ascorbate peroxidase (APX) activity was measured as previously reported [36]. The reaction buffer solution contained 50 mM of NaH_2PO_4 buffer (pH 7), 0.1 mM of EDTA, 0.5 mM of ascorbate and 1.2 mM of H_2O_2. The reaction was started by adding sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 290 nm for 1 min when the extinction coefficient was 2.8 mM^{-1} cm^{-1}.

Glutathione reductase: Glutathione reductase (GR) was measured as previously reported [15]. The reaction buffer solution contained 50 mM of Tris-Cl buffer (pH 7.5), 3 mM of $MgCl_2$, 0.15 mM of NADPH and 0.5 mM of GSSG. The reaction was started by adding sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 340 nm for 2 min when the extinction coefficient was 6.2 mM^{-1} cm^{-1}.

Dehydroascorbate reductase: Dehydroascorbate reductase (DHAR) was done according to [36] with some modification. The reaction buffer solution contained 50 mM of NaH_2PO_4 buffer (pH 7), 2.5 mM of GSH, and 0.1 mM of DHA. The reaction was started by adding sample solution to the reaction buffer

solution. The activity was calculated from the change in absorbance at 265 nm for 2 min when the extinction coefficient was 0.18 mM^{-1} cm^{-1}.

Determination of protein: Protein contents of the crude extract was measured as described by [11] using bovine serum albumin as a standard.

Statistical Analysis: Data were analyzed by ANOVA, and mean values were compared by the Tukey test. Differences at $p < 0.05$ were considered significant.

3. Results and Discussion

Plant's treatment with osmolytes like proline, betaine can remarkably improve their tolerance to stress condition [5]. In our previous investigation, we found that application of exogenous proline and betaine mitigate the detrimental effects of salt stress on rice plants by reducing H_2O_2 and oxidation of membrane lipid [49]. A lower lipid peroxidation resulting from elevated activities of antioxidants under salt stress was reported on salt-tolerant wild tomato, *Lycopersicum pennellii* [47] and Wild beet, *Beta maritime* [10]. To make it clear the causes of lower lipid peroxidation by proline and betaine, it is necessary to investigate the antioxidant enzymes. SOD is the major O_2^-scavenger and its enzymatic action results in H_2O_2 and O_2 [17]. Results in this study showed that SOD activity slightly increased under salt stress (Fig. 1), a similar increase in SOD activity was observed in salt-stressed *P. maritima*, maize and tomato [45] [6] [31]. However, application of exogenous proline and betaine decreased the SOD activity (6.32% and 12.64%, respectively) under salt stress conditions; this result might imply that there was lower accumulation of superoxide anion in rice plant. Similar to our observation, external supply of proline to plants under stress conditions was reported to suppress SOD activity in paraquat-induced ice plant [48] and salt-induced cucumber plant [29]. These result suggest that neither proline nor betaine could enhance H_2O_2 production through scavenge O_2^-under salt stress.

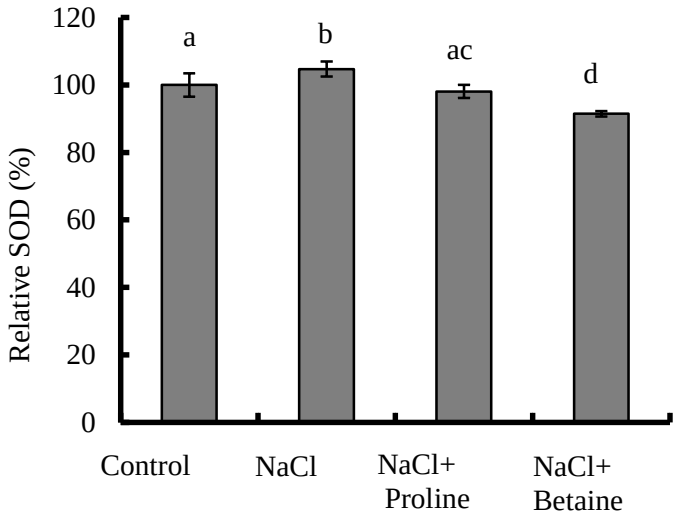

Figure 1: SOD activity in the rice plants treated with 25 mM NaCl in the presence and the absence of 1 mM proline or betaine for 12 h. Each value was obtained from more than three experiments. Error bars represent standard deviation. Bars with different letters are significantly different at $p < 0.05$.

Catalase is one of the vital enzymes in scavenging H_2O_2 in plant cells exposed to various abiotic stresses due its higher turnover rate of reaction [18]. Hydrogen peroxide is eliminated by several classes of peroxidases [3]. The results of this study show that CAT, POX and APX activity increased significantly under salt stress conditions (Figs. 2A, B, C). Similar increases in the activities of these enzymes have been reported in rice, barley and beet cultivars subjected to salt stress [14] [52] [46] [10]. Exogenous proline and betaine treatment significantly increased CAT activity (35.53% and 30.55%, respectively) (Fig. 2A) in response to NaCl stress, while POX (12.55% and 20.50%, respectively) and APX (7.74% and 6.16%, respectively) activities decreased (Fig. 2B, C), which was in agreement with previous reports [46] [42] [1]. This result indicated that catalase is more important

than APX and POX to detoxification of H_2O_2 induced by salt stress.

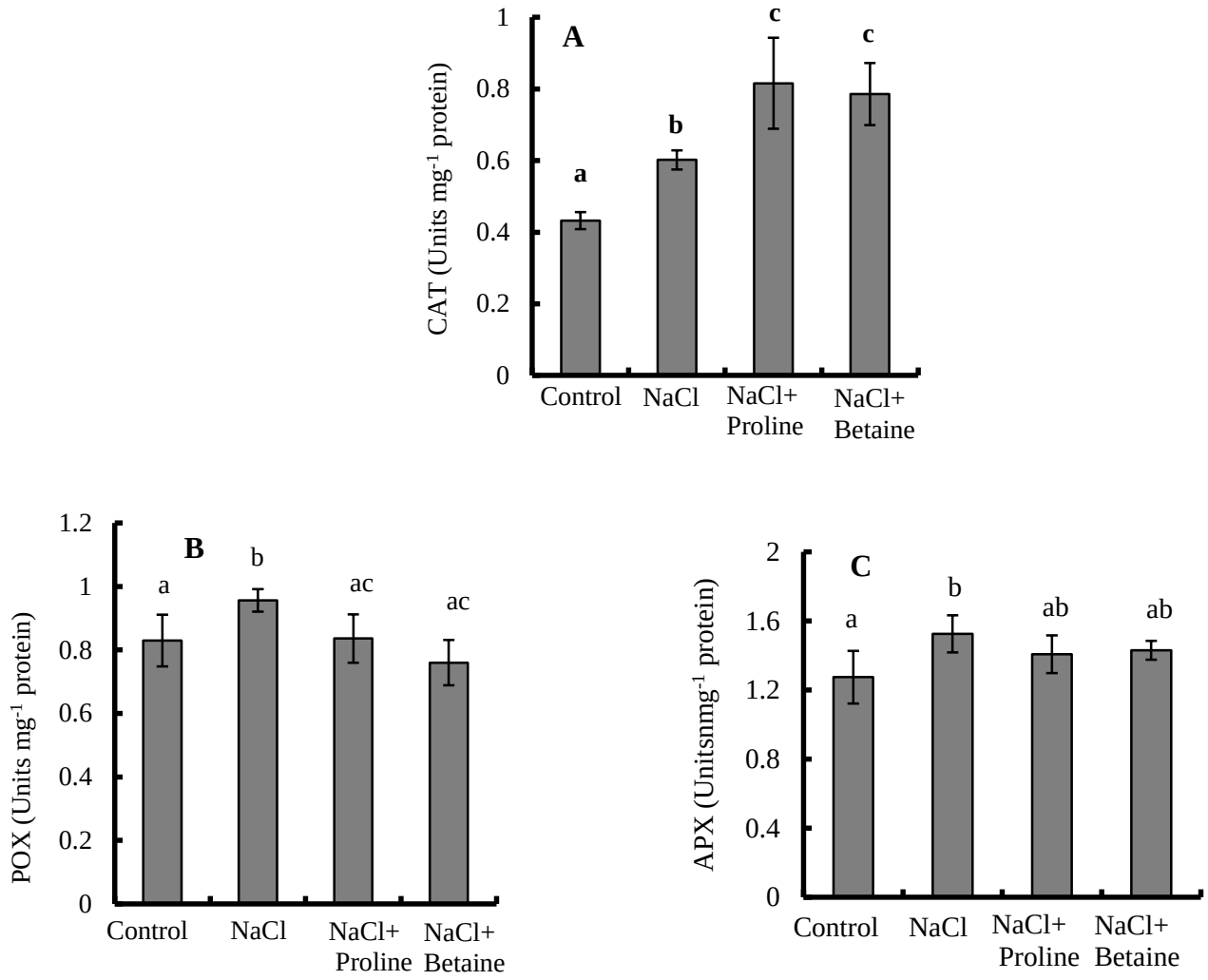

Figure 2: CAT activity (A), POX activity (B) and APX activity (C) in the rice plants treated with 25 mM NaCl in the presence and the absence of 1 mM proline or betaine for 12 h. Each value was obtained from more than three experiments. Error bars represent standard deviation. Bars with different letters are significantly different at $p < 0.05$.

Glutathione (GSH) has vital roles in development of plant stress tolerance to adverse environmental conditions [7]. GSH, as a potential scavenger of $^1O_2$, H_2O_2 and OH^+, counteracts the inhibitory effects of ROS-induced oxidative stress and maintains the normal reduced state of cells [32] [12]. GR, the last enzyme of ascorbate–glutathione cycle, catalyzes NADPH-dependent reduction of oxidized glutathione. GR is important in protecting many plants from oxidative stress [16]. In the present study, GR and DHAR activity were significantly increased in Nipponbare rice plants in response to salt stress (Fig. 3A, B). These results were well agreed with other reports [20] [43]. Both proline and betaine increased GR activity (31.72% and 30.12%, respectively) induced by salt stress (Fig. 3A). However, application of exogenous proline and betaine decreased DHAR activity (8.21% and 17%, respectively) in salt stressed seedlings (Fig. 3B). These results were corroborated with other recent findings where exogenous proline and betaine up-regulated the GR activity and decreased DHAR activity under salt stress [46] [43] [28] which could maintain a high GSH pool [27]. It can be suggested that exogenously applied proline and betaine could help plants to deal with stress conditions via scavenge ROS.

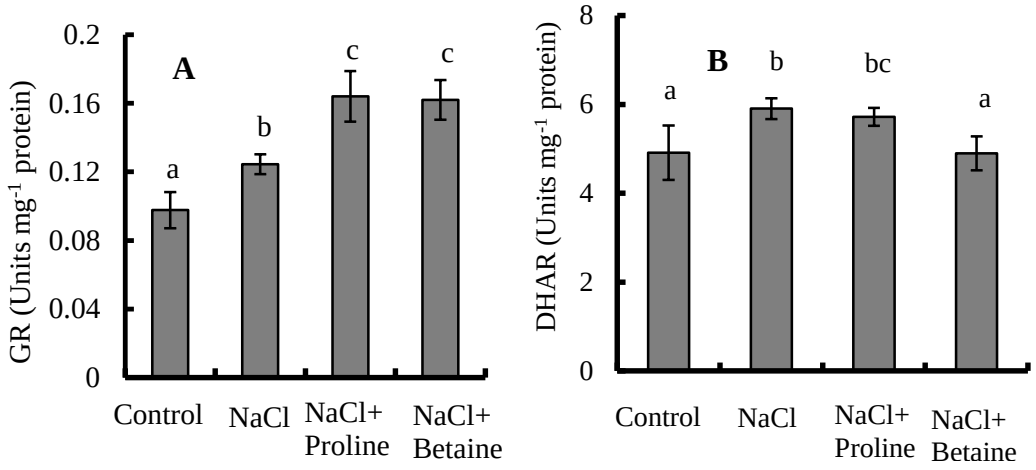

Figure 3: GR activity (A) and DHAR activity (B) in the rice plants treated with 25 mM NaCl in the presence and the absence of 1 mM proline or betaine for 12 h. Each value was obtained from more than three experiments. Error bars represent standard deviation. Bars with different letters are significantly different at $p < 0.05$.

4. Conclusion

Exogenous proline- and betaine was effective in mitigating the detrimental effects of salt stress on rice plants by the elevation of CAT and GR activity under salt stress. Therefore, application of exogenous proline and betaine might be promising approach for salt stress management in the era of climatic changes.

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