Australian Journal of Crop Science AJCS

AJCS 10(1):50-56 (2016) ISSN:1835-2707

Improvement of salt tolerance in rice (*Oryza sativa* L.) by increasing antioxidant defense systems using exogenous application of proline

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Abstract

Proline accumulation contributes to the protection of plants against salt stress by inducing antioxidant defense systems. To investigate the protective effects of proline against salt stress, salt-sensitive (BRRI dhan29) and moderately salt-tolerant (BRRI dhan47) rice cultivars were grown in pots. Rice plants were exposed to different concentrations of NaCl at active tillering stage, and proline solutions (0, 25 and 50 mM) were sprayed on the leaves. Salt stress caused a significant reduction in growth and yield of both rice cultivars. Growth was drastically reduced and both cultivars failed to produce grains at 50 and 100 mM NaCl stresses, and even application of proline failed to compensate the adverse effects at those high salt stresses. Exogenous proline showed a significant increase in plant growth of both cultivars at 25 mM NaCl stress, and that proline increased grain yield of only salt-sensitive rice at same salt stress. Salt stress significantly decreased chlorophyll and ascorbate contents, straw K^+/Na^+ ratio and activity of antioxidant enzyme guaiacol peroxidase (POX) in both cultivars. Intracellular proline content increased in salt-sensitive rice but decreased in salt-tolerant rice under salt stress. Exogenous proline increased chlorophyll, intracellular proline and ascorbate contents, K^+/Na^+ ratio and activities of antioxidant enzymes in salt-sensitive rice at 25 mM NaCl stress although most of those data were not affected in salt-tolerant rice. The present study; therefore, suggests that exogenous proline confers tolerance to salt stress in salt-sensitive rice by maintaining higher K^+/Na^+ ratio and enhancing proline accumulation and antioxidant defense systems.

Keywords: Antioxidant enzymes, chlorophyll, proline, rice growth, salt stress.
Abbreviations: CAT_catalase, POX_guaiacol peroxidase, APX_ascorbate peroxidase, ROS_reactive oxygen species.

Introduction

Salinity is one of the major abiotic factors limiting crop productivity worldwide. More than 6% of the world's land and one third of the irrigated land are significantly affected by soil salinity (FAO, 2008). In Bangladesh, about 1.06 million hectares of arable lands are affected by soil salinity (SRDI, 2010). Rice is one of the most important cereals in the world and number one in Bangladesh. Rice is a salt-sensitive crop and its sensitivity is variable at different growth stages. Salinity is a serious threat to rice production in Southern Bangladesh. Therefore, the improvement of salinity tolerance in rice has come to the fore. Salinity imposes both ionic toxicity and osmotic stress to plants (Hasegawa et al., 2000; Zhu, 2003). Salt stress disturbs cytoplasmic K^+/Na^+ homeostasis, causing an increase in Na^+/K^+ ratio in the cytosol (Zhu, 2003). Accumulation of excess Na^+ and Cl^- causes ionic imbalances that may impair the selectivity of root membranes and induce K^+ deficiency (Gadallah, 1999). Plants develop a variety of adaptive mechanisms in response to salt stress. Accumulation of compatible solutes in plants is one of the main adaptive mechanisms to salt stress. Proline is the most common compatible solute that plays a pivotal role in the process of osmotic adjustment in various plants (Hasegawa et al., 2000; Ashraf and Foolad, 2007). Proline contributes to the protection of membranes, proteins and enzymes from damaging effects of various stresses (Ashraf and Foolad, 2007; Hossain et al., 2014). Moreover, proline provides a protection against salt stress via maintaining redox homeostasis (Hoque et al., 2008). Under salt stress, exogenous proline up-regulates stress-protective proteins (Khedr et al., 2003), and reduces protein oxidation (Hoque et al., 2008) and lipid peroxidation (Banu et al., 2009). Proline also suppresses production of free radicals (Hasegawa et al., 2000; Okuma et al., 2004) and reactive oxygen species (ROS) (Banu et al., 2009, 2010). Salinity induces the production of ROS in plant cells (Hasegawa et al., 2000; Apel and Hirt, 2004; Banu et al., 2009, 2010). The excess production of ROS is toxic to plants and causes oxidative damage to cellular constituents, leading to cell death (Noctor and Foyer, 1998; Apel and Hirt, 2004; Banu et al., 2009, 2010; Hossain et al., 2014) although they act as signaling molecules, mediating many key physiological processes. Plants possess enzymatic and non-enzymatic antioxidant defense systems to protect cells against the damaging effects of ROS (Noctor and Foyer, 1998; Apel and Hirt, 2004). The major ROS-scavenging antioxidant enzymes are catalase (CAT), guaiacol peroxidase (POX) and ascorbate peroxidase (APX). Ascorbate directly scavenges ROS and functions as an electron donor to APX for scavenging H_2O_2 (Noctor and Foyer, 1998). Numerous authors have reported that salt stress differentially affects the components of antioxidant defense system in plants (Noctor and Foyer, 1998; Hasegawa et al., 2000; Mittova et al., 2003; Demiral and Türkan, 2005; El-Shabrawi et al., 2010; Nounjan and Theerakulpisut, 2012; Hasanuzzaman et al., 2014).

There are increasing evidences that proline enhances antioxidant defense mechanism and improves stress tolerance in plants (Khedr et al., 2003; Hossain and Fujita, 2010; Nounjan and Theerakulpisut, 2012; Hasanuzzaman et al., 2014; Hossain et al., 2014). Exogenous proline increases intracellular proline content (Okuma et al., 2004), which leads to suppression of cell death and improvement of salt tolerance in tobacco cultured cells via increment of the antioxidant defense systems (Hoque et al., 2007a, b, 2008; Banu et al., 2009, 2010). Recent studies have shown that exogenous proline application improves salt tolerance in rice by increasing K^+/Na^+ ratio (Sobahan et al., 2012), endogenous proline (Hasanuzzaman et al., 2014) and activities of antioxidant enzymes (Nounjan and Theerakulpisut, 2012; Hasanuzzaman et al., 2014). However, protective mechanisms of proline in plant responses to salt stress remain to be clarified. In order to clarify the role of proline in salt tolerance, we investigated the effects of exogenous proline on the growth, chlorophyll, intracellular proline and ascorbate contents, and activity of antioxidant enzymes in two contrasting rice genotypes exposed to salt stress.

Results

Protective effects of proline on growth and yield of rice against salt stress

Salt stress caused a significant decrease in growth and yield of salt-sensitive and salt-tolerant rice (Table 1). Salt stress (25 mM NaCl) decreased grain yield by 2-fold and 1.5 fold of salt-sensitive and salt-tolerant rice, respectively. Drastic reduction of growth was observed when plants of both cultivars were exposed to 50 and 100 mM NaCl, and finally both cultivars failed to produce grains (Table 1). Exogenous proline (25-50 mM) application significantly increased root and shoot growth as well as grain yield of salt-sensitive rice at 25 mM NaCl stress. At 25 mM NaCl stress, exogenous proline significantly increased root and shoot growth of salt-tolerant rice although that proline did not affect grain yield. It was also noted that application of exogenous proline failed to produce grains in both rice cultivars under 50 and 100 mM NaCl stress conditions (Table 1).

Effects of proline on chlorophyll contents under salt stress

Salt stress caused a significant reduction in chlorophyll-b and total chlorophyll contents of both rice cultivars although chlorophyll-a content was significantly higher in salt-stressed plants than in non-stressed plants (Fig 1a). Exogenous proline did not result in increase in chlorophyll-a content either in salt-sensitive or in salt-tolerant cultivar under salt stress. On the other hand, proline application (25 and 50 mM) significantly increased chlorophyll-b and total chlorophyll contents in salt-sensitive rice at 25 mM NaCl stress. In salt-tolerant rice, application of 50 mM proline resulted in a significant increase in chlorophyll-b and total chlorophyll contents under salt stress condition (Fig 1a).

Intracellular proline content induced by exogenous proline under salt stress

Intracellular proline content was higher in salt-tolerant rice than in salt-sensitive rice (Fig 1b). Salt stress showed a significant increase in intracellular proline content in salt-sensitive rice but a decrease in salt-tolerant rice (Fig 1b). Exogenously applied proline resulted in a significant increase in intracellular proline content in salt-sensitive rice at 25 mM NaCl stress. On the contrary, intracellular proline content in salt-tolerant rice was not affected by exogenous proline under salt stress (Fig 1b).

Effects of proline on ascorbate content under salt stress

Ascorbate content in salt-tolerant rice was approximately 1.4-fold higher than that in salt-sensitive rice (Fig 2a). A significant reduction in ascorbate content was observed in both rice cultivars in response to NaCl stress. Exogenous proline resulted in a significant increase in ascorbate content in both cultivars under NaCl stress. Surprisingly, ascorbate content was higher in 25 mM proline-treated plants than in 50 mM proline-treated plants at NaCl stress (Fig 2a).

Activity of antioxidant enzymes induced by proline under salt stress

To investigate whether proline enhances antioxidant defense system in rice under salt stress, activities of major ROS-scavenging antioxidant enzymes viz. CAT, POX and APX were measured. Salt stress caused significant decreases in POX and APX activities in salt-sensitive rice (Fig 2c, d). CAT activity also decreased due to salt stress but this decrease was not significant (Fig 2b). Upon exposure to salt stress, significant decreases in POX activity and significant increases in CAT and APX activities were observed in salt-tolerant rice (Fig 2b-d). Exogenous proline did not result in significant increase in CAT activity either in salt-sensitive or in salt-tolerant cultivar under salt stress although the CAT activity in salt-tolerant rice was always higher compared to non-stress condition. Proline application showed significant increases in both POX (85 to 139%) and APX (96 to 190%) activities in salt-sensitive rice in response to NaCl stress (Fig 2c, d). Enhanced APX and POX activities in salt-tolerant rice were observed by 25 mM and 50 mM proline application, respectively, under NaCl stress (Fig 2c, d).

K^+/Na^+ ratio influenced by proline under salt stress

Salinity caused a significant reduction in straw K^+/Na^+ ratio in both cultivars (Fig 3a). At 25 mM NaCl stress, exogenously applied proline showed a significant increase in straw K^+/Na^+ ratio in salt-sensitive but not in salt-tolerant rice. Proline application did not increase straw K^+/Na^+ ratio at higher salinity stresses (50 and 100 mM NaCl) irrespective of salinity tolerance in rice (Fig 3a). The grain K^+/Na^+ ratio was higher in salt-tolerant rice than in salt-sensitive rice (Fig 3b). Proline application significantly increased grain K^+/Na^+ ratio in salt-sensitive but not in salt-tolerant rice at 25 mM NaCl stress (Fig 3b).

Discussion

The protective role of proline against NaCl-induced oxidative damage in plants was widely reported. We previously reported that exogenous proline suppressed NaCl-induced cell death and improved salt tolerance in cultured tobacco cells (Okuma et al., 2004; Hoque et al., 2007a, b; Banu et al., 2009). In the present study, we clarified the protective effects of exogenous proline on rice against NaCl stress. Exogenous proline improved NaCl-induced growth inhibition of salt-sensitive and salt-tolerant rice although the grain yield was found to be improved in salt-sensitive rice but not in salt-tolerant rice (Table 1). Sobahan et al. (2012) also showed that exogenous proline improved salt tolerance in salt-sensitive

Table 1. Effect of exogenous proline on the growth and yield of BRRI dhan29 and BRRI dhan47 rice under salt stress. "ND" (not detected) indicates no plants survived during the data recording. ND samples were not considered for statistical analysis. Values represent the mean of four replications. Same letter in a column represents insignificant difference at $P \leq 0.05$.

Treatments	BRRI dhan29					BRRI dhan47				
	Root dry weight (g/pot)	Plant dry weight (g/pot)	No. of effective tillers/ hill	No. of filled grains/ panicle	Grain weight (g/pot)	Root dry weight (g/pot)	Plant dry weight (g/pot)	No. of effective tillers/ hill	No. of filled grains/ panicle	Grain weight (g/pot)
T_0: Control	70.2b	89.1a	21.0a	195a	72.1a	60.0a	70.0a	20.0a	151a	76.0a
T_1: 25 mM NaCl	27.8d	42.3d	11.0c	167c	36.3d	36.8c	31.0d	16.0c	142b	50.6b
T_2: 25 mM NaCl+25 mM proline	52.3c	64.3c	17.0b	178bc	46.2c	36.3c	46.9c	16.3bc	143b	49.2b
T_3: 25 mM NaCl+50 mM proline	72.9a	85.0b	16.0b	190ab	49.8b	46.9b	58.0b	17.6b	137c	51.3b
T_4: 50 mM NaCl	5.38e	10.3e	ND	ND	ND	5.06d	12.2e	ND	ND	ND
T_5: 50 mM NaCl+25 mM proline	3.10g	5.68f	ND	ND	ND	4.00de	11.1f	ND	ND	ND
T_6: 50 mM NaCl+50 mM proline	4.30f	4.30f	ND	ND	ND	4.25de	7.00g	ND	ND	ND
T_7: 100 mM NaCl	1.96h	3.00g	ND	ND	ND	2.50ef	6.50gh	ND	ND	ND
T_8: 100 mM NaCl+25 mM proline	2.00h	3.50g	ND	ND	ND	2.50ef	5.80h	ND	ND	ND
T_9: 100 mM NaCl+50 mM proline	2.01h	3.55g	ND	ND	ND	2.00f	5.61h	ND	ND	ND
SE(±)	4.31	5.20	0.59	2.22	2.05	3.26	3.55	0.28	0.85	1.74
CV%	17.81	16.73	3.62	1.21	4.01	16.25	13.96	1.57	0.59	3.07

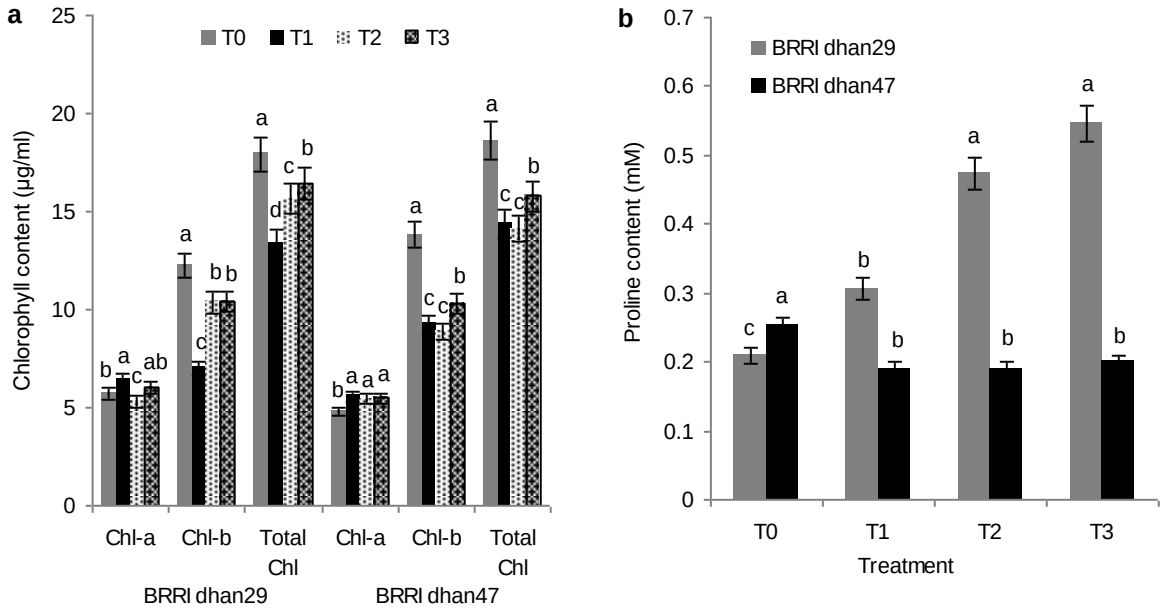

Fig 1. Chlorophyll (a) and intracellular proline (b) contents in salt-sensitive and salt-tolerant rice induced by exogenous proline under salt stress. Values represent the mean±SE of four replications. For the same rice cultivar, bars with the same letters are not significantly different at $P \leq 0.05$. No chlorophyll and proline contents were detected in the treatments T_4, T_5, T_6, T_7, T_8 and T_9. ND samples were not considered for statistical analysis. Treatment details are shown in the Table 1.

rice more effectively than salt-tolerant rice. It was also observed that none of the plants of both rice genotypes survived when exposed to 50 and 100 mM NaCl stresses even with the application of proline (Table 1). A large body of evidences reported that exogenous proline improved growth of various plants including rice in response to salt stress (Khedr et al., 2003; Hossain and Fujita,2010; Ahmed et al., 2011; Nounjan and Theerakulpisut, 2012; Hasanuzzaman et al., 2014). Taken together, results suggested that the detrimental effects of salt stress within a certain range of concentrations on plants could be minimized by foliar application of proline. Chlorophyll is one of the most important pigment components, providing photosynthetic ability of a plant. Chlorophyll content varies due to salinity levels, eventually affecting plant growth and development. Chlorophyll-b and total chlorophyll contents decreased in both rice cultivars due to salt stress (Fig 1a). The decreased chlorophyll content under saline condition could be due to impaired biosynthesis or increased pigment degradation. On the other hand, chlorophyll-a content was not affected by salt stress (Fig 1a) probably due to conversion of chlorophyll-b into chlorophyll-a during chlorophyll degradation process, consequently leading to increased chlorophyll-a (Fang et al., 1998; Eckardt, 2009). Exogenous

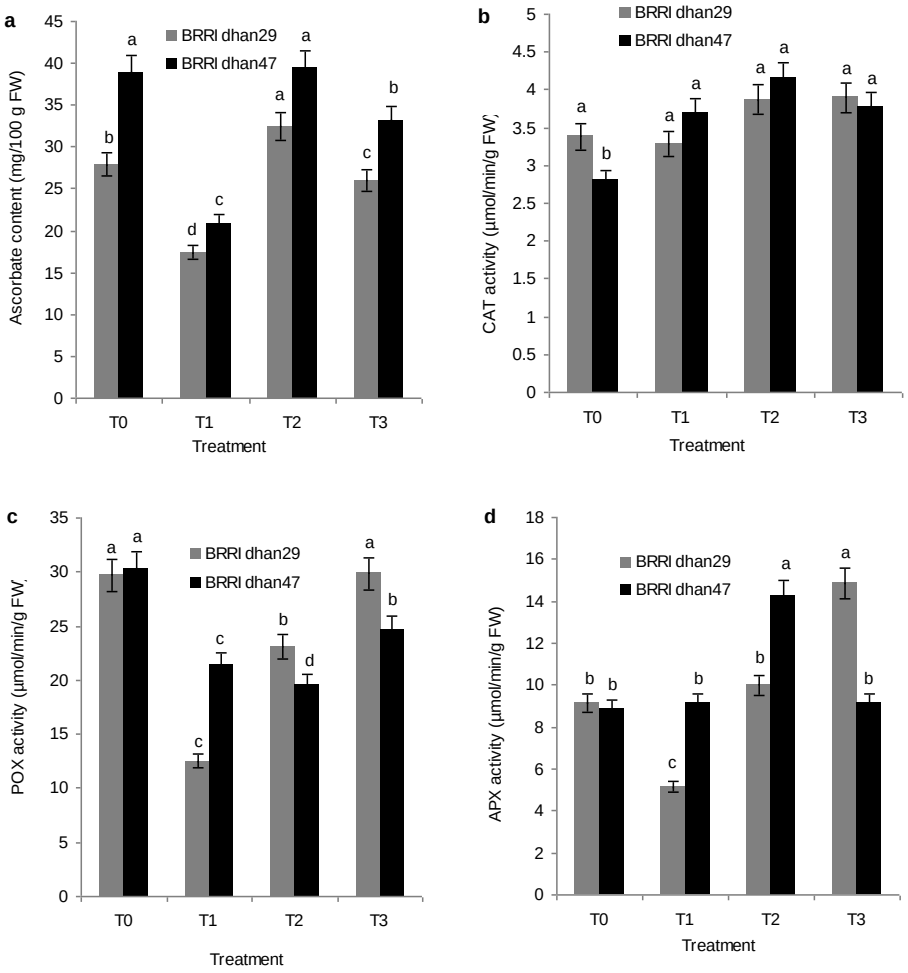

Fig 2. Ascorbate content (a) and activities of antioxidant enzymes CAT (b), POX (c) and APX (d) in salt-sensitive and salt-tolerant rice induced by exogenous proline under salt stress. The activity was expressed in µmol/min/g FW. Values represent the mean±SE of four replications. For the same rice cultivar, bars with the same letters are not significantly different at $P \leq 0.05$. Other details are shown in the legend of Fig 1.

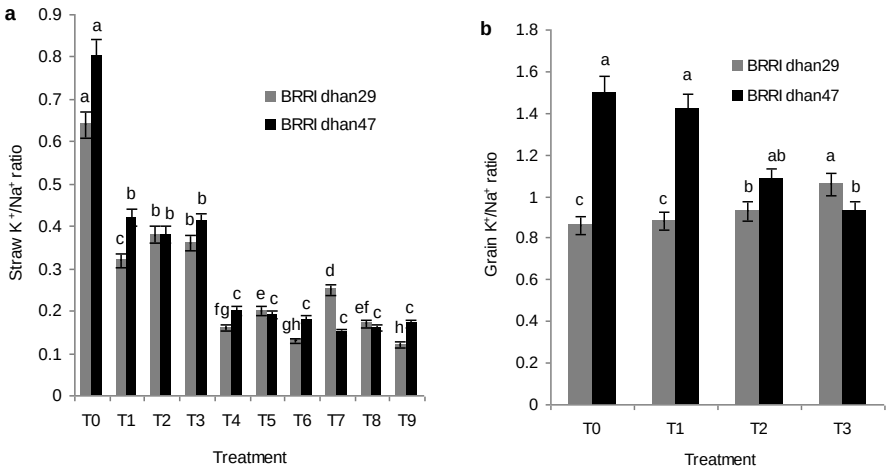

Fig 3. Straw K^+/Na^+ (a) and grain K^+/Na^+ (b) ratio in salt-sensitive and salt-tolerant rice influenced by exogenous proline under salt stress. Values represent the mean±SE of four replications. For the same rice cultivar, bars with the same letters are not significantly different at $P \leq 0.05$. Other details are shown in the legend of Fig 1.

proline alleviated the negative effect of salt stress and improved the chlorophyll content; however, the effect was more prominent in salt-sensitive cultivar compared to salt tolerant cultivar (Fig 1a). Some authors demonstrated that salt stress led to a decrease in chlorophyll content in rice genotypes whereas exogenous proline mitigated the harmful effects of salt stress on rice by increasing photosynthetic activity and chlorophyll contents (Sobahan et al., 2012; Hasanuzzaman et al., 2014). These results indicated that increased growth of rice plants during salt stress in the presence of proline was positively correlated with enhanced rates of photosynthetic capacity. The increased accumulation of proline in plants was correlated with improved salinity tolerance (Ashraf and Foolad, 2007; Hasanuzzaman et al., 2014). Our previous studies in cultured tobacco cells showed that exogenous proline increased intracellular proline levels and improved salt tolerance (Okuma et al., 2004; Hoque et al., 2007a), indicating that accumulation of proline played pivotal role in adaptation to osmotic stress caused by salinity. Similar to the results of Hasanuzzaman et al. (2014), salt-tolerant cultivar contained higher proline content as compared to salt-sensitive cultivar (Fig 1b). However, an increased level of intracellular proline in salt-sensitive but not in salt-tolerant rice was observed in response to salt stress (Fig 1b). Salt-sensitive rice IR-28 also produced higher amount of proline under salt stress than in salt-tolerant Pokkali (Demiral and Türkan, 2005), suggesting that salt-sensitive genotypes needed to synthesize high levels of proline for osmotic adjustment under saline conditions. Exogenous proline application further increased intracellular proline level in salt-sensitive rice which was positively associated with the improvement of salinity tolerance (Fig 1b). In rice plants, similar effects of exogenous proline were demonstrated by Hasanuzzaman et al. (2014). The concentration of proline, however, was not high enough to adjust the osmotic potential in some plants under salt stress. Despite the low proline accumulation, salt tolerance of salt-tolerant cultivar could be attributed to increased antioxidant defense system. In parallel with our results, Demiral and Türkan (2005) reported that the enhanced salt tolerance of rice was correlated with increased capacity of antioxidant system. Proline was suggested to function as an antioxidant in protecting cells against various abiotic stresses since proline scavenged free radicals and suppressed ROS accumulation (Hasegawa et al., 2000; Okuma et al., 2004; Banu et al., 2009, 2010).

Increased antioxidant defense mechanism was positively associated with the decreased oxidative damage and improved salinity tolerance (Hasegawa et al., 2000; Mittova et al., 2003; Demiral and Türkan, 2005; Hoque et al., 2007a, b, 2008; Banu et al., 2009; El-Shabrawi et al., 2010). Salinity impaired antioxidant defense system in plants whereas proline enhanced this defense system against NaCl-induced damage, thereby improving salt tolerance (Khedr et al., 2003; Hossain and Fujita, 2010; Nounjan and Theerakulpisut, 2012; Hasanuzzaman et al., 2014). In order to elucidate the role of proline in antioxidant defense mechanism, ascorbate content and antioxidant enzyme activities were measured.

In plant cells, ascorbate is a major antioxidant that directly scavenges ROS (Noctor and Foyer, 1998). Salt stress significantly decreased ascorbate content in both rice cultivars whereas the decreased ascorbate due to salt stress was alleviated by exogenously applied proline (Fig 2a). Salt stress led to a decrease in ascorbate content in salt-sensitive cultivars (Mittova et al., 2003; Hasanuzzaman et al., 2014). Conversely, an increased amount of ascorbate was observed in NaCl-stressed cells in the presence of exogenous proline (Hoque et al., 2007b; Hasanuzzaman et al., 2014). The biosynthetic capacity of ascorbate is impaired under stress conditions because the ascorbate pool is generally determined by not only rate of regeneration but also synthesis (Song et al., 2005). However, increased amount of ascorbate in proline-treated plants under salt stress was probably due to its regeneration or synthesis process accelerated by external proline. The metabolism of H_2O_2 is mainly dependent on the activity of antioxidant enzymes CAT and POXs localized in all compartments of plant cells. Salinity decreased the activities of antioxidant enzymes including CAT in salt-sensitive rice but these activities increased in salt-tolerant rice (Demiral and Türkan, 2005; El-Shabrawi et al., 2010; Hasanuzzaman et al., 2014). In our experiment, decreased activities of CAT and POX in salt-sensitive and POX in salt-tolerant rice were observed due to salt stress (Fig 2b, c). In the presence of proline, enhanced CAT and POX activities were found in both rice cultivars under salt stress (Fig 2b, c). Similarly, some authors found that exogenous proline enhanced the activity of CAT and POX in the both salt-sensitive and salt-tolerant rice during salt stress (Nounjan and Theerakulpisut, 2012; Hasanuzzaman et al., 2014). These results suggested that higher CAT and POX activities offered by exogenous proline could effectively detoxify H_2O_2 accumulated in salt-stressed plants. APX is considered as a major H_2O_2-scavenging enzyme in plants. In this study, activity of APX was approximately 3-fold higher than that of CAT (Fig 2b, d), suggesting that detoxification of H_2O_2 was mostly activated by APX. Salt stress caused a remarkable decrease in APX activity in salt-sensitive but a slight increase in salt-tolerant rice (Fig 2d). Under salt stress, exogenous proline significantly increased APX activity in both salt-sensitive and salt-tolerant rice cultivars (Fig 2d). Demiral and Türkan (2005) reported that APX activity decreased in IR-28 (salt-sensitive) but increased in Pokkali (salt-tolerant rice) under salt stress. Similar results were also reported in other rice genotypes under salt stress conditions (Nounjan and Theerakulpisut, 2012; Hasanuzzaman et al., 2014). Furthermore, they demonstrated that exogenous proline enhanced the activity of APX in both salt-sensitive and salt-tolerant rice during salt stress (Nounjan and Theerakulpisut, 2012; Hasanuzzaman et al., 2014). Taken together, results suggested that proline could effectively detoxify H_2O_2 by increasing APX activity in salt-stressed plants. A high cytosolic K^+/Na^+ ratio is essential for reducing salinity-induced oxidative damage. Enhanced influx of Na^+ and inhibition of K^+ uptake by plants under salt stress caused disturbances in K^+/Na^+ homeostasis, leading to toxic effect on plants (Zhu, 2003). Exogenous proline was shown to reduce Na^+ accumulation and increase K^+/Na^+ ratio under salt stress (Ahmed et al., 2011; Sobahan et al., 2012). In the present study, exogenous proline showed an increase in K^+/Na^+ ratio in salt-sensitive rice under salt stress (Fig 3a, b). These results illustrated that exogenous proline improved salt tolerance in rice by reducing Na uptake and increasing K uptake.

Materials and methods

Soil collection and pot preparation

Pot experiments were carried out in the net house of the Department of Soil Science, Bangladesh Agricultural University, Mymensingh. Soils were collected from 0-15 cm depth from Bangladesh Agricultural University farm. A total of 80 plastic pots were prepared with 8 kg soils in each pot. The soil was silt loam having pH 6.15, electrical conductivity

0.17 dS m^{-1}, exchangeable Na 0.35 meq 100 g^{-1} soil, total N 0.11% and organic matter 1.90%.

Plant materials and treatments

Two rice (*Oryza sativa* L.) cultivars viz. BRRI dhan29 (salt-sensitive) and BRRI dhan47 (moderately salt-tolerant) were used in the experiment. Ten treatment combinations viz. control (no NaCl or proline), 25 mM NaCl, 25 mM NaCl+25 mM proline, 25 mM NaCl+50 mM proline, 50 mM NaCl, 50 mM NaCl+25 mM proline, 50 mM NaCl+50 mM proline, 100 mM NaCl, 100 mM NaCl +25 mM proline, and 100 mM NaCl +50 mM proline were used for the two rice cultivars. Three healthy seedlings of 30-day-old were transplanted in a single hill in each pot. NaCl was used as per treatments for developing salinity. Rice plants were exposed to different concentrations of NaCl at 30 days after transplanting (active tillering stage). Active tillering refers to a stage when tillering rate is high with leaf emergence at regular intervals. On the same day, different doses of proline containing 0.1% Tween-20 were sprayed on the leaves at a volume of 25 mL per plant as per treatments. The experiment was laid out in a completely randomized design with four replications.

Management practices, crop harvesting and data recording

Normal water was used as irrigation. Fertilization and other management practices were performed as and when required. At 15 days after proline application, healthy green leaves were collected from rice plants to determine chlorophyll, proline and ascorbate contents, and activity of antioxidant enzymes. The crop was harvested at full maturity. Grain and straw yields and plant parameters were recorded. The K and Na contents from grain and straw samples were analyzed.

Assay of chlorophyll contents

Chlorophyll content was measured according to Porra et al. (1989). An aliquot amount of fresh green leaf was suspended in 10 mL of 80% acetone, mixed well and kept at room temperature in the dark for 7 days. The supernatant was collected after centrifugation at 5000 rpm for 15 min. The absorbance was recorded at 645 nm and 663 nm using a spectrophotometer (model 336001, Spectronic Instruments, USA).

Assay of intracellular proline contents

Proline content was determined following the method of Bates et al. (1973). An aliquot amount of fresh green leaf was homogenized in 10 mL of 3% sulfosalicylic acid and then the homogenate was centrifuged at 5000 rpm for 15 min. Other details of the assay were followed as described by Islam et al. (2009).

Assay of ascorbate contents

Ascorbate content was determined by 2,6-dichlorophenolindophenol visual titration method where ascorbate stoichiometrically reduced the dye 2,6-dichlorophenolindophenol to colorless compound. An aliquot amount (0.5 g) of green leaf was blended in 10 mL of 3% metaphosphoric acid using a blender to yield homogenous extract. The whole extract was filtered through a piece of cheese cloth and washed with 3% metaphosphoric acid solution. Ten mL of aliquot of the filtrate was titrated against the standardized dye.

Preparation of enzyme extract and assay of antioxidant enzymes

An aliquot amount of fresh green leaf was homogenized with 5 mL of 50 mM Tris–HCl buffer (pH 8.0) for CAT, and 50 mM KH_2PO_4 buffer (pH 7.0) for POX and APX. The homogenate was centrifuged at 5000 rpm for 20 min and the supernatant was then used as enzyme extract. CAT (EC: 1.11.1.6) activity was assayed as described by Islam et al. (2009). POX (EC: 1.11.1.7) and APX (EC: 1.11.1.11) activities were assayed as described by Hoque et al. (2007a, b).

Digestion of plant samples for K and Na determination

Grain and straw samples were dried in an oven at about 65°C for 48 h and then ground in a grinding machine to pass through a 20-mesh sieve. Ground sample (0.5 g) was taken into a 100 mL digestion vessel. Ten mL of diacid mixture (HNO_3:$HClO_4$ = 2:1) was added into the vessel. After leaving for a while, the vessels were heated at a temperature slowly raised to 200°C. Heating was stopped when the dense white fume of $HClO_4$ occurred. After cooling, the content was transferred into a 50 mL volumetric flask and the volume was made with distilled water. The digest was diluted to make a desired concentration. The K and Na contents in the digest were determined using a flame photometer (Jencon PFP 7, JENCONS-PLS, UK) following to Brown and Lilleland (1946).

Statistical analysis

Data were analyzed statistically using analysis of variance with the help of software package MSTAT-C. The significant differences between mean values were compared by Duncan's Multiple Range Test. Differences at $P \leq 0.05$ were considered significant.

Conclusions

Salt-tolerant rice maintained higher antioxidant defense systems without elevated levels of proline accumulation than salt-sensitive rice during salt stress. On the contrary, exogenous proline improved the growth and grain yield of salt-sensitive rice under salt stress by increasing proline accumulation and antioxidant defense systems as well as maintaining higher K^+/Na^+ ratio. However, the biomolecular mechanisms of proline in plant responses to salinity remain to be elucidated. Further studies are required to elucidate the biomolecular mechanisms and signaling pathways underlying the roles of proline in salt tolerance of plants.

Acknowledgements

This work was supported by a grant from the Bangladesh Agricultural University Research System.

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