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Chitosan mitigates salt stress in rice by enhancing antioxidant defense system

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ABSTRACT

Salinity is one of the major constraints for rice (*Oryza sativa* L.) cultivation globally, as rice is highly sensitive to salinity than other cereal crops. In order to elucidate the improvement mechanism of salinity tolerance by chitosan (CS), we analyzed the morphological, biochemical and antioxidant enzymatic activities of salt-stressed rice at germination and seedling stages of four cultivars: BRRI dhan28 and BRRI dhan29 as salt-sensitive and Pokkali and Pengek as salt-tolerant. Salinity inhibited seed germination as well as growth of rice seedlings. At the germination stage, CS increased the germination percentage by enhancing amylase activity and significantly accelerated shoot growth but not root growth under salt stress condition. In the seedling stage, 50 ppm of CS mitigated the growth inhibition of seedling of the sensitive cultivars and the reduction of chlorophyll (both chl 'a' and chl 'b') contents. Moreover, CS increased the activities of catalase (CAT) and ascorbate peroxidase (APX) but not the activities of peroxidase (POX). However, the accumulation of proline and phenols in the seedlings was increased under saline condition but not under saline condition supplemented with CS. Principal component analysis revealed that a relatively stronger association between 50 ppm CS treatment with the non-stress condition than that of 25 ppm CS treatment both in sensitive and tolerant cultivars which suggested that 50 ppm CS treatment was more effective. Our results suggest that exogenous CS can be used to mitigate salt-induced adverse effects in rice as well as in other crop plants.

Keywords: Salinity, chitosan, antioxidant system, salinity tolerance, rice

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1 Introduction

Salinity is a serious threat to the crop production in the southern region of Bangladesh (Haque et al., 2014) and it is one of the adverse environmental factors that affect plants spanning from seed germination to productivity. More than 6% of the world's total cultivable area is affected by over accumulated salt (Munns and Tester, 2008), and this problem prolongs to worsen due to secondary salt, which accumulates in irrigated soils as water evaporates (Yang and Guo, 2017).

Seed germination plays important roles in the establishment of seedlings and their subsequent growth. Salinity impairs plant growth and development via water scarcity, cytotoxicity due to excessive uptake of ions such as sodium (Na^+) and chloride (Cl^-), and nutritional imbalance (Hasegawa et al., 2000). However, salinity hampers seed germination by affecting major events of germination such as imbibition, metabolism activation, the emergence of embryonic tissues and seedling establishment (Hasanuzzaman et al., 2013;

Wahid et al., 2011). It has been revealed that salinity inhibits seed germination by reducing α-amylase activity, which is the major reason for poor hydrolysis or imbibitions of stored substances (Hua-long et al., 2014; Liu et al., 2018). Other studies reported that salt stress reduced root length, root dry weight, shoot length and shoot dry weight in both sensitive and tolerant rice cultivars where the magnitude of growth reduction is much higher in sensitive cultivar compared to tolerant (Mahmud et al., 2016; Kumar and Khare, 2016).

Salinity causes oxidative stress in plants through the production of reactive oxygen species (ROS) or free radicals, such as superoxide ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) (Noctor and Foyer, 1998). Plants encounter salinity stress through enzymatic and non-enzymatic antioxidant mechanisms (Gupta and Huang, 2014; Bose et al., 2013; Kibria et al., 2017; Dreyer and Dietz, 2018). Non-enzymatic antioxidants catalyze redox reactions and rely on electron donation via reduction of low-molecular-weight antioxidants, such as glutathione, flavonoids, phenols and free proline (Noctor and Foyer, 1998; Symes et al., 2018). Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) have been previously reported as the major antioxidant enzymes that serve as an antioxidant defense system in many plants under salt stress (Parida et al., 2004; Chawla et al., 2012; Mahmud et al., 2017; Wang et al., 2017; Foyer, 2018).

Various exogenous compounds are applied to promote plant growth and induce abiotic resistance (Yasmeen et al., 2012). Chitosan similar to plant growth regulators, is a partially deacetylated form of chitin, a natural biopolymer from the exoskeleton of crustaceans and fungal cell walls, which is biocompatible, biodegradable and a sustainably renewable cheap resource that has many applications, including plant defense (Katiyar et al., 2014; Malerba and Cerana, 2015; Ray et al., 2016b), confer resistance against abiotic stresses such as water deficit, salinity, heat stress, and heavy metal toxicity (Malerba and Cerana, 2015). It has been revealed that exogenous chitosan could mitigate salt stress by increasing antioxidant enzyme (SOD, POX, and CAT) and also enhancing photosynthetic pigments i.e. chlorophyll a and b (Ma et al., 2011). Despite all this knowledge, there are still many points that need to be clarified about the stage, organ, and variety-specific alleviating capability of chitosan under salt stress.

The present study aims to elucidate the chitosan's dose-specific response to the antioxidative capacity of the salt-sensitive and salt-tolerant rice cultivars at the seedling stage.

2 Materials and Methods

2.1 Experimental layout

The experiment was conducted using two salt-sensitive (BRRI dhan28 and BRRI dhan29) and two salt-tolerant rice cultivars (Pokkali and Pengek). The uniform-sized rice seeds were sorted out and then seeds were sterilized using 5% sodium hypochlorite + 2% Tween-20 for 25 min, followed by five times washing with distilled water. Subsequently, the seeds were presoaked in distilled water for 24 h and kept for 3 days on a wet filter paper covered with aluminum foil at 27±2 °C to induce germination. Three days after germination, seeds were transferred to the mentioned treatments and the number of seeds germinated was counted until the next 5th day. For seedlings parameters, uniformly germinated seeds were placed in plastic pots (10.5 cm × 12.5 cm) containing 8 kg of soil (silt loam, pH of 6.18, 0.15 dS m^{-1} EC, 8.4% CEC, exchangeable Na^+ of 0.35 milliequivalents per 100 g soil, exchangeable K^+ of 0.14 cmol kg^{-1}) in a net-house (average temperature 25 °C and relative humidity 60%). The 21-day-old seedlings were separated into four treatment groups as follows "C", foliar spray with distilled water (non-stress condition); "S", 9 dS m^{-1} NaCl + foliar spray with distilled water; "S+25CS" 9 dS m^{-1} NaCl + foliar spray with 25 ppm chitosan; and "S+50CS" 9 dS m^{-1} NaCl + foliar spray with 50 ppm chitosan. In the case of salt treatment, 2 L of desired concentrations of NaCl was added to the top surface of the soil. Leaf of the 21 days old seedlings was sprayed with 25 mL of chitosan once with the desired concentration. The seedlings were grown for seven days more before harvesting, and then the fresh leaves were collected to assess the parameters mentioned. CS was dissolved in 1.0% acetic acid to get a stock solution. The pH of the solution was adjusted to 6.5 with NaOH. Both the germination and seedling stage experiments were conducted in completly randomized design with three independent replications for each treatment.

2.2 Germination percentage (GM%)

To test CS-induced salinity tolerance, 20 seeds of each cultivar were placed in a 9 cm Petri dish with an imbibed filter paper containing the four groups of treatment as follows "C", with distilled water (non-stress condition); "S", 12 dS m^{-1} NaCl; "S+25CS" 12 dS m^{-1} NaCl + 25 ppm chitosan; and "S+50CS" 12 dS m^{-1} NaCl + 50 ppm chitosan. Germination percentage is calculated from the following formula:

$$\text{GM (\%)} = \frac{G}{T} \times 100 \quad (1)$$

where GM (%) = germination percentage, G = total number of seeds germinated, and T = total number of seeds placed for germination.

2.3 Determination of α-amylase activity

Alpha-amylase was determined spectrophotometrically by using 3, 5 dinitrosalicylic acid (DNSA) following the modified procedure of Bernfeld (1955). Endosperm tissues were used for amylase extraction. Five hundred milligrams of endosperm tissue were crushed with 10 mL of 0.02 M phosphate buffer at pH 6.0. One gram soluble starch as a substrate was dissolved in 100 mL of 0.02 M phosphate buffer, pH 6.9 containing 0.0067 M NaCl. One milliliter of enzyme extract and 1.0 mL of substrate solution were taken in a test tube. The reaction mixture was incubated at 30 °C for 15 minutes and the reaction was stopped by adding 5 mL of DNSA reagent. The homogenate was centrifuged at 3000×g for 20 minutes. The supernatant was used to determine the enzyme activity. The optical density of the color developed was read at 540 nm and maltose liberated (mg) were determined.

2.4 Analysis of plant growth parameters

The length from the shoot base to the leaf tip was measured for shoot length (SL) determination. Likewise, the root length (RL) was determined by measuring the length from the root base to the root tip. From each experiment, 25 seedlings were collected and weighed to determine the dry weight (DW). The DW of shoot and root was determined after oven drying at 60 °C for four days. The DWs of shoots and roots were expressed as mg seedling^{-1}.

2.5 Determination of antioxidant enzyme activity

Antioxidative defense mechanisms by supplemented CS were assessed by examining major antioxidant enzymes such as CAT (EC 1.11.1.6) (Aebi, 1984), POX (EC 1.11.1.7) and APX (EC 1.11.1.11) (Nakano and Asada, 1981). Fresh leaf sample (50 mg) was collected and homogenized with 3 mL of 50 mM phosphate buffer (pH 8.0) (PB) in an ice-chilled mortar and pestle. The homogenate was centrifuged at 11,000×g for 10 min. The clear supernatant was used for assaying the enzyme activity. For the CAT activity assay, the reaction mixture was made of 0.7 mL of PB, 0.1 mL of EDTA and 0.1 mL of H_2O_2. For POX, 0.6 mL of 50 mM PB, 0.1 mL of EDTA, 0.1 mL of H_2O_2 and 0.1 mL of guaiacol and for APX assay, 0.6 mL of PB, 0.1 mL of EDTA, 0.1 mL of H_2O_2, and 0.1 mL of ascorbate. All the mixture was made in an Eppendorf tube. The reaction was started by adding 0.1 mL of enzyme extract and changes in absorbance were recorded immediately at 240 nm, 470 nm, and 290 nm for CAT, POX, and APX assay, respectively, at 30 s intervals for two min. The activity of CAT and APX was calculated from the decrease whereas POX activity was from the increase in absorbance.

2.6 Determination of proline

Proline content was determined following the modified method of Bates et al. (1973). Leaf sample (50 mg) was homogenized in a chilled mortar with pestle using 10 mL of 3% sulfosalicylic acid. The homogenate was centrifuged (4500×g for 10 min) and then filtered through filter paper. Two milliliters of the filtered extract were pipette into the test tube followed by 2 mL acid ninhydrin and 2 mL glacial acetic acid and then incubated for 1 hour at 100 °C. The reaction was started by adding 4 mL of toluene. The absorbance of the collected toluene was measured at 520 nm in a vis/uv spectrophotometer (Shimadzu, UV-1201; Japan).

2.7 Determination of total phenol

Total phenol was determined according to the modified method developed by Sadsivam and Manickam, (1996). Freeze-dried leaf sample (50 mg) was mixed with 10 times a volume of 80% ethanol and centrifuged at 10,000×g for 20 min. Dissolved pellets were mixed with 0.5 mL of folin-ciocalteau reagent followed by 2 ml of 20% sodium carbonate. The material was mixed thoroughly and tubes were placed in boiling water exactly for one minute. The tubes were then cooled and the absorbance was measured at 650 nm spectrophotometer.

2.8 Determination of chlorophyll content

Chlorophyll content was determined according to the method developed by Coombs et al., (1985). Fresh leaf sample (50 mg) was taken into a test tube containing 10 mL of 80% acetone and was covered by aluminum foil, and preserved in the dark for 7 to 10 days. Spectrophotometric reading was taken at 645 nm and 663 nm wavelengths.

2.9 Statistical analysis

The data were subjected to a one-way or two-way analysis of variance using Minitab 17 (Pennsylvania, USA), followed by Tukey's test ($P < 0.05$). The heatmap with hierarchical clustering and principal component analysis (PCA) was performed by R 3.6.1 using 'heatmap', 'ggplot2' and 'factoMineR' packages.

3 Results

3.1 Alpha-amylase activity of rice seeds

The protective role of CS on the germination capacity of rice seeds under salinity stress was evaluated by determining the GM% and α-amylase activity (Fig. 1a-b). The GM% was recorded higher under non-stress

condition compared to saline condition. The highest GM% was observed in Pokkali (100%), whereas the lowest (75%) was in BRRI dhan28 under saline condition. However, there were significant increases of GM% in all sensitive and tolerant cultivars with supplementation of 25 ppm and 50 ppm of CS compare to that of saline condition. Salinity drastically reduced the α-amylase activity in all cultivars compared to non-stress conditions. The highest amylase activity was observed in Pokkali under non-stress condition. However, the lowest amylase activity was observed in the saline condition in BRRI dhan28. Supplementation of CS with saline condition remarkably increased the amylase content compared to that of saline condition. There was a significant increase in amylase content in all cultivars supplemented with CS while no variations were found between two different concentrations.

3.2 Growth and biomass of rice plants

To determine whether CS could alleviate the toxic effect of salinity, we monitored the growth and biomass of rice seedlings in terms of shoot length (SL), shoot dry weight (SDW), root length (RL) and root dry weight (RDW) (Fig. 2a-d). The SL as well as SDW, was recorded higher in the tolerant cultivars under non-stress conditions. Salinity induced the reduction of these parameters both in the tolerant as well as sensitive cultivars. Supplementation of CS with saline condition significantly increased SL and SDW in the tolerant cultivars. In BRRI dhan28, both 25 and 50 ppm CS treatments enhanced SL and SDW, whereas only 50 ppm CS treatment improved the SL and SDW in BRRI dhan29 significantly compared to saline condition. Salinity reduced RL and RDW significantly in sensitive cultivars but these values remain unchanged in the tolerant cultivars under salt stress conditions. Supplementation of CS at both concentrations could not alleviate the salt-induced reduction of RL and RDW. From these points, we suppose that CS might not interact with salt stress in the root growth zone.

3.3 Phenotype of rice plants

Salinity caused a significant reduction in plant growth, leaf expansion and variation in leaf color in all the treatments compared with their respective control plants during growth stages (Fig. 3). However, the tolerant cultivars showed lesser degree of reduction in these traits that indicating their considerable adaptability in stressed conditions. Supplementation of CS on salt-stressed rice seedlings improved the growth in all cultivars. Saline condition supplemented with 50 ppm of CS displayed improved growth of rice seedlings than 25 ppm of CS. Among the cultivars, Pengek and Pokkali showed superiority over the other two cultivars.

3.4 Photosynthetic pigments in rice plants

We determined the Chl content of rice leaves to know the role of CS in protecting photosynthetic machinery under salt stress (Fig. 4a-b). The content of Chl 'a' and Chl 'b' was found higher in the sensitive cultivars compared to tolerant cultivars. Salt stress caused a drastic reduction in Chl 'a' and Chl 'b' contents compared to non-stress condition in the sensitive cultivars, whereas tolerant cultivars showed a slight reduction. On the contrary, supplementation of CS showed significant alleviation of Chl 'a' and Chl 'b' content more likely in the sensitive cultivars. Saline condition supplemented with 50 ppm of CS enhanced the upregulation of chlorophyll content.

3.5 Proline and phenolic contents

Salt stress increased the proline content compared with that of the non-stress condition (Fig. 5a). Interestingly, salt-sensitive cultivars had higher proline content compared to tolerant cultivars under non-stress condition. Supplementation of CS could significantly reduce the proline content in all the cultivars. Saline conditions supplemented with both 25 and 50 ppm of CS had a similar trend of decrease in proline content in BRRI dhan28. However, saline condition supplemented with only 50 ppm of CS reduced the proline content in BRRI dhan29 compare to saline condition. Salinity increased total phenolic contents in all the cultivars (Fig. 5b). Conversely, supplementation of both 25 and 50 ppm of CS with the saline condition could significantly lower the phenolic contents to its basal level in the tolerant cultivars, whereas supplementation of 50 ppm of CS with the saline condition could significantly decrease the phenolic contents in sensitive cultivars.

3.6 Antioxidant activities in rice plants

The rice seedlings exposed to salt stress had increased CAT and APX activity in salt-tolerant cultivars whereas sensitive cultivars unable to increase CAT and APX activity (Fig. 6a, b). In contrast, CS supplementation to saline condition showed enhancement of CAT and APX activity in sensitive cultivars as compared with only salt-stressed seedlings. Supplementation of 50 ppm of CS showed more effect on CAT and APX activity. Salt stress increased POX activity in salt-tolerant cultivars as compared with the non-stress condition, whereas sensitive cultivars were unable to increase POX activity under salt-stress conditions (Fig. 6c). Supplementation of CS with saline condition did not affect POX activity in salt-tolerant as well as salt-sensitive cultivars as compared with saline condition.

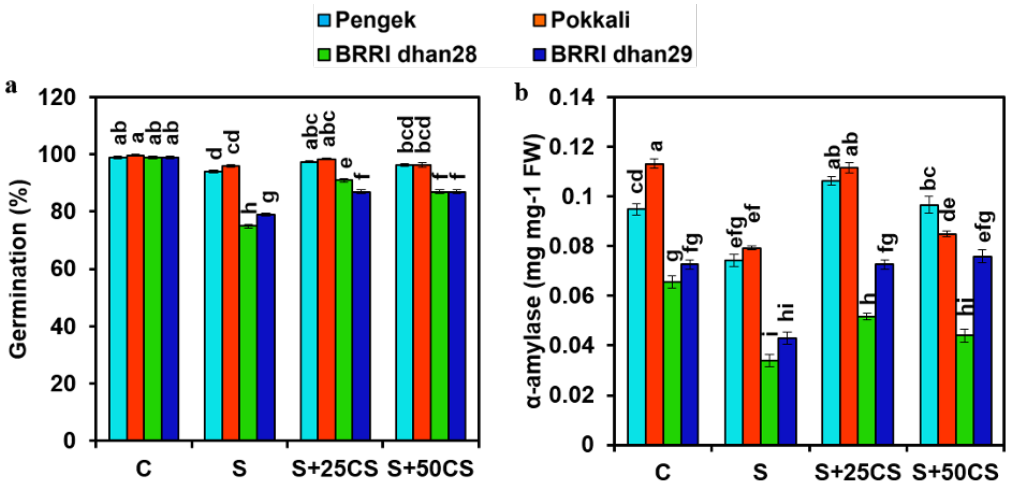

Figure 1. Effect of CS supplementation on (a) germination percentage (GM%) and (b) α-amylase activity of rice seed grown under normal or salt stress condition. Data represented as the means of three independent replicates for each treatment (n = 3). The vertical bars indicate standard errors of means. Different letters on the top of each bar denote statistically significant differences at $P < 0.05$, based on Tukey's test. "C", with distilled water (non-stress condition); "S", 12 dS m^{-1} NaCl; "S+25CS" 12 dS m^{-1} NaCl + 25 ppm chitosan; and "S+50CS" 12 dS m^{-1} NaCl + 50 ppm chitosan

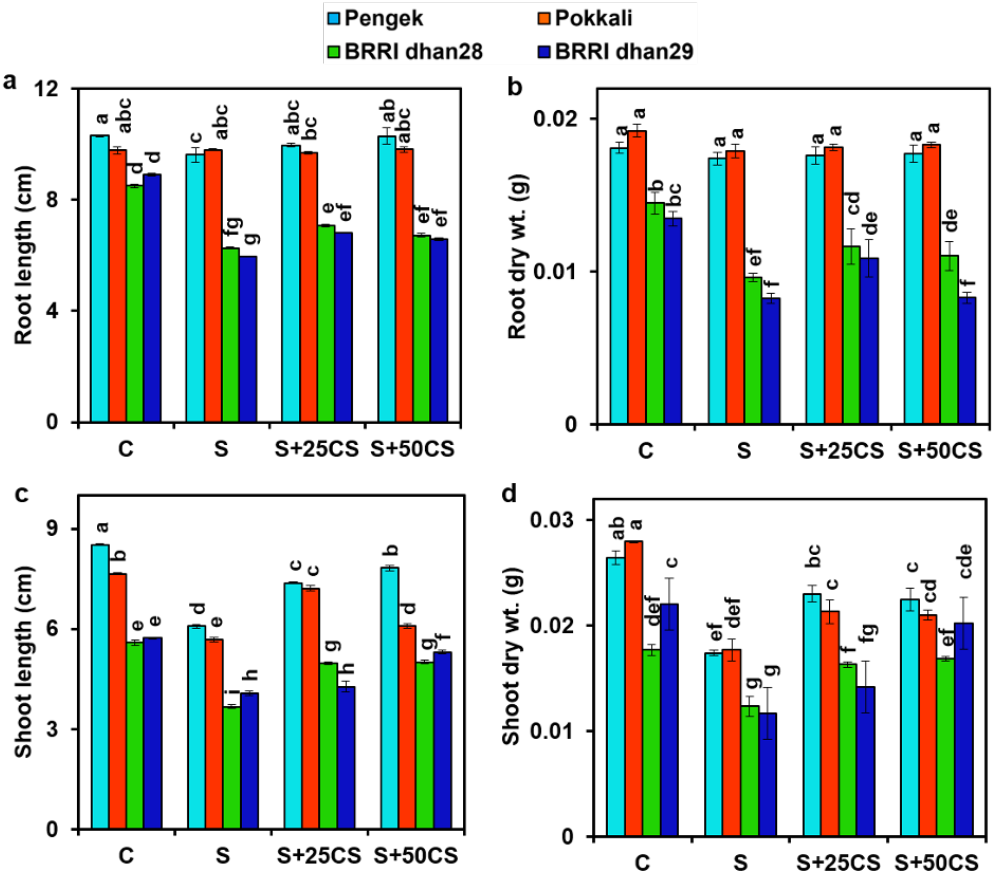

Figure 2. Effect of CS supplementation on (a) root length, (b) root dry wt., and (c) shoot length and d) shoot dry wt. of rice seedlings grown under normal or salt stress condition. Data represented as the means of three independent replicates for each treatment (n = 3). The vertical bars indicate standard errors of means. Different letters on the top of each bar denote statistically significant differences at $P < 0.05$, based on Tukey's test. "C", with distilled water (non-stress condition); "S", 12 dS m^{-1} NaCl; "S+25CS" 12 dS m^{-1} NaCl + 25 ppm chitosan; and "S+50CS" 12 dS m^{-1} NaCl + 50 ppm chitosan

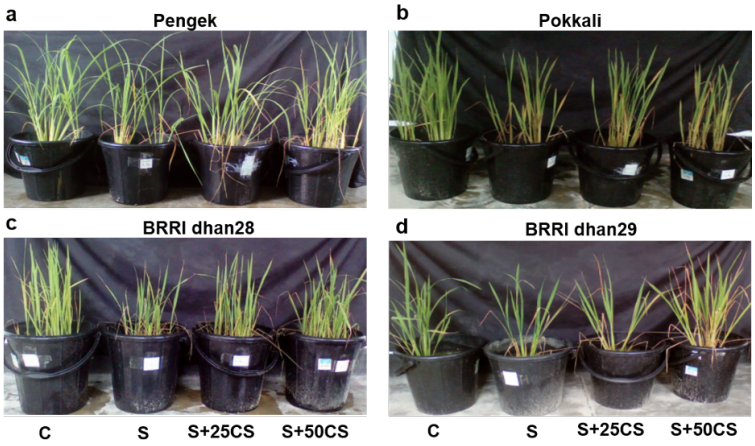

Figure 3. Effect of CS supplementation on the phenotypes of (a) Pengek, (b) Pokkali, (c) BRRI dhan28, and (d) BRRI dhan29 rice seedlings grown under normal or salt stress condition. "C", foliar spray with distilled water (non-stress condition); "S", 9 dS m^{-1} NaCl + foliar spray with distilled water; "S+25CS" 9 dS m^{-1} NaCl + foliar spray with 25 ppm chitosan; and "S+50CS" 9 dS m^{-1} NaCl + foliar spray with 50 ppm chitosan

3.7 Heatmap with hierarchical clustering and PCA

Mean values of all morpho-physiological and biochemical parameters were used to perform heatmap with hierarchical clustering and PCA. Hierarchical clustering categorized different treatment × cultivars into two clusters (Cluster-A and Cluster-B) (Fig. 7a). Cluster-A contains all treatments combination with salt-sensitive BRRI dhan28 and BRRI dhan29 except 'S+50CS'×BRRI dhan29. On the other hand, cluster-B is composed of all treatments combined with salt-tolerant Pokkali and Pengek. It is visible that the hierarchical clustering separated tolerant and sensitive genotypes in separate clusters irrespective of treatments.

The first two components of PCA combinedly explained 74.2% of the data variability (Fig. 7b). The PCA biplot revealed the association of the different morpho-physiological and biochemical parameters with the treatment groups of different cultivars. The growth-related morpho-physiological parameters were strongly associated with 'C', 'S+25CS' and 'S+50CS' treatments of Pokkali and Pengek compared to that of all treatments of BRRI dhan28 and BRRI dhan29. The 'S' treatment of Pokkali and Pengek strongly interlinked with CAT, APX and POX activity, and phenol content.

4 Discussion

Improvement of crop cultivation technologies is focused on increasing crop productivity and improving yield quality, and at the same time on minimizing risks posed to the natural environment. The use of biostimulants contributes to the increase in the productivity of plants, especially under their exposure to stress induced by negative environmental stimuli such as salinity, drought, high and low temperature etc. It is well reported that CS supplementation upregulate many physiological processes, and here we investigated the effect of CS supplementation on salt stress mitigation in rice. Salinity hampers seed germination by affecting major events of germination such as imbibition, metabolism activation, the emergence of embryonic tissues and seedling establishment. However, the negative effect of salinity on seed germination may vary due to various factors, such as varietal variation, level of salinity and other external factors. Poor hydrolysis also limits the translocation of food reserve from storage tissue to develop embryo that also negatively affects germination and seedling establishment. Salinity inhibits amylase activity, which is the major reason for poor hydrolysis or imbibition of stored substances. In this study, salt-stress reduced germination percentage as well as α-amylase activity whereas both GM% and α-amylase activity were increased with the supplementation of CS in salt-stressed rice seedlings. (Fig. 1a-b). Increased α-amylase activity by supplemented CS is probably due to the increment of GM% as well as the survival of rice seedlings under salt stress.

Determination of morphological parameters such as growth and biomass of plants are used to assess the adverse effects of abiotic stress (Akram et al., 2019). In the present investigation, the RL, RDW, SL and SDW were reduced in salt-stressed rice seedlings compared with that of non-stress plants (Fig. 2a-d) which might happen due to the reduced chlorophyll contents (Fig. 4a-b) (Roy et al., 2019; Tahjib-Ul-Arif et al., 2019).

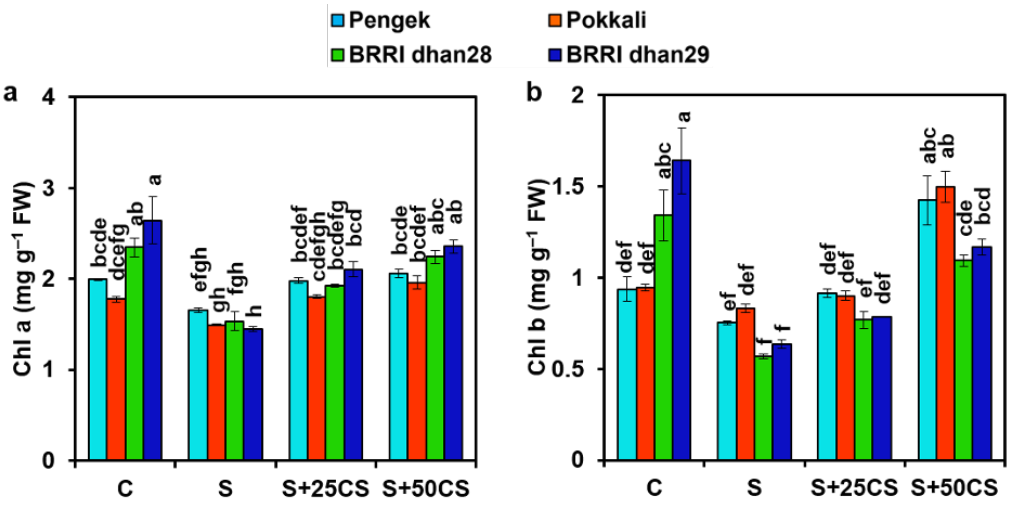

Figure 4. Effect of CS supplementation on photosynthetic pigments (a) Chl a and (b) Chl b contents of the leaves of rice seedlings grown under normal or salt stress condition. Data represented as the means of three independent replicates for each treatment (n = 3). The vertical bars indicate standard errors of means. Different letters on the top of each bar denote statistically significant differences at $P < 0.05$, based on Tukey's test. "C", foliar spray with distilled water (non-stress condition); "S", 9 dS m^{-1} NaCl + foliar spray with distilled water; "S+25CS" 9 dS m^{-1} NaCl + foliar spray with 25 ppm chitosan; and "S+50CS" 9 dS m^{-1} NaCl + foliar spray with 50 ppm chitosan

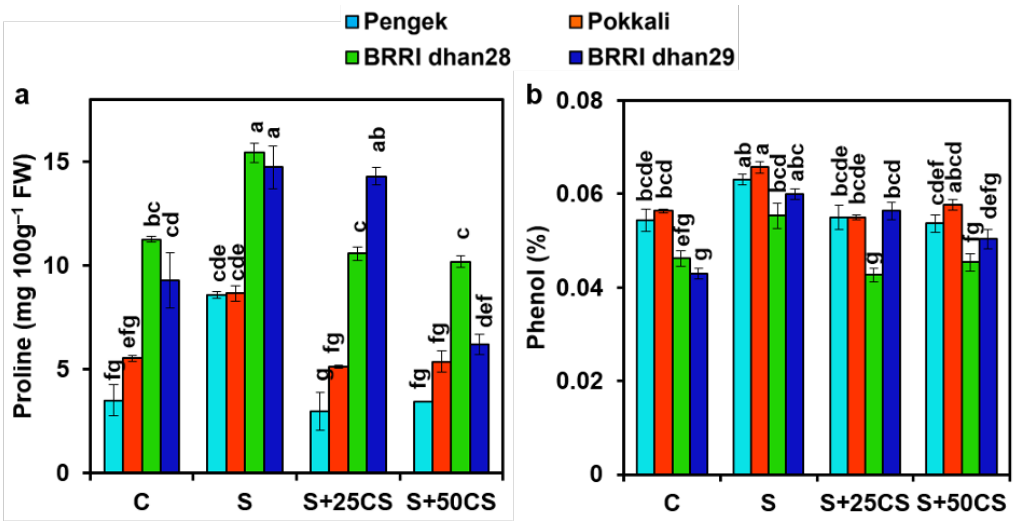

Figure 5. Effect of CS supplementation on (a) proline and (b) phenol contents of the leaves of rice seedlings grown under normal or salt stress condition. Data represented as the means of three independent replicates for each treatment (n = 3). The vertical bars indicate standard errors of means. Different letters on the top of each bar denote statistically significant differences at $P < 0.05$, based on Tukey's test. "C", foliar spray with distilled water (non-stress condition); "S", 9 dS m^{-1} NaCl + foliar spray with distilled water; "S+25CS" 9 dS m^{-1} NaCl + foliar spray with 25 ppm chitosan; and "S+50CS" 9 dS m^{-1} NaCl + foliar spray with 50 ppm chitosan

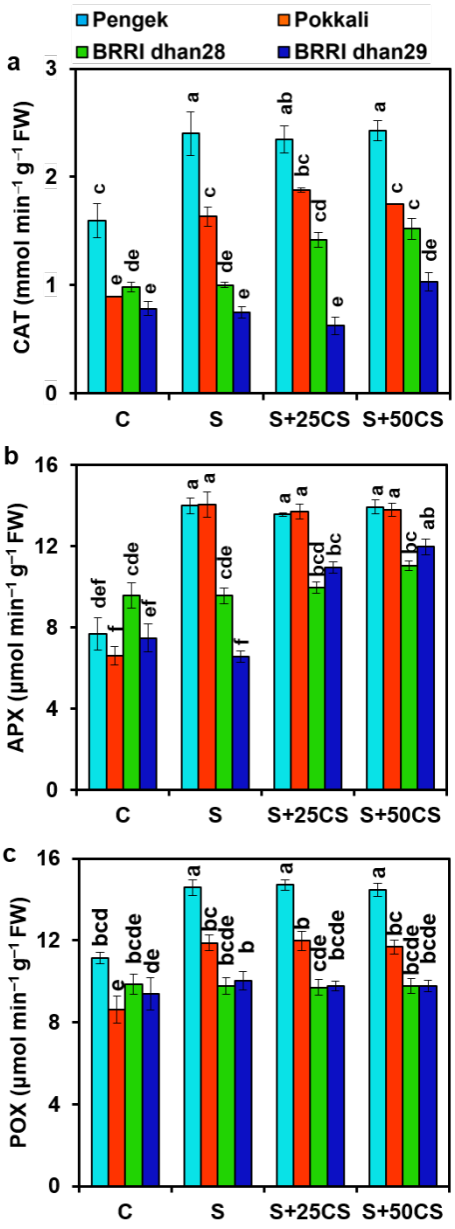

Figure 6. Effect of CS supplementation on (a) catalase (CAT) activity, (b) ascorbate peroxidase (APX) activity, and (c) peroxidase (POX) activity of the leaves of rice plants grown under normal or salt stress condition. Data represented as the means of three independent replicates for each treatment (n = 3). The vertical bars indicate standard errors of means. Different letters on the top of each bar denote statistically significant differences at $P < 0.05$, based on Tukey's test. "C", foliar spray with distilled water (non-stress condition); "S", 9 dS m^{-1} NaCl + foliar spray with distilled water; "S+25CS" 9 dS m^{-1} NaCl + foliar spray with 25 ppm chitosan; and "S+50CS" 9 dS m^{-1} NaCl + foliar spray with 50 ppm chitosan

On the contrary, supplementation of CS with saline condition improved the growth performance of salt-stressed rice seedlings as evident by their improved phenotypic appearance (Fig. 3a-d) and growth-related features, including RL, RDW, SL and SDW (Fig. 2a-d), perhaps by increasing chlorophyll contents of rice plants (Fig. 4a-b). Moreover, our results were verified by PCA, which indicated that salt-stressed rice plants supplemented with CS exhibited a stronger correlation with chlorophyll content compared with salt-stressed plants in all four rice cultivars (Fig. 7b). Consistent with our results, it has also been reported that exogenous CS increased root and shoot length as well as root and shoot dry weights in wheat and maize plants (jing Guan et al., 2009; Lizárraga-Paulín et al., 2011; Ma et al., 2011; Peykani and Sepehr, 2018), rice (Ruan and Xue, 2002; Boonlertnirun et al., 2008), beans (*Phaseolus vulgaris* L) (Zayed et al., 2017) and improves the tolerance of seedlings to stress conditions.

To withstand salt stress, plants accumulate compatible solutes such as proline, which decreases the cytoplasmic osmotic potential, facilitating water absorption, and scavenges reactive oxygen species (ROS) (Qureshi et al., 2013; Pottosin et al., 2014). As an osmoprotectant with potential antioxidant activity, proline plays a vital role in abiotic stress tolerance in plants (Hasanuzzaman et al., 2014; Nahar et al., 2016; Ray et al., 2016b). Proline is accumulated preferentially in leaves to maintain chlorophyll level and cell turgor to protect photosynthetic activity under salt stress (Silva-Ortega et al., 2008). In this investigation, proline content was increased in salt-stressed rice seedlings compared to that of non-stressed plants which is more pronounced in salt-sensitive cultivars than in salt-tolerant cultivars (Fig. 5a). This was also supported by PCA where proline content was more closely associated with saline-stressed sensitive species than in other treatment groups (Fig. 7b). The accumulation of proline in plants under salt stress is caused either by the induction of expression of proline biosynthesis genes (P5CS, P5CR) or by the repression of the genes of its degradation pathway (PDH silencing) (Marco et al., 2015). The elevated proline content due to salt stress may also be an adaptation to compensate the energy for growth and survival and thereby help plant tolerate stress, as reported earlier in *Crotalaira striata* Chandrashekar and Sandhyarani (1996) and spinach leaves (Ozturk and Demir, 2003). However, in salt-stressed rice seedlings, foliar supplementation of CS decreased the proline content which suggested that CS somehow prevent water imbalance in plants thus reducing the need for excess proline accumulation. Similar results were found in salt-affected rice seedlings (Hasanuzzaman et al., 2014; Rahman et al., 2016; Shams Peykani and Farzami Sepehr, 2018; Sen et al., 2020).

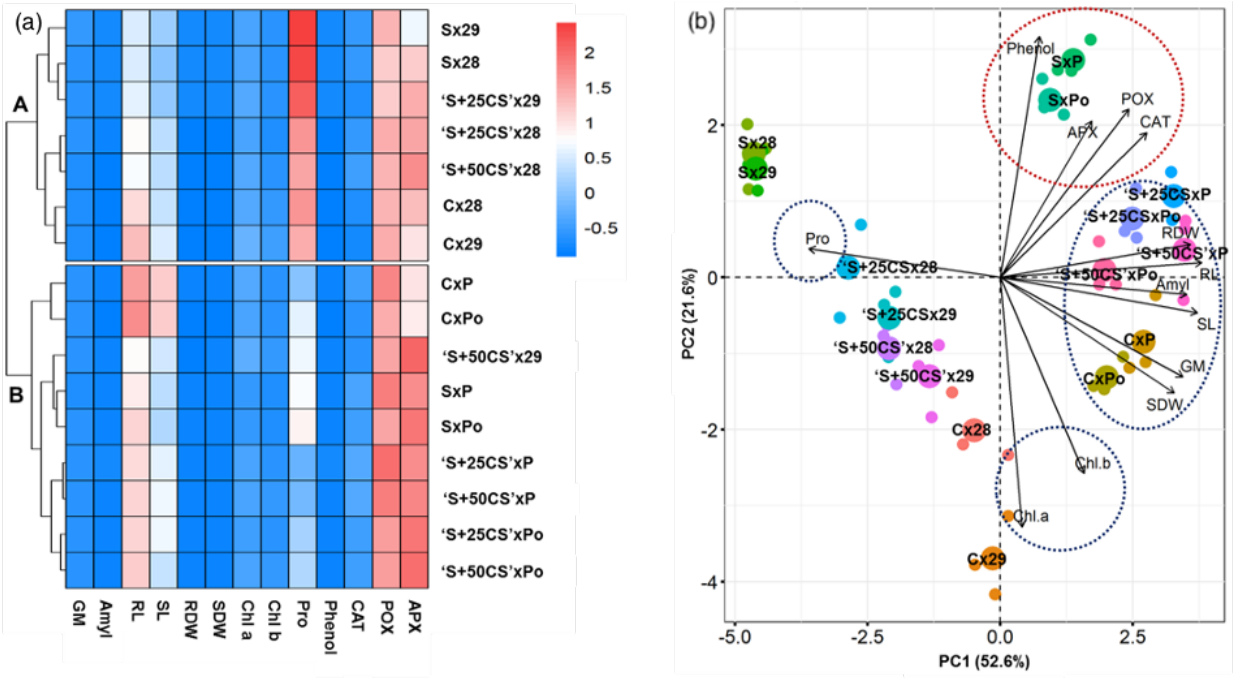

Figure 7. (a) Heatmap with hierarchical clustering and (b) principal component analysis (PCA) show the treatment-variable interaction of different cultivars. In hierarchical clustering two distinct clusters (cluster-A and -B) were identified at the treatment × cultivars level. The colour scale of heatmap displays the intensity of normalized mean values of different parameters. The entire dataset was analyzed using PCA. The variables included germination percentage (GM), α-amaylase content (Amyl), shoot length (SL), root length (RL), shoot dry weight (SDW), root dry weight (RDW), chlorophyll a (Chl a), chlorophyll b (Chl b), proline (Pro), total phenol content (Phenol), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX). "C", foliar spray with distilled water (non-stress condition); "S", NaCl + foliar spray with distilled water; "S+25CS" NaCl + foliar spray with 25 ppm chitosan; and "S+50CS" NaCl + foliar spray with 50 ppm chitosan. '28', BRRI dhan28; '29', BRRI dhan29, 'P', Pengek; and 'Po', Pokkali

The non-enzymatic antioxidants such as phenolic compounds play a significant role in the mitigation of excessive cellular ROS activities caused by salt stress. Plants need to adapt different abiotic stresses and polyphenols accumulate in response to these stresses helping plants to acclimatize to unfavorable environments (Lattanzio, 2013; Pereira, 2016). Plants under stress can biosynthesize more phenolic compounds in comparison to plants growing under normal conditions (Selmar, 2008). These compounds have antioxidative properties and are capable of scavenging free radicals and reducing cell membrane peroxidation (Schroeter et al., 2002), hence protecting plant cells from adverse effects of oxidative stress. In this study, salt stress increased total phenolics contents in both rice genotypes compared with that of non-stress condition (Fig. 5b). The increased phenolics contents in salt stress conditions might be regulated by the altered activities of key enzymes of phenolic biosynthetic pathways like PAL (phenylalanine ammonia lyase) (Mrázová et al., 2017). On the contrary, supplementation of CS reduced the phenolic contents compared with that of salt-stressed rice seedlings (Fig. 5b).

The increase of antioxidant activity is related to better oxidant management under stressed conditions (Ahmad et al., 2010; Engwa, 2018). In this investigation, CAT and APX activities were increased in salt-tolerant cultivars but not in salt-sensitive cultivars under salt stress conditions. The PCA also revealed that tolerant cultivars had a stronger positive association with POX, APX, and CAT activity than sensitive cultivars (Fig. 7b). However, supplementation of CS increased CAT and APX activities in salt-stressed rice seedlings of salt-sensitive cultivars (Fig. 6a-b). Turk (2019) recently reported that CS increased CAT and APX activities in salt-stressed wheat seedlings in comparison to non-stress seedlings and increase tolerance to salt stress. It has been revealed that low concentration CS treated seeds of safflower and sunflower can alleviate the oxidative stress caused by salt stress by reducing enzyme (CAT and POX) activities in both crops (Jabeen and Ahmad, 2012). Several studies have also reported that pretreatment with CS during salinity stress results in increased antioxidant enzyme activities which ultimately reduces the negative effect caused by salt stress in *Oryza sativa*

(Martínez González et al., 2015), (Al-Tawaha et al., 2018), *Vigna radiata* (Ray et al., 2016a), *Trachyspermum ammi* (Mahdavi and Rahimi, 2013) and *Plantago ovata* (Mahdavi, 2013). Further, a hydroponic experiment done on wheat showed that CS treated seed resulted in positive effects by significantly increasing antioxidant enzymzes (POX and CAT) during salt-induced stress and was able to alleviate the oxidative stress (Ma et al., 2011).

5 Conclusion

Increasing the tolerance of rice against salinity is an important task for rice researchers to reduce the effect of climate change on rice production. In summary, our study provides the evidence that supplementation of CS comparatively ameliorated salt stress-induced growth inhibition and biomass loss by regulating several physiological and biochemical mechanisms, including (i) improvement of germination by increasing α-amylase activity, (ii) protection of photosynthetic pigments, (iii) maintaining osmoprotection and stimulation of phenolics productions, and (iv) enhancement of the activities of ROS-scavenging enzymatic antioxidants (CAT, APX and POX). Taken together, these results suggest that CS supplementation could be a viable cost-effective technology that can be employed for the mitigation of salt stress-caused adverse effects on crop performance to ensure sustainable agriculture in saline-prone areas. However, further studies at the field level using different modes of CS supplementation to a range of crop species under various regimes of salinity should be needed to ascertain the beneficial roles of CS in the management of salinity. Additionally, it would be fascinating to identify whether CS application positively alters seed biochemical and nutrient contents of rice that may help us deal with the undernutrition problems of the people living in many developing countries.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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