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Effect of Soil Salinity and Exogenous Proline Application on Rice Growth, Yield, Biochemical and Antioxidant Enzyme Activities

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Abstract

Pot experiments were conducted to investigate the potential effects of exogenous proline on morphological and biochemical features, growth and yield of two inbred rice cultivars viz. BRRI dhan29 (salt-sensitive) and Binadhan-8 (moderately salt-tolerant) under salt stress conditions. Rice plants were exposed to NaCl stress at vegetative stage. Proline solutions were sprayed over rice leaves at vegetative and panicle initiation stages. Salt stress caused a significant reduction in growth and yield of both rice cultivars. NaCl stress at 50 mM caused a drastic decrease in growth of both cultivars. In salt-sensitive rice, salt stress significantly decreased chlorophyll content, K^+/Na^+ ratio and activities of H_2O_2-scavenging antioxidant enzymes catalase (CAT), guaiacol peroxidase (POX) and ascorbate peroxidase (APX), and increased intracellular proline levels. In salt-tolerant rice, significant decreases in chlorophyll and intracellular proline contents, K^+/Na^+ ratio and POX activity, and significant increases in ascorbate content and CAT and APX activities were observed in response to salt stress. Exogenous proline resulted in an increase in chlorophyll, intracellular proline and ascorbate contents, K^+/Na^+ ratio and activities of antioxidant enzymes, thereby increased growth and yield of both rice cultivars at 25 mM NaCl stress. Salt-tolerant cultivar produced significant amount of grains at 50 mM NaCl stress under 100 mM proline application, although other plants of both cultivars died due to 50 mM NaCl stress under 25-100 mM proline application. The present study suggests that exogenous proline confers tolerance to salinity in rice by increasing chlorophyll content, intracellular proline levels, K^+/Na^+ ratio and antioxidant defense mechanisms.

Keywords: *Ascorbate peroxidase; antioxidant enzymes catalase; chlorophyll; proline; salt stress; rice*

Introduction

Soil salinity is a major concern to agriculture all over the world because it affects almost all plant functions. More than 6% of the world's land and one third of the world's irrigated land are significantly affected by soil salinity [1,2]. Moreover, soil salinization due to irrigation is becoming increasingly detrimental to agriculture. Agriculture is the single most important sector of Bangladesh's economy. About one million hectare of arable lands is affected by soil salinity [3]. Rice, the most important cereal crop worldwide, is highly sensitive to salinity. Sensitivity of rice to salinity stress varies with the growth stage. Bangladesh is the fourth largest rice producing country in the world. Rice production is seriously hampered in saline areas of southern Bangladesh due to lack of salt-tolerant high yielding variety and improper management practices.

Salinity imposes both ionic toxicity and osmotic stress to plants, leading to nutrition disorder and oxidative stress [4,5]. Salt stress disturbs cytoplasmic K^+/Na^+ homeostasis, causing an increase in Na^+ to K^+ ratio in the cytosol [5]. Accumulation of excess Na^+ and Cl^- causes ionic imbalances that may impair the selectivity of root membranes and induce K^+ deficiency [6]. Plants have evolved a variety of adaptive mechanisms to respond to environmental stress including salt stress. One of the main adaptive mechanisms to salt stress in plants is the accumulation of compatible solutes. Proline is the most common compatible solute that occurs in a wide variety of plants. Increased levels of proline accumulated in plants correlate with enhanced salt tolerance [4,7-9]. Proline contributes to the osmotic adjustment [4,8] as well as to the protection of membranes, proteins and enzymes from the damaging effects of various stresses [8,10]. Moreover, proline provides a protection against salt stress via maintaining redox homeostasis [11]. Under salt stress, exogenous application of proline up-regulates stress-protective proteins [12], and reduces lipid peroxidation [7,13] and protein oxidation [11]. Proline also suppresses production of free radicals [4,7,10] and reactive oxygen species (ROS) [13-15]. Hong., *et al*. [16] suggest that the role of proline as a free radical scavenger is more important in overcoming stress than its role as a simple osmolyte.

Environmental stresses including salinity induce the production of ROS including hydrogen peroxide (H_2O_2) in plant cells [4,13-15,17]. These ROS are highly reactive and toxic to plants, and can lead to cell death by causing damage to proteins, lipids, DNA and carbohydrates [13,17,18,] although they act as signaling molecules that mediate many key physiological processes. Plants possess an array of enzymatic and non-enzymatic antioxidant defense systems to protect their cells against the damaging effects of ROS [17,18]. The major ROS-scavenging antioxidant enzymes include catalase (CAT), guaiacol peroxidase (POX) and ascorbate peroxidase (APX). Ascorbate, one of the key components of ascorbate-glutathione cycle, directly scavenges ROS and functions as an electron donor to APX for scavenging H_2O_2 [18,19]. There are reports on the changes in content and activity of different components of the antioxidant defense systems in plant responses to salt stress [4,9,11,18,20-24].

Up-regulation of the components of the antioxidant defense mechanisms offered by proline is expected to protect plants from NaCl-induced oxidative damage. There are increasing evidences that proline enhances antioxidant defense mechanisms and improves stress tolerance in plants and cultured cells [12,15,25-27] We have shown that exogenous application of proline increased intracellular proline contents [7], which lead to suppression of cell death and improvement of salt tolerance in tobacco cultured cells via increment of the antioxidant defense systems [11,13,14,23,24]. Further, exogenous proline induced the expression of ROS-scavenging antioxidant defense genes in response to salt stress [13,14]. Recent studies in rice have shown that exogenous proline improves salt tolerance by increasing K^+/Na^+ ratio [9,28], endogenous proline and antioxidant enzyme activities viz. CAT, POX and APX [9]. However, protective mechanisms of proline in plant responses to salt stress remain to be clarified. In order to clarify the potential role of proline in salt tolerance, we investigated the effects of exogenous proline on growth, chlorophyll, intracellular proline and ascorbate contents, and activity of antioxidant enzymes in two contrasting rice genotypes exposed to salt stresses.

Materials and Methods

Soil collection, pot preparation, plant materials and treatments

The pot experiments were carried out at Net-house of the Department of Soil Science, Bangladesh Agricultural University (BAU), and Mymensingh. Equal size plastic pots were prepared with 8 kg soils. Characteristically, the soil was silt loam having pH 6.15, electrical conductivity 0.17 dS/m, exchangeable Na 0.35 meq/100 g soil, total N 0.11% and organic matter 1.90%. Two high yielding rice (*Oryza sativa* L.) cultivars *viz*. BRRI dhan29 (salt-sensitive) and Binadhan-8 (moderately salt-tolerant) were used as plant materials. Nine treatment combinations *viz*. control (no NaCl or proline), 25 mM NaCl, 25 mM NaCl + 25 mM proline, 25 mM NaCl + 50 mM proline, 25 mM NaCl + 100 mM proline, 50 mM NaCl, 50 mM NaCl + 25 mM proline, 50 mM NaCl + 50 mM proline, 50 mM NaCl + 100 mM proline were used for the two rice cultivars. Rice seedlings were grown in non-saline silt loam soils. Three healthy seedlings of thirty-day-old were transplanted in each hill of each pot. The pure salt (NaCl) was used for developing salinity. Rice plants were exposed to different concentrations of NaCl at 30 days after transplanting (vegetative stage). 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 treatment. Similarly, proline was applied at 65 days after transplanting (panicle initiation stage) as per treatment. 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 45 days after transplanting (15 days after first proline application), healthy green leaves were detached from the plants for the determination of chlorophyll, proline and ascorbate contents, and activity of antioxidant enzymes such as CAT, POX and APX. The crop was harvested at full maturity. Grain and straw yields and plant parameters were recorded. K and Na contents were measured from grain and straw samples.

Assay of chlorophyll contents

Chlorophyll content was measured according to Porra., *et al*. [29]. 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. After that, the absorbance was recorded at 645 nm and 663 nm using a spectrophotometer.

Assay of intracellular proline contents

Proline content was measured according to the method of Bates., *et al*. [30]. An aliquot amount of fresh green leaf was homogenized in 10 mL of 3% sulfosalicylic acid and the homogenate was centrifuged at 5000 rpm for 15 min. Two milliliters of the supernatant were reacted with 2 mL of acid ninhydrin (1.25 g ninhydrin dissolved in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid) and 2 mL of glacial acetic acid for 1 hr at 100°C and the reaction was then terminated in an ice bath. The colored reaction mixture was extracted with 4 mL of toluene and the absorbance was recorded at 520 nm. Proline content was calculated from a standard curve.

Assay of ascorbate contents

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

Preparation of enzyme extract

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.

Assay of antioxidant enzymes

CAT (EC: 1.11.1.6) activity was determined according to the method of Aebi [31]. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 8.0), 0.25 mM EDTA, 20 mM H_2O_2 and 25 µL of enzyme extract. The reaction was started by the addition of H_2O_2. The activity was calculated from the decrease in absorbance at 240 nm for 2 min when the extinction coefficient was 40 mM^{-1} cm^{-1}. POX (EC: 1.11.1.7) activity was determined according to Nakano and Asada (1981). The reaction buffer solution contained 50 mM KH_2PO_4 buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H_2O_2, and 10 mM guaiacol. The reaction was started by adding enzyme extract to the reaction buffer solution. The activity was calculated from change in absorbance at 470 nm for 30 sec where an extinction coefficient is 26.6 mM^{-1} cm^{-1}. APX (EC: 1.11.1.11) activity was measured following the method of Nakano and Asada [32]. The reaction buffer solution contained 50 mM KH_2PO_4 buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H_2O_2, and 0.5 mM ascorbate. The reaction was started by the addition of enzyme extract 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}.

Preparation of plant samples for the determination of K and Na

Grain and straw samples were dried in an oven at about 65°C for 48 hours and then ground in a grinding machine to pass through a 20 mesh sieve. Grinding samples of 0.5 g (grain and straw, separately) were transferred into 100 mL digestion vessel. Ten milliliters of diacid mixture (HNO_3:$HClO_4$ = 2:1) were added into the vessel. After leaving for a while, the flasks 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 taken into a 50 mL volumetric flask and the volume was made with distilled water. This digest was used for the determination of K and Na.

Determination of K and Na

Five milliliters digest for grain and 2 mL digest for straw were taken, and both digests are diluted to make a desired concentration. Then K and Na contents were determined using a flame photometer according to Brown and Lilleland [33].

Statistical Analysis

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

Results

Growth and yield of rice

Salt stress caused a significant reduction in growth and yield of salt-sensitive (BRRI dhan29) and salt-tolerant (Binadhan-8) rice (Table 1). NaCl stress at 50 mM caused a drastic reduction in growth parameters of both rice cultivars. Neither salt-sensitive nor salt-tolerant rice produced effective tillers as well as grains after exposure to 50 mM NaCl stress. It was also observed that all plants of both cultivars died within 15 days after exposure to 50 mM NaCl stress. Foliar application of proline (25-100 mM) resulted in an increase in growth, yield contributing characters and yield of both rice cultivars in response to 25 mM NaCl stress. It is important to note that salt-tolerant (Binadhan-8) rice cultivar produced effective tillers and grains at 50 mM NaCl with 100 mM proline application, although salt-sensitive rice cultivar (BRRI dhan29) failed to do so (Table 1).

Chlorophyll contents in rice

Chlorophyll a content was significantly decreased in salt-tolerant rice in response to salt stress, although chlorophyll b and total chlorophyll contents were significantly lower in both salt-sensitive and salt-tolerant rice cultivars during salt stress than non-stress (Table 2). On the contrary, foliar application of proline showed significant increases in chlorophyll a, b and total chlorophyll contents in both rice cultivars with 25 mM NaCl stress condition. At 50 mM NaCl stress, exogenous proline also offered a significant amount of chlorophyll contents in salt-tolerant rice (Table 2).

Intracellular proline contents in rice

Salt stress significantly increased intracellular proline levels in salt-sensitive rice but decreased in salt-tolerant rice (Figure 1A). Exogenous proline showed a significant increase in proline accumulation in both rice varieties at 25 mM NaCl stress. The proline accumulation was much higher in salt-tolerant rice than salt-sensitive when 50 or 100 mM proline was exogenously applied at NaCl-stressed plants. Additionally, the intracellular proline level was higher in plants treated with 50 mM NaCl + 100 mM proline than 25 mM NaCl (Figure 1A).

Ascorbate contents in rice

Ascorbate contents were measured in rice to investigate whether exogenous proline influenced antioxidant defense system (Figure 1B). Ascorbate contents in salt-sensitive rice was not affected by salt stress, but significantly increased in salt-tolerant rice. Exogenous application of proline showed a significant increase in ascorbate contents in salt-sensitive rice at 25 mM NaCl stress, however, no increase of ascorbate contents was observed in salt-tolerant rice. On the contrary, 100 mM proline resulted in a significant increase in ascorbate content in salt-tolerant rice responses to 50 mM NaCl stress (Figure 1B).

Treatments	BRRI dhan29				Binadhan-8			
	Plant dry weight (g/pot)	No. of effective tillers /hill	No. of filled grains/ panicle	Grain 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	49.3a	20a	141a	45.3a	57.1a	22a	135a	45.0a
T_1: 25 mM NaCl	15.0c	9d	119c	20.1e	15.5d	8d	81e	16.5d
T_2: 25 mM NaCl+25 mM proline	32.5b	13c	139a	36.9b	24.2c	13b	99d	27.4c
T_3: 25 mM NaCl+50 mM proline	33.8b	15b	126b	32.8d	28.1b	12c	120b	32.5b
T_4: 25 mM NaCl+100 mM proline	32.4b	13c	139a	34.5c	28.2b	13b	105c	29.6c
T_5: 50 mM NaCl	4.51d	ND	ND	ND	2.79f	ND	ND	ND
T_6: 50 mM NaCl+25 mM proline	4.65d	ND	ND	ND	4.50e	ND	ND	ND
T_7: 50 mM NaCl+50 mM proline	4.69d	ND	ND	ND	3.58ef	ND	ND	ND
T_8: 50 mM NaCl+100 mM proline	5.58d	ND	ND	ND	14.8d	6e	59f	12.9e
SE (±)	5.65	2.63	23.44	6.33	5.75	2.52	18.11	5.48
CV%	4.92	4.31	2.62	4.65	3.87	4.06	3.17	8.65

Table 1: *Effect of proline on the growth and yield of BRRI dhan29 and Binadhan-8 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 from four replications. Same letter in a column represents insignificant difference at P < 0.05.

Treatments	BRRI dhan29			Binadhan-8		
	Chl-a (µg/ml)	Chl-b (µg/ml)	Total Chl (µg/ml)	Chl-a (µg/ml)	Chl-b (µg/ml)	Total Chl (µg/ml)
T_0: Control	5.51d	9.98a	15.5a	5.40b	8.98c	14.4b
T_1: 25 mM NaCl	5.66d	7.20c	12.9b	4.96c	6.46e	11.4c
T_2: 25 mM NaCl + 25 mM proline	6.66b	8.42b	15.1a	5.45b	9.31bc	14.7ab
T_3: 25 mM NaCl + 50 mM proline	6.97a	8.32b	15.4a	5.58b	9.88a	15.5a
T_4: 25 mM NaCl + 100 mM proline	6.25c	8.19b	14.4a	6.89a	8.19d	15.1ab
T_5: 50 mM NaCl	ND	ND	ND	ND	ND	ND
T_6: 50 mM NaCl + 25 mM proline	ND	ND	ND	ND	ND	ND
T_7: 50 mM NaCl + 50 mM proline	ND	ND	ND	ND	ND	ND
T_8: 50 mM NaCl + 100 mM proline	ND	ND	ND	5.67b	9.57ab	15.2ab
SE (±)	1.10	1.50	2.59	0.96	1.49	2.43
CV%	4.60	6.19	10.21	6.55	4.79	6.84

Table 2: *Chlorophyll contents in salt sensitive and salt-tolerant rice influenced by proline under salt stress.*
"ND" (not detected) indicates no plants survived during the estimation. Values represent the mean from four replications. Same letter in a column represents insignificant difference at p < 0.05.

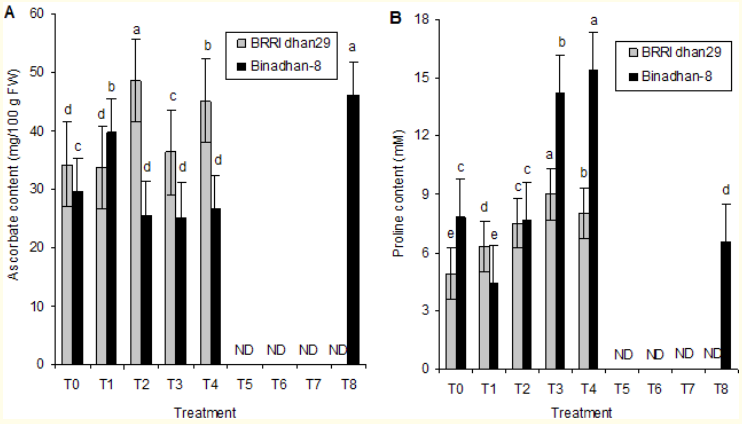

Figure 1: *Ascorbate (A) and intracellular proline (B) contents in salt-sensitive and salt-tolerant rice induced by exogenous proline under salt stress. "ND" (not detected) indicates no plants survived during the estimation. Values represent the mean ± SE from four replications. For the same rice cultivar, bars with the same letters are not significantly different at P < 0.05.*

Treatment Details

T_0: control (no NaCl or proline), T_1: 25 mM NaCl, T_2: 25 mM NaCl+25 mM proline, T_3: 25 mM NaCl+50 mM proline, T_4: 25 mM NaCl+100 mM proline, T_5: 50 mM NaCl, T_6: 50 mM NaCl+25 mM proline, T_7: 50 mM NaCl+50 mM proline, T_8: 50 mM NaCl+100 mM proline.

Activity of antioxidant enzymes in rice

We investigated whether proline enhanced the activities of H_2O_2-scavenging antioxidant enzymes (Figure 2B-D). Salt stress caused significant reductions in CAT, POX and APX activities in salt-sensitive rice, and POX activity in salt-tolerant rice. Proline application showed significant increases in CAT, POX and APX activities of salt-sensitive rice in response to salt stress. In salt-tolerant rice, salt stress resulted in an increase in CAT activity, and this increase was more prominent in the presence of proline. It was also observed that CAT activity was significantly higher at 50 mM NaCl + 100 mM proline than non-stress. POX activities were significantly increased in salt-tolerant rice cultivar under different concentrations of proline application at 25 mM NaCl or even 50 mM NaCl stress condition. APX activity was also increased in salt-tolerant rice cultivar under salt stress, although exogenous application of proline did not show any increase in APX activity (Figure 2D).

K^+/Na^+ ratio in rice

Salt stress significantly decreased K^+/Na^+ ratio in both salt-sensitive and salt-tolerant rice cultivars (Figure 3A, B). Exogenous proline increased straw K^+/Na^+ ratio in both cultivars but these increases were not consistent. The straw K^+/Na^+ ratio was also high in salt-tolerant rice when exposed to 50 mM NaCl with 100 mM proline (Figure 3A). The grain K^+/Na^+ ratio was higher in salt-tolerant rice than salt-sensitive rice even exposure to NaCl and proline (Figure 3B). However, exogenous proline resulted in a significant increase in grain K^+/Na^+ ratio in both cultivars (Figure 3B).

Changes in soil properties

We investigated the changes in soil properties such as pH, EC, exchangeable Na, organic matter and total N of post-harvest soils (Table 3). A considerable increase in soil pH, EC and exchangeable Na was observed under NaCl stress. No remarkable changes in soil organic matter and total N were observed due to NaCl stress. Foliar proline treatments on rice cultivars had no influence on soil properties under salt stress conditions (Table 2).

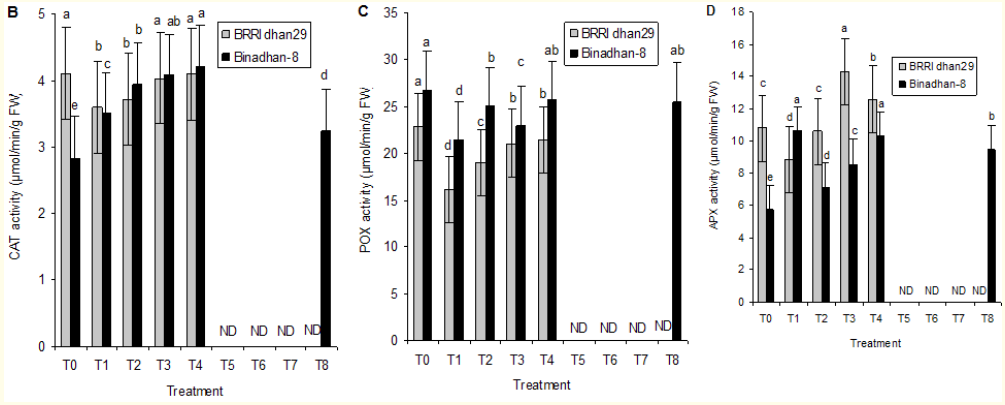

Figure 2: *Activities of antioxidant enzymes CAT (B), POX (C) and APX (D) in salt-sensitive and salt-tolerant rice induced by exogenous proline under salt stress. The activity was expressed in µmol/min/g FW. "ND" (not detected) indicates no plants survived during the estimation. Values represent the mean ± SE from four replications. For the same rice cultivar, bars with the same letters are not significantly different at P < 0.05.*

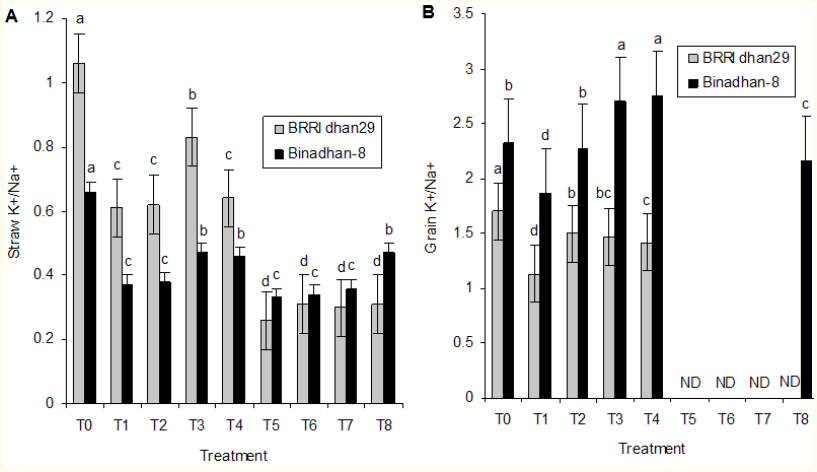

Figure 3: *Straw K^+/Na^+ (A) and grain K^+/Na^+ (B) ratio in salt-sensitive and salt-tolerant rice influenced by exogenous proline under salt stress. "ND" (not detected) indicates no plants survived during the estimation. Values represent the mean ± SE from four replications. For the same rice cultivar, bars with the same letters are not significantly different at P < 0.05.*

Discussion

Protective effects of proline on growth and yield

In the present study, we investigated the protective effects of exogenous proline on rice against NaCl-induced damage. Previously we have shown that exogenous proline improves cell growth and suppresses cell death in tobacco cultured cells induced by salt stress [7,13,14,23,24,]. Sobahan., *et al*. [28] have shown that exogenous proline improves salt tolerance in salt-sensitive rice more effectively than salt-tolerant rice. In this experiment, exogenous proline increased growth and yield of both salt-sensitive and salt-tolerant rice at 25 mM NaCl stress (Table 1). Further, salt-tolerant rice plants survived and produced effective tillers and grains in response to 50 mM NaCl stress in the presence of 100 mM proline. In contrast, none of plants of both cultivars survived in response to 50 mM NaCl stress even application of 25-100 mM proline (Table 1). There are increasing evidences that exogenously supplied proline improves growth of a variety of plant species in response to salt stress [9,12,26,27,34]. These results suggest that the adverse effects of salt stress on plants could be alleviated by application of proline. These results also suggest that salt-tolerant plant over expressing proline accumulation could contribute to the improvement of salinity tolerance.

Chlorophyll contents

Chlorophyll is one of the most important pigment components of a plant. Measurement of chlorophyll content provides the quantitative information about photosynthesis. Chlorophyll content may vary due to varying salt stress levels, affecting plant growth and development. The reduction in plant growth in the present investigation subjected to salinity is associated with a decreased rate of photosynthetic capacity. Chlorophyll contents in both salt-sensitive and salt-tolerant rice decreased due to salt stress whereas this decrease was alleviated by exogenous application of proline (Table 2). Similarly, there are evidences that salinity leads to a decrease in chlorophyll contents in rice genotypes [28,35,36]. Some authors have also shown that exogenous proline reduces the adverse effects of salt stress by increasing photosynthetic activity and chlorophyll contents in a variety of plants including rice [28,34,37], indicating that improved plant growth of rice cultivars due to exogenous proline under salt stress might be positively correlated with an increased rate of photosynthetic capacity.

Intracellular proline contents

Increased levels of proline accumulated in plants correlate with improved salt tolerance [7-9]. Exogenous application of proline remarkably increases proline accumulation under NaCl stress and mitigates NaCl-induced growth inhibition [7,23], indicating that uptake of compatible solutes plays an important role in adaptation to osmotic stress caused by salinity. A significant increase in intracellular proline content was observed in salt-sensitive rice but not in salt-tolerant rice responses to salt stress (Figure 1B). Exogenous proline increased the intracellular proline levels in both rice cultivars which were positively associated with the improvement of salt tolerance (Figure 1B). Similar to our results, Summart., *et al*. [38] and Nounjan., *et al*. [9] showed that salt stress caused an increase in proline accumulation in rice. Nounjan., *et al*. [9] also showed that exogenous application of proline increased endogenous proline in rice plants. The concentration of proline, however, is not high enough to adjust the osmotic potential in some plants under salt stress [39]. Proline has been suggested to function as an antioxidant in protecting cells against the damaging effects of various stresses since proline scavenges free radicals and suppresses ROS accumulation [4,7,10,13,14,25].

Antioxidant defense mechanisms

Up-regulation of the antioxidant defense mechanisms correlates with the alleviation of oxidative damage and improved tolerance to salinity [4,11,20-24]. Plants employ both enzymatic and non-enzymatic antioxidant defense systems against NaCl-induced oxidative damage. This defense system is impaired due to salt stress. On the contrary, proline enhances antioxidant defense mechanisms against NaCl-induced damage and improves salt tolerance in various plants as well as in cultured cells [7,9,11-14,23-27]. In order to elucidate the antioxidant defense mechanism of proline, we measured ascorbate content and activities of major H2O2-scavenging antioxidant enzymes CAT, POX and APX. In plant cells, ascorbate is a major antioxidant that directly scavenges ROS and act as an electron donor to

APX for scavenging H_2O_2 involved in the ascorbate-glutathione cycle [18,19]. A significant increase in ascorbate content was observed in salt-tolerant but not in salt-sensitive rice responses to salinity (Figure 2A). The opposite results were observed in the presence of proline at 25 mM NaCl stress although 100 mM proline significantly increased ascorbate content in salt-tolerant rice at 50 mM NaCl stress (Figure 2A). Several authors have shown that salt stress leads to a decrease in ascorbate content in salt-sensitive cultivars [20-24,40,41]. An increase in ascorbate contents has been reported in tobacco cultured cells under NaCl stress in the presence of exogenous proline [24]. The biosynthetic capacity of ascorbate is impaired under stress conditions because the ascorbate pool is generally determined by its rates of not only regeneration but also synthesis [42]. However, increased ascorbate contents in proline-treated plants under salinity are probably due to its regeneration or synthesis process accelerated by proline or decreased activity of APX where ascorbate is used as a reductant.

The metabolism of H_2O_2 is mainly dependent on antioxidant enzymes such as CAT and POXs localized in almost all compartments of plant cells. It has been reported that the increase in antioxidant enzyme activities is involved in eliminating H_2O_2 from salt-stressed roots [43]. In this experiment, salt stress caused a reduction in CAT and POX activities in salt-sensitive and POX activity in salt-tolerant rice whereas these reductions in both rice cultivars were suppressed by exogenous application of proline (Figure 2B) (Figure C). Similar results have also been demonstrated by several authors [9,12,23] who observed that CAT and POX activities decreased under salt stress but increased in the presence of proline. The increased CAT and POX activities in the presence of proline suggest that exogenous proline is able to effectively detoxify H_2O_2 generated by salt stress.

APX is widely considered as a major H_2O_2-scavenging enzyme in plants [32] when CAT activity is suppressed and endogenous level of H_2O_2 is enhanced [44]. Similar to our previous results in tobacco cultured cells [23,24], APX activity in rice plants was higher than that of CAT activity (Figure 2B,D), indicating that H_2O_2 was mostly detoxified by APX using ascorbate as a reductant. In the present study, salt stress leads to a decrease in APX activity in salt-sensitive rice but an increase in salt-tolerant rice (Figure 2D). On the other hand, exogenous proline results in an increase in salt-sensitive but not in salt-tolerant rice induced by salt stress (Figure 2D). These results are in agreement with our previous results observed in NaCl-unadapted tobacco cultured cells [24], suggesting that exogenous proline could contribute to the detoxification of H_2O_2 by increasing APX activity in salt-sensitive plant during salt stress.

K^+/Na^+ ratio

A low ratio of Na^+ to K^+ in the cytosol is essential for normal cellular functions of plants. Na^+ competes with K^+ uptake, causing an increase in Na^+ to K^+ ratio in the cytosol under salt stress, resulting in accumulation of toxic levels of Na^+ and insufficient K^+ concentrations for enzymatic reactions and osmotic adjustment [5,45]. Exogenously supplied proline reduces Na^+ accumulation and increases K^+/Na^+ ratio under salt stress [9,28,34]. In contrast, exogenous proline has been shown to alleviate the inhibition of NaCl-induced cell growth without improving a ratio of K^+ to Na^+ [7]. Salt stress significantly reduced K^+/Na^+ ratio in both rice cultivars whereas this K^+/Na^+ ratio was increased by proline application under NaCl stress (Figure 3A,B). These results indicate that exogenous proline contributes to the reduction of Na uptake as well as increment of K uptake under salt stress condition.

Changes in soil properties

Saline soils have detrimental effects on soil physical and chemical properties, and cause nutrient deficiencies [46]. It was observed that salt stress considerably increased soil pH, EC and exchangeable Na, whereas no remarkable changes in organic matter and total N were observed after harvest of rice in soil (Table 3). Similar results have also been found in our previous report [47]. Under salt stress, foliar application of proline did not affect soil properties after post-harvest (Table 3).

Treatment	Soil properties				
	Soil pH	EC (dS m^{-1}) (soil:water = 1:5)	Exchangeable Na (me/100g)	Organic matter (%)	Total N (%)
T_0: Control	6.17	0.180	0.35	1.88	0.108
T_1: 25 mM NaCl	6.50	0.583	0.63	1.84	0.106
T_2: 25 mM NaCl + 25 mM proline	6.48	0.582	0.64	1.84	0.106
T_3: 25 mM NaCl + 50 mM proline	6.48	0.580	0.64	1.85	0.106
T_4: 25 mM NaCl + 100 mM proline	6.49	0.582	0.63	1.85	0.105
T_5: 50 mM NaCl	6.62	1.971	1.28	1.82	0.104
T_6: 50 mM NaCl+25 mM proline	6.61	1.970	1.27	1.83	0.104
T_7: 50 mM NaCl+50 mM proline	6.61	1.968	1.28	1.83	0.104
T_8: 50 mM NaCl+100 mM proline	6.62	1.970	1.28	1.83	0.105

Table 3: *Changes in soil properties after rice harvest. Values represent the mean from four replications.*

Conclusion

Under salt stress, salt-tolerant plants maintained higher antioxidant defense mechanisms without elevated accumulation of proline while salt-sensitive plants contributed to the accumulation of proline, suggesting that two rice cultivars differentially responded to salinity. Exogenous proline confers tolerance to salt stress in rice by maintaining higher K^+/Na^+ ratio, and increasing chlorophyll contents, proline accumulation and antioxidant defense mechanisms. Additionally, Binadhan-8 (salt-tolerant rice) confers tolerance to high salt stress in the presence of high concentration of exogenous proline, probably due to maintaining higher antioxidant defense mechanisms. However, the molecular mechanisms of proline in plant responses to salinity remain to be elucidated. Further studies are needed to elucidate the molecular mechanisms and signaling pathways underlying the role of proline in salt tolerance in plants.

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Volume 2 Issue 1 July 2015