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RESEARCH ARTICLE

Exogenous Calcium Supplementation Improves Salinity Tolerance in *BRRI Dhan28*; a Salt-Susceptible High-Yielding *Oryza Sativa* Cultivar

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Abstract

Salinity is one of the most brutal abiotic stressors, commencing a great stumbling block in the way of attaining food security in Bangladesh. Cultivation of rice in saline soils can be possible after enhancing its salt tolerance. This study aimed to examine the potential impact of exogenous calcium (3 and 5 mM Ca^{2+} in $CaCl_2$) on conferring salt tolerance in rice (*cv.* BRRI dhan28). At the germination stage, Ca^{2+}-primed seeds were grown under 100 mM NaCl stress conditions for nine days. At the seedling stage, rice seedlings were grown in a sandponic culture with Hoagland′s nutrient solution amended or not amended with 100 mM NaCl for 20 days with or without Ca^{2+} supplementation. Our results revealed that NaCl-stressed rice plants showed highly compromised germination indices and growth parameters, which could be attributed to reduced shoot and root growth, decreased photosynthetic pigments, increased H_2O_2 accumulation, and elevated levels of lipid peroxidation measured as malondialdehyde (MDA). On the other hand, exogenous Ca^{2+} application noticeably improved germination indices, growth and biomass-related parameters under salt stress. Ca^{2+}-treated salt-stressed plants displayed amplified chlorophyll content, as well as suppressed the accumulation of H_2O_2, contributing to oxidative damage protection. Ca^{2+} supplementation for salt-stressed rice seedlings elevated relative water content without increasing excess proline, indicating the role of Ca^{2+} in maintaining water balance under stressful conditions. Furthermore, exogenous Ca^{2+} decreased membrane injury under NaCl stress, as mirrored by notably diminished levels of MDA in stressed seedlings. The defensive role of Ca^{2+} counter to oxidative stress was connected with the elevated activities of antioxidant enzymes such as catalase, ascorbate peroxidase, and peroxidase. In general, the best results in terms of growth at both germination and seedling stages were obtained in response to 3 mM Ca^{2+} treatment. Finally, Ca^{2+} supplementation can be an effective practice to cultivate rice in saline soils.

Key words : Antioxidant enzymes, calcium, environmental stress, germination indices, reactive oxygen species, osmolytes, rice

Introduction

Frequent climate change triggers various abiotic stressors, such as such salinity, high and low temperature, water deficit, and nutrient deficiency which cause prime constraints to crop production (Mittler and Blumwald 2010). A serious one, salt stress, is an overwhelming stressor because the majority of crop plants are vulnerable to salinity (Hasanuzzaman et al. 2013). A considerable amount of the world's irrigated land has been affected by salinity and it has been predicted that the extent of salt-affected arable land will be enhanced by 50% by 2050 (Mahajan and Tuteja 2005). During salt stress, plants are subjected to three major challenges, like increasing osmotic pressure, misbalancing ion uptake, and oxidative stress, which cause metabolic disturbance and impairment of various physiological processes of plants (Gupta and Huang 2014). A high extent of salt uptake by the plant results in

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increasing osmotic pressure in cytosol and reducing water potential which leads to the disruption of normal homeostasis of plant cells and a generation of "physiological drought" (Munns and Tester 2008). Similarly, excess Na^+ in the growing medium due to increased salinity counterattacks K^+ uptake and triggers K^+ leakage from plant cells and as a result, Na^+ content increases and K^+ content decreases in plants that causes inhibition of metabolic enzymes and protein synthesis (Munns 2005).

Furthermore, high salinity stress leads to an imbalance between the production and scavenging of ROS (H_2O_2) that eventually results in excessive oxidative stress (Hasanuzzaman et al. 2013). This excess oxidative stress triggers the peroxidation of polyunsaturated fatty acids which results in the production of various secondary products including toxic malondialdehyde (MDA) (Das and Roychoudhury 2014; Gill and Tuteja 2010). Consequently, various detrimental phenomenon can be displayed such as an oxidative alteration of essential biomolecules, membrane damage, enzymatic inhibition, cell death, and the inhibition of plant growth and development (Mahajan and Tuteja 2005; Munns and Tester 2008; Parvaiz and Satyawati 2018). On the other hand, plants evolved distinct biochemical and physiological defense mechanisms to protect it from the adversity of various abiotic stressors (Hasegawa et al. 2000; Parida and Das 2005). Furthermore, plants acquired inherent mechanisms to counteract stress-induced ROS-generation, which includes antioxidant enzymes such as catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) which neutralize stress-induced excess H_2O_2 by converting it into H_2O (Mostofa et al. 2016; Sofo et al. 2015). It is a well-known fact that the basal or foliar application of different signaling molecules or protectants can mitigate the adverse effects of salinity through activating these defense mechanisms.

Exogenous calcium (Ca^{2+}) has been shown to mitigate adverse effects of salinity on different plants (Kaya et al. 2002; Rahman et al. 2016; Tuna et al. 2007; White and Broadley 2003). It is not only a signaling molecule but also a secondary messenger, which plays a significant role in extenuating environmental stressors (Jaleel et al. 2007; Kader and Lindberg 2010). In addition, Ca^{2+} enhances Ca, Mg, and K contents in plants under sodium stress (Hussain et al. 2017) and controls the ion homeostasis pathways in plants by restricting the entry of Na^+, thus it plays a pivotal role in salt stress signaling (Yokoi et al. 2002). It plays a vital role to protect the structural and functional integrity of plant membranes (Tuna et al. 2007) and cell wall structure stabilization (Neves-Piestun 2001), regulating ion transport and selectivity, and controlling ion-exchange behavior as well as cell wall enzyme activities (Ashraf and Harris 2004). Moreover, Ca^{2+} stimulates the activities of antioxidant enzymes activity by binding to calmodulin (CaM), a ubiquitous calcium-binding protein (Gong et al. 1998; Yang and Poovaiah 2002). It has been suggested that exogenous calcium can mitigate the sodium toxicity in plants (Arshi et al. 2010; Cha-um et al. 2012; Manivannan et al. 2007; Nasir Khan et al. 2010).

In Bangladesh, salinity is the second most serious abiotic stress that limits the crop production. The coastal region of Bangladesh covers 32% of the country which comprised about 2.86 million hectares (Mha) land of which 1.056 Mha has been affected by salinity during 2009 (Soil Resource Development Institute (SRDI) 2010). About 0.223 Mha (26.7%) new land is affected by various extent of salinity during the last four decades (Soil Resource Development Institute (SRDI) 2010; Alam el al. 2017). Besides, due to global warming, the rising sea level will pose threat by expanding the severity of salinity (Institute of Water Modeling (IWM) and Center for Environmental and Geographic Information Services (CEGIS) 2007). It is predicted that a 0.3 m rise in the sea level will lead to increased salinity which will ultimately cause 0.5 million metric tons of net loss in rice (*Oryza sativa* L.) production in Bangladesh (World Bank 2000). Rice is the staple food for Bangladeshi people and in the near future, there will be a need to produce more rice to feed the ever-increasing population. Therefore, there will be a need to cultivate rice in the salinity affected lands. Several studies have also revealed that exogenous application of Ca^{2+} in plant growth medium helps to develop abiotic-stress tolerance (Wu and Wang 2012), by enhancing the antioxidant defense system and other physiological and biochemical attributes (Manivannan et al. 2007; Srivastava et al. 2015). However, alleviation of salt stress in rice by exogenous application of Ca^{2+} has rarely been examined in Bangladesh. Considering the above, this research aimed to shed light on the potential role of Ca^{2+} in mitigating salinity stress in rice (i.e. BRRI dhan28 cultivar) at both germination and seedling stages. In this connection, changes in terms of growth performances, ROS accumulation, antioxidant enzymes activities, photosynthetic pigments, and proline accumulation were measured in Ca^{2+}-supplemented salt-exposed rice seedlings and only salt-exposed rice seedlings.

Materials and Methods

Plant material and germination stage experiment

Rice (*cv.* BRRI dhan28, salt susceptible high-yielding cultivar) seeds were surface sterilized with 70% ethanol for 8-10 min and subsequently washed several times with sterilized distilled water. In the next step, the disinfected seeds were soaked in calcium (0, 3, and 5 mM Ca^{2+} using $CaCl_2$) solution for 24 h. The treated seeds were then air-dried back to normal moisture conditions. After that, about 100 Ca^{2+}-treated or untreated seeds were placed on blotting paper per Petri dish and 10 ml of distilled water or 10 ml 100 mM NaCl solution was used daily for non-saline and saline treatments, respectively. These Petri dishes were placed in an incubator at 30 ± 2°C in dark conditions for germination. Seeds were considered as germinated when the radical reached 2 mm in length. From the 2nd day after incubation (DAI), numbers of germinated seeds were recorded up to 4th DAI and by using these germination

counts, several germination indices were calculated, including final germination percentage (FGP), germination rate index (GRI), and mean germination time (MGT) (Kader 2005), as well as the coefficient of velocity of germination (CVG) (Kader and Jutzi 2004).

$$FGP = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds\ placed\ in\ a\ Petri\ dish} \times 100$$

$$CVG\,(\%\,day^{-1}) = \frac{\sum N_i}{\sum (N_i T_i)} \times 100$$

$$CRI\,(\%\,day^{-1}) = \frac{\sum N_i}{I}$$

Where, N is the number of seeds germinated on day, i and T_i are the numbers of days from sowing. The CVG gives an indication of the rapidity of germination: it is increased by increasing the number of germinated seeds and reducing the time required for germination. GRI is reflected the percentage of germination on each day of the germination period, where higher GRI values are indicate higher and faster germination, which in turn indicates lower MGT. From the 6th to 9th DAI, radicle and plumule length (RL and PL) were measured and at the 9th day radicle and plumule fresh weight (RFW and PFW) were recorded. The radicle and plumule dry weight (RDW and PDW) were recorded after 4 days of oven-drying at 60°C.

Seedling stage experiment, growth conditions, and treatments

Uniformly germinated 25 rice seeds were transferred to a 6 L plastic tray filled with sand, and allowed to grow in distilled water for 3 d. On the beginning of 4th day, the distilled water was replaced by 0.5× modified Hoagland nutrient solution (Hoagland and Arnon 1950). The nutrient solution was renewed twice a week. To ensure better availability of nutrients the pH was maintained at 5.6 using a pH meter (Hanna HI 2211, Padova, Italy). Later, 12-day-old rice seedlings were exposed to salt stress (100 mM NaCl) in the presence and absence of exogenous Ca^{2+} (3 mM and 5 mM Ca^{2+} as $CaCl_2$) with nutrient solution to verify the protective role of Ca^{2+} under salt stress conditions. The control plants were grown in nutrient solution only. Therefore, our experiment consisted of six treatments as follows: Control (C), 100 mM NaCl (S), 3 mM Ca^{2+} (Ca1), 5 mM Ca^{2+} (Ca2), 100 mM NaCl in combination with 3 mM Ca^{2+} (S+Ca1) and 100 mM NaCl in combination with 5 mM Ca^{2+} (S+Ca2). The experiment was repeated three times under the same conditions in a completely randomized design (CRD). Finally, the rice seedlings were grown for 20 d after exposure to salt stress, and different morphological, physiological, and biochemical data were then measured.

Assessment of salt toxicity symptoms and seedling growth

The growth of rice seedlings was assessed by measuring shoot length (SL), root length (RL), shoot dry weight (SDW), and root dry weight (RDW). Shoot length was determined by measuring the length from the bottom of the main stem to the end of the emerging third leaf. Root length was determined by measuring the length from bottom of the stem to the tip of the root. To determine dry weight, 10 seedlings from each treatment were separated to shoots and roots and were then oven-dried at 60°C for 4 d. Dry shoots and roots were weighed and expressed as mg $shoot^{-1}$ and mg $root^{-1}$.

Salt toxicity symptoms in rice seedlings were determined at the 16th day after salinization following the Standard Evaluation System (SES) proposed by IRRI (International Rice Research Institute (IRRI) 2002).

Relative water content determination

The relative water content of leaves was calculated according to (Barrs and Weatherley 1962). Immediately after collection, leaf laminas were weighed (FW), then immediately placed between two layers of filter paper and immersed in distilled water in a Petri dish for 24 h. Turgid weight (TW) was measured after gently removing excess water with blotting paper. Dry weight (DW) was measured after 48 h oven-drying at 70°C. Finally, RWC was calculated using the following formula:

$$RWC\,(\%) = \frac{FW - DW}{TW - DW} \times 100$$

Determination of chlorophyll content

Chlorophyll content was determined according to the method developed by Coombs et al. (1985). A weight of 0.05 g fresh leaf sample was taken into a screw-capped test tube containing 10 ml of 80% acetone and was covered by aluminum foil, and preserved in the dark for 7 d. After that, spectrophotometric (T80, UV-Visible Spectrophotometer, PG Instruments, China) reading was taken at 645 and 663 nm wavelengths and the result was expressed as mg g^{-1} FW of leaf.

Determination of proline content

Proline content was determined according to Bates et al. (1973) with some modifications. Leaf samples (0.5 g) were homogenized with 10 ml 3% sulfosalicylic acid and the homogenate was centrifuged at 11,500 ×g for 10 min. Supernatant (2 ml) was mixed with 2 ml glacial acetic acid and 2 ml acid Ninhydrin solution. After 1 h incubation at 100°C, the mixture was cooled. The developed color was extracted with 4 ml toluene and the optical density of the chromophore was observed spectrophotometrically (T80, UV-Visible Spectrophotometer, PG Instruments, China) at 520 nm. Proline content was determined by preparing a standard curve of known concentrations of proline.

Determination of lipid peroxidation and hydrogen peroxide contents

Lipid peroxidation (as MDA) and hydrogen peroxide (H_2O_2) of 20 d salt-treated rice seedlings were measured following the method of (Zhang and Huang 2013) and (Velikova et al. 2000), respectively. A weight of 0.1 g plant leaves was homogenized with 0.1% Trichloroacetic acid (TCA) and centrifuged at 11,500 ×g for 15 min at 4°C. The supernatant (1.0 mL) was mixed with 4 mL 20% TCA containing 0.5% of Thiobarbituric acid (TBA) and incubated at 80°C for 15 min. After cooling immediately, the samples were centrifuged again at 11,500 ×g for 12 min. The absorbance was recorded at 532 nm in a UV-VIS spectrophotometer (T80, PG Instruments, China) and MDA content was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$.

The supernatant of 0.1% TCA mediated extract (0.5 ml) was mixed with 1.0 ml of 1.0 M KI and 0.5 ml of 10 mM potassium phosphate buffer (PPB) (pH 7.0). After 1 h incubation in the dark, the absorbance was recorded at 390 nm in a UV-VIS spectrophotometer (T80, PG Instruments, China) and H_2O_2 content was calculated by using the extinction coefficient of 0.28 $\mu M^{-1}cm^{-1}$.

Antioxidant enzyme extraction and assays

Fifty mg of fresh leaf sample was collected and homogenized with 3 ml of 50 mM ice-cold PPB (pH 8.0) in a pre-cooled mortar and pestle. The homogenate was centrifuged at 12,000 ×g for 10 min. The clear supernatant was used for assaying the CAT (EC: 1.11.1.6), APX (EC: 1.11.1.11) and POD (EC: 1.11.1.7) activity. All procedures were performed at 0-4°C.

CAT activity was measured as described by Aebi (1984) using an extinction coefficient of 40 M^{-1} cm^{-1}. To 0.7 ml of 50 mM PPB (pH 8.0) in an Eppendorf tube, 0.1 ml of EDTA, and 0.1 ml of H_2O_2 were added and mixed thoroughly. The reaction was started by adding 0.1 ml of plant extract. POD and APX activities were determined by following the method of Nakano and Asada (1981) using the extinction coefficients of 26.6 M^{-1} cm^{-1} and 2.8 M^{-1} cm^{-1}, respectively. For APX assay, 0.6 ml of 50 mM PPB (pH 8.0), 0.1 ml of EDTA, 0.1 ml of H_2O_2, and 0.1 ml of ascorbate were added in an Eppendorf tube. As well as for POD assay, 0.6 ml of 50 mM PPB (pH 8.0), 0.1 ml of EDTA, 0.1 ml of H_2O_2, and 0.1 ml guaiacol were added in an Eppendorf tube. Reaction was initiated by adding 0.1 ml of enzyme extract. The changes of absorbance were recorded using a spectrophotometer (T80, UV-Visible Spectrophotometer, PG Instruments, China) immediately at 240 nm, 290 nm, and 470 nm for CAT, APX and POD assay, respectively, at 30 s intervals for two min.

Statistical analysis

The statistical analysis was performed using the General Linear Model procedure of Minitab 17.0. Data were subjected to one-way analysis of variance (ANOVA) to compare the effects of salt stress treatments. The differences between the means were compared using the Fisher's least significant difference (LSD) test (LSD, $P \leq 0.01$ and 0.05).

Results

Germination indices of rice under salt stress and Ca^{2+} treatments

Germination indices of BRRI dhan28 rice cultivar were evaluated under normal and saline conditions both in integration with Ca^{2+} at two levels (3 and 5 mM) by measuring several key germination-related parameters such as FGP, CVG, GRI, and MGT (Table 1). Treatment with both 3 and 5 mM Ca^{2+} did not affect these germination indices compared to the controls (without Ca^{2+}). On the other hand, 100 mM NaCl-salt stress significantly reduced FGP, CVG, and GRI by 10.8, 6.9, and 14.7%, respectively, while it significantly increased MGT by 7.0% compared to the unstressed control values. However, the 3 mM and 5 mM Ca^{2+} treatment signifi cantly increased FGP (by 13.6 and 12.2%, respectively), CVG (by 4.9 and 2.8%, respectively), and GRI (by 17 and 14.2%, respectively), while it decreased MGT (by 4.3 and 2.6%, respectively) in rice seedlings under salt stress compared to the corresponding controls (100 mM NaCl without Ca^{2+}). Under both normal and saline conditions, the

Table 1. Exogenous calcium (Ca^{2+}) effect on germination indices under 100 mM NaCl stress at germination stage.

Treatments	Germination indices			
	FGP (%)	CVG (% day^{-1})	GRI (% day^{-1})	MGT (days)
Control (C)	98.7±1.3^{a}	46.3±1.3^{a}	47.0±0.8^{a}	2.16±0.06^{b}
Salt (S)	88.0±2.3^{b}	43.1±0.6^{b}	40.1±1.4^{b}	2.31±0.03^{a}
3 mM Ca^{2+}	100.0±0.0^{a}	46.6±0.8^{a}	47.8±0.5^{a}	2.14±0.03^{b}
5 mM Ca^{2+}	98.7±1.3^{a}	46.0±1.0^{a}	46.8±1.0^{a}	2.17±0.04^{b}
S+3 mM Ca^{2+}	100.0±0.0^{a}	45.2±0.7^{ab}	46.9±0.5^{a}	2.21±0.03^{ab}
S+5 mM Ca^{2+}	98.7±1.3^{a}	44.3±0.6^{ab}	45.8±0.6^{a}	2.25±0.01^{ab}
Sig. level	*	*	*	*

Data are represented as mean ± SE from three replications. Within each column, same letter indicates no significant difference among treatments. '*' and '**' indicate significant at 5% and 1% level of probability applying LSD, respectively. FGP means final germination percent, CVG means coefficient of velocity of germination, GRI means germination rate index, and MGT means mean germination time.

level of 3 mM Ca^{2+} was more effective than the level of 5 mM Ca^{2+}.

Growth of rice at germination stage under salt stress and Ca^{2+} treatments

Effects of Ca^{2+} on growth and biomass (i.e. height, FW, and DW of plumule and radicle) of rice seedlings under NaCl stress are presented in Fig. 1 and Table 2. The lowest plumule and radicle lengths were observed in salt-treated conditions. Plumule and radicle lengths showed an increasing trend over time. But due to salt stress, in comparison with the control, the plumule length was decreased by 41-72%, whereas the radicle length was decreased by 55-41% over time. However, 3 mM or 5 mM Ca^{2+} application alleviated the salt stress effects and increased plumule and radicle lengths by 40 or 25% and 63 or 58%, respectively, compared to the corresponding control (Fig. 1).

In addition, Ca^{2+} application also enhanced plumule and radicle growth and even more than the control conditions. Plumule fresh weight (PFW), radicle fresh weight (RFW), plumule dry weight (PDW), and radicle dry weight (RDW) were reduced by 58, 32, 65, and 37%, respectively, in salt-stressed seedlings when compared with the unstressed controls. However, treatment with 3 mM Ca^{2+} significantly increased PFW, RFW, PDW, and RDW in salt-stressed seedlings by 137, 87, 146, and 95%, respectively, in comparison with salt-stressed controls (without Ca^{2+}). Although 5 mM Ca^{2+} also significantly increased these parameters, the obtained values were lower than those obtained with 3 mM Ca^{2+} treatment. Under normal conditions, applying the Ca^{2+} significantly enhanced these parameters (Table 2).

Seedling growth of rice under salt stress and Ca^{2+} treatments

Under normal conditions, application of 3 mM Ca^{2+} significantly increased plant growth in terms of shoot length (SL), root length (RL), shoot fresh weight (SFW), and shoot dry weight (SDW), while 5 mM Ca^{2+} treatment reduced these growth parameters compared to the controls (Table 3). Salt-induced stress significantly decreased SL, RL, RDW, and SDW by 36.8, 32.6, 73.4, and 87.6%, respectively, compared to the non-stressed controls. However, supplementation with 3 mM Ca^{2+} to the salt-treated seedlings markedly restored seedlings SL, RL, RDW, and SDW by 35.8, 26.1, 50.6, and 572.5%, respectively, compared with the NaCl-stressed control seedlings. Similarly, 5 mM Ca^{2+} treatment significantly improved these growth parameters but the obtained values were lower than those obtained with 3 mM Ca^{2+} treatment.

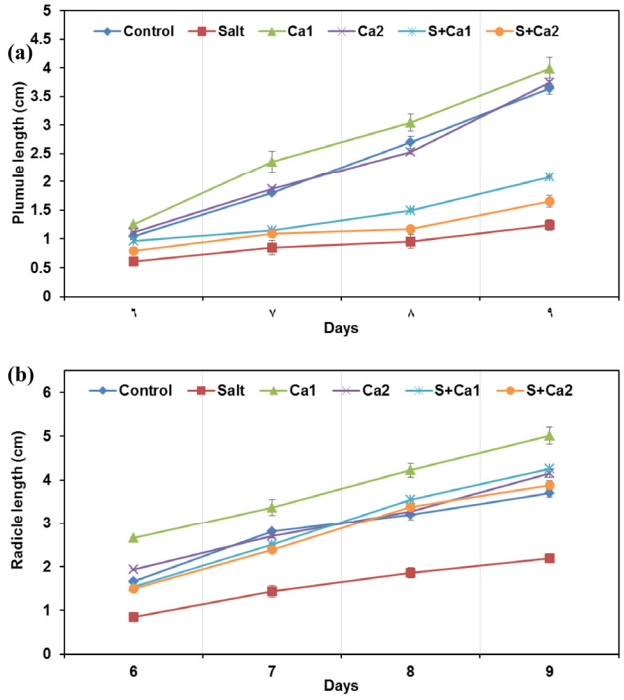

Fig. 1. Effects of exogenous calcium (Ca^{2+}) on (a) plumule length and (b) radicle length under 100 mM NaCl stress at germination stage. Vertical bars indicate the standard errors (SEs). Control (complete nutrient solution without additions), salt (100 mM NaCl added to nutrient solution), Ca1 (3 mM Ca^{2+} added to nutrient solution), Ca2 (5 mM Ca^{2+} added to nutrient solution), S+Ca1 (100 mM NaCl + 3 mM Ca^{2+} added to nutrient solution), S+Ca2 (100 mM NaCl + 5 mM Ca^{2+} added to nutrient solution).

Table 2. Exogenous calcium (Ca^{2+}) effect on growth and relative water content of BRRI dhan28 under 100mM NaCl stress at germination stage.

Treatments	Morphological parameters			
	RFW (mg)	PFW (mg)	RDW (mg)	PDW (mg)
Control (C)	17.2±0.6^{c}	14.3±0.8^{b}	2.13±0.3^{c}	2.86±0.1^{ab}
Salt (S)	11.7±0.5^{d}	5.9±0.5^{c}	1.33±0.1^{d}	1.00±0.2^{c}
3 mM Ca^{2+}	25.3±1.1^{a}	19.1±1.1^{a}	3.86±0.4^{a}	3.06±0.2^{a}
5 mM Ca^{2+}	22.7±1.7^{ab}	14.7±1.2^{b}	2.86±0.1^{b}	2.73±0.1^{ab}
S+3 mM Ca^{2+}	21.9±2.1^{abc}	14.0±0.6^{b}	2.60±0.2^{bc}	2.46±0.2^{b}
S+5 mM Ca^{2+}	18.7±2.5^{bc}	12.7±0.7^{b}	2.26±0.1^{bc}	2.53±0.1^{b}
Sig. level	*	*	*	*

Data are represented as mean ± SE from three replications. Within each column, same letter indicates no significant difference among treatments. '*' and '**' indicate significant at 5% and 1% level of probability applying LSD, respectively. RFW means radicle fresh weight, PFW means plumule fresh weight, RDW means radicle dry weight and PDW means plumule dry weight.

Table 3. Exogenous calcium (Ca^{2+}) effect on growth and relative water content of BRRI dhan28 under 100 mM NaCl stress at seedling stage.

Treatment	Growth parameters					RWC (%)
	SL (cm)	RL (cm)	SDW (mg)	RDW (mg)	SES	
Control (C)	19.0±0.2^{b}	7.07±0.42^{b}	147.0±3.2^{ab}	32.7±1.2^{b}	1	86.00±1.6^{a}
Salt (S)	12.0±0.4^{e}	4.79±0.23^{e}	18.2±0.5^{e}	8.7±0.5^{e}	7	69.15±1.5^{c}
3 mM Ca^{2+}	20.9±0.1^{a}	7.98±0.27^{a}	151.0±0.7^{a}	40.8±1.4^{a}	1	88.25±1.8^{a}
5 mM Ca^{2+}	17.0±0.1^{c}	6.28±0.38^{c}	145.2±1.4^{b}	28.5±1.4^{c}	3	86.35±0.6^{a}
S+3 mM Ca^{2+}	16.3±0.0^{c}	6.04±0.35^{c}	122.4±0.7^{c}	13.1±0.6^{d}	3	78.55±1.2^{b}
S+5 mM Ca^{2+}	14.1±0.2^{d}	5.45±0.35^{d}	107.9±0.6^{d}	9.1±0.3^{e}	5	74.60±1.1^{b}
Sig. level	*	*	*	**		

Data are represented as mean ± SE from three replications. Within each column, same letter indicates no significant difference among treatments. '*' and '**' indicate significant at 5% and 1% level of probability applying LSD, respectively. SL means shoot length, RL means root length, SDW means shoot dry weight, RDW means root dry weight, SES means Standard Evaluation System Score (proposed by IRRI) and RWC means relative water content.

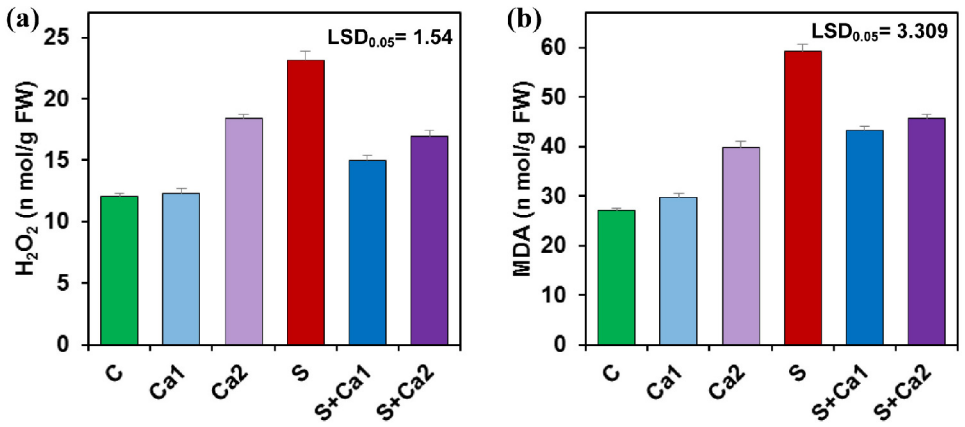

Fig. 2. Effects of exogenous calcium (Ca^{2+}) on (a) H_2O_2 content and (b) MDA content of 20-d old 100 mM NaCl-treated rice seedlings. Different letters on histogram indicate significant differences at $p < 0.05$ applying Fisher's LSD and vertical bars indicate the standard deviation (SDs). C (Control, complete nutrient solution without additions), Ca1 (3 mM Ca^{2+} added to nutrient solution), Ca2 (5 mM Ca^{2+} added to nutrient solution), S (100 mM NaCl added to nutrient solution), S+Ca1 (100 mM NaCl + 3 mM Ca^{2+} added to nutrient solution), S+Ca2 (100 mM NaCl + 5 mM Ca^{2+} added to nutrient solution)..

Phenotypic appearance of rice under salt stress and Ca^{2+} treatments

Under non-stressed conditions, exogenously-applied 3 mM Ca^{2+} had no visual effect on rice seedlings (SES score was 1), but 5 mM Ca^{2+} showed some leaf damage (SES score was 3) (Table 3). Salt stress severely damaged the rice seedlings phenotypically, resulting in rolling and burning of leaf tips and the whole plant acquired a yellow color (SES score was 7). Supplementation with 3 mM Ca^{2+} was more effectively, reversing the salt-induced damage and improved the phenotypic appearance of rice seedlings (SES score was 3) than supplementation with 5 mM Ca^{2+} (SES score was 5).

Proline content and RWC of rice under salt stress and Ca^{2+} treatments

Under stress-free conditions, both 3 mM and 5 mM Ca^{2+} application did not show any significantly change of proline content in rice seedlings compared with the controls (Fig. 3d). Interestingly, NaCl-salt treatment significantly enhanced proline content by 93% compared to that of control seedlings. Under salt stress, both 3 and 5 mM Ca^{2+} treatments significantly decreased proline content by 25.66 and 22.5%, respectively, compared with the salt-stressed only rice seedlings.

Furthermore, salt stress significantly reduced RWC by 19.6% in comparison with the control conditions. However, 3 mM or 5 mM Ca^{2+} application in salt-stressed seedlings significantly increased RWC compared to the salt-stressed only plants. Application of Ca^{2+} in non-stressed seedlings did not show any significant change of RWC compared with that of non-stressed control seedlings (Table 3).

H_2O_2 and MDA accumulation in rice seedlings under salt stress and role of Ca^{2+} treatment

In this study, the H_2O_2 content in salt-stressed rice leaves remarkably increased by 91.8%, compared with that of control seedlings (Fig. 2a). Both 3 mM and 5 mM exogenous Ca^{2+} treatment in salt-stressed rice seedlings significantly reduced H_2O_2 content compared with salt-stressed only plants, but 3 mM Ca^{2+} was more effective than 5 mM Ca^{2+} to prevent excess H_2O_2 accumulation. Under stress-free condition, application

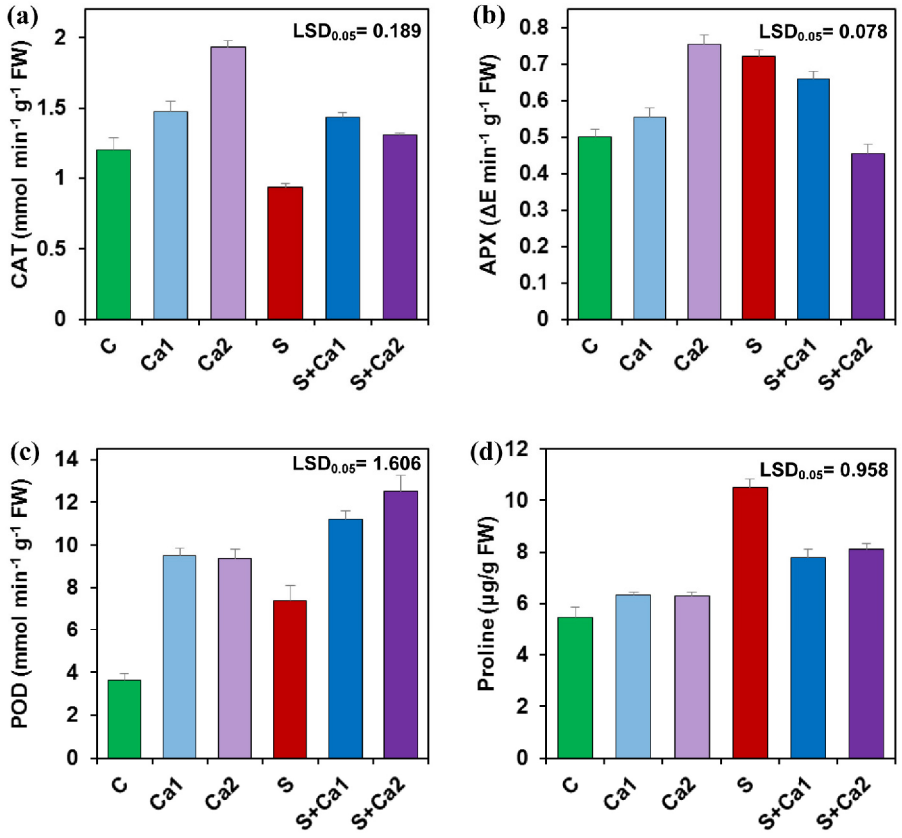

Fig. 3. Effects of exogenous calcium (Ca^{2+}) on (a) CAT activity, (b) APX activity, (c) POD activity and (b) proline content of 20-d old 100 mM NaCl-treated rice seedlings. Different letters on histogram indicate significant differences at $p < 0.05$ applying Fisher's LSD and vertical bars indicate the standard deviations (SDs). C (Control, complete nutrient solution without additions), Ca1 (3 mM Ca^{2+} added to nutrient solution), Ca2 (5 mM Ca^{2+} added to nutrient solution), S (100 mM NaCl added to nutrient solution), S+Ca1 (100 mM NaCl + 3 mM Ca^{2+} added to nutrient solution), S+Ca2 (100 mM NaCl + 5 mM Ca^{2+} added to nutrient solution).

of 3 mM Ca^{2+} did show any change of H_2O_2 content whereas 5 mM Ca^{2+} significantly enhanced H_2O_2 content compared with that of 3 mM Ca^{2+} treated and stress-free control seedlings.

MDA is a lipid peroxidation product, often regarded as an indicator of membrane damage. In our study, MDA content significantly enhanced by 118.4% in rice seedling subjected to salt stress when compared with that of control seedlings (Fig. 2b). On the other hand, when salt-stressed rice seedlings were treated with 3 mM and 5 mM Ca^{2+}, the MDA content significantly reduced compared to that of salt-stressed only plants. It is worth mentioning that 5 mM Ca^{2+} treatment in salt stress-free plants significantly increased MDA content in comparison with 3 mM Ca^{2+} treated and non-stressed control seedlings.

Antioxidant enzyme activities of rice under salt stress and Ca^{2+} treatments

The regulatory role of Ca^{2+} was assessed on the activities of various antioxidant enzymes involved in ROS metabolism such as CAT, APX, and POD (Figs. 3a-c). Under non-stressed conditions, 3 mM and 5 mM Ca^{2+} application significantly increased the activities of CAT, APX, and POD compared with that of control condition. NaCl-salt treatment displayed increases in APX and POD activities by 44 and 103.5%, respectively, as compared with NaCl-free controls. Whereas the CAT activity decreased, by 22.1%, in salt-stressed only plants compared with stress-free control plants. Under salt stress conditions, Ca^{2+} treatments significantly increased CAT and POD activity whereas decreased APX activities compared with the salt-stressed plants.

Effects of Ca^{2+} treatments on photosynthetic pigments of rice under salt stress

The highest values of chlorophyll contents [i.e. chl-a, chl-b, and chl-(a+b)] were observed in stress-free control seedlings which is statistically similar to the 3 mM Ca^{2+} treated non-stressed plants. Whilst the lowest values were observed in NaCl-salt treated plants (Fig. 4). NaCl-salt stress decreased chl-a, chl-b, and chl-(a+b) contents by 72.5, 80.7, and 73.4%, respectively, compared with stress-free control seedlings. Compared to only NaCl-stress treatments, Ca^{2+} treatments at both levels (3 or 5 mM) significantly increased chl-a, chl-b, and chl-(a+b) contents under salt stress conditions. Importantly, 3 mM Ca^{2+} treatments showed significantly higher chl-a, chl-b, and chl-(a+b) contents compared to that of 5

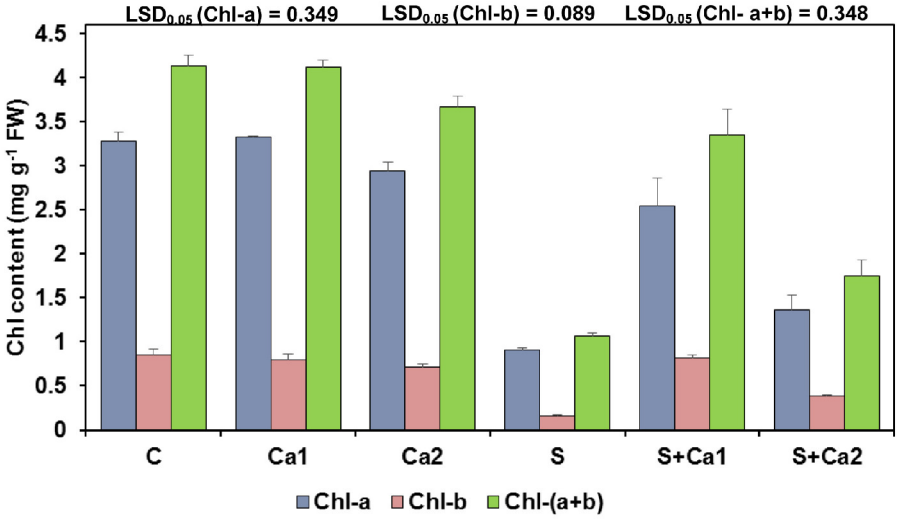

Fig. 4. Effects of exogenous calcium (Ca^{2+}) on chlorophyll content of 20-d old 100 mM NaCl-treated rice seedlings. Different letters of same color on histogram indicate significant differences at $p < 0.05$ applying Fisher's LSD and vertical bars indicate the standard deviation (SDs). C (Control, complete nutrient solution without additions), Ca1 (3 mM Ca^{2+} added to nutrient solution), Ca2 (5 mM Ca^{2+} added to nutrient solution), S (100 mM NaCl added to nutrient solution), S+Ca1 (100 mM NaCl + 3 mM Ca^{2+} added to nutrient solution), S+Ca2 (100 mM NaCl + 5 mM Ca^{2+} added to nutrient solution).

mM Ca^{2+} treatments. Moreover, under stress-free condition, 3mM Ca^{2+} application showed significantly higher chl-a and chl-(a+b) contents than 5 mM Ca^{2+} application.

Discussion

Salinity stress suppresses growth and development of mesophytes (Ashraf and Harris 2004). Among cereal crops, rice is the most susceptible crop to salt stress (Munns and Tester 2008). Thus, growth and development of rice can be hampered by slight accumulation of salts in cells due to the impairment of various physiological and biochemical processes (Gupta and Huang 2014; Munns and Tester 2008; Parihar et al. 2015). Subsequently, plants have developed complex mechanisms to adapt themselves to defeat salinity-induced damage. On the other hand, under continuous stressful conditions, the basal capacity of plants may annihilate and plants need to boost more protective mechanisms to overcome the injurious consequences. Our research distinctly indicated that the supplementation of Ca^{2+} alleviated salt-induced toxic effects in rice both at germination and seedling stage.

In the present study, salt stress adversely affected germination indices (i.e. FGP, CVG, and GRI, Table 1). Our results are correspond with those of Ruan and Xue (2002) and Khan et al. (2015). Salinity lowers the osmotic potential of growing medium, hampering water imbibition by seeds (Acosta-Motos et al. 2017), causing ionic toxicity that alters the activity of enzymes of nucleic acid metabolism (Gomes-Filho et al. 2008), disturbing hormonal balance, and reducing the utilization of seed reserve food (Othman et al. 2006). It also enhances the ABA content in pre-germinated seeds, inhibiting the seed germination process (Mokhtari et al. 2008). Thus, salinity negatively influences the germination indices. However, supplemental Ca^{2+} alleviated the deleterious salt effects and improved the germination indices (Table 1). Seed priming with Ca^{2+} might increase water absorption by seeds from the growing media to activate various metabolic processes of germination to start much earlier (Elouaer and Hannachi 2012). Further, Ca^{2+} neutralizes the binding of Na^{+} in cell walls, assuages membrane leakiness, and prevents salt-induced decline in cell production and cell elongation (Flowers et al. 2015; Hadi and Karimi 2012). Salt stress causes interruption in seed germination and consequently extends the mean germination time (MGT) (Tilaki et al. 2011). This finding is similar to our finding, where salt stress increased MGT and exogenously-applied Ca^{2+} decreased it (Table 1). Germinated seed radicles are primarily affected by excess salts due to water deficiency and specific ionic toxicity (Rengel 1992), increasing the time taken until radicle emergence and decreasing the germination rate (GRI) (Panuccio et al. 2014). Calcium is found to adjust the transmission and permeability of ions and control ion exchange, playing an important role in improving germination indices (Rengel 1992).

Salt-affected rice growth at both germination (e.g. radicle, plumule length, fresh and dry weights) and seedling (e.g. shoot, root length and total dry weight) stages (Tables 2 and 3), as observed by Panuccio et al. (2014) and Alom et al. (2014), in halophyte quinoa and wheat under salt stress. On the other hand, exogenous Ca^{2+} improved overall growth and biomass of salt-stressed rice seedlings which could be attributed to its role in protecting chl-a and chl-b contents from salt-induced damage (Tables 2, 3 and Fig. 4). Like other abiotic stresses, salt-induced stress destabilizes the pigment-protein complex and decreases photosynthetic pigments by increasing the activity of chlorophyllase enzyme and/or overproduction of

ROS (Hasanuzzaman et al. 2014). Similar results were observed in our study where salt stress drastically reduced chlorophyll content of rice seedlings (Fig. 4). The loss of chlorophyll leads to a decrease in the photosynthetic efficiency and ultimately lower growth under stress condition (El-Tayeb 2005). On the contrary, Ca^{2+} supplementation prevented the reduction of chlorophyll contents of the rice seedlings due to NaCl-stress (Fig. 4). The restoration of chlorophyll contents might be due to lower ROS production by Ca^{2+} supplementation under salt-stress (Fig. 2). This result is in agreement with the findings of some previous studies in *Brassica juncea* and rice under Cd and salt stress (Ahmad et al. 2015; Rahman et al. 2016).

Interestingly, in this study plumule length was affected more than radicle length at germination stage and root length was also less affected than shoot length at seedling stage by NaCl-salt stress (Fig. 1 and Table 3). Similar responses were reported earlier by Kapoor and Pande (2015) and Mehrafarin et al. (2011) in fenugreek under salinity stress conditions who reported that soil salinity suppresses shoot growth more than the root growth. Roots play an important role in shoot growth under saline conditions. Better growth of root may be due to active translocation of salt and ions from root to shoot (Reddy et al. 2017). However, exogenous treatment with Ca^{2+} counterattacked the adverse consequences of salinity of growth (Fig. 1 and Tables 2, 3). Ca^{2+} ions are interconnected with several vital mitotic events, including breakdown of nuclear envelope and metaphase-anaphase transition (Hepler 1994). It also activates calmodulins and increases cytokinine production, which can augment cell division (Snedden and Fromm 2001), plant growth, and biomass production. Excess Na^{+} ions in root medium inhibit elongation of root cells by reducing the cellular division (Teerarak et al. 2009). Accumulation of Na^{+} in the apoplasm may disrupt ionic interactions between cell wall constituents such as pectin and extensin, and may affect, adversely, the apoplasmic enzymes (Flowers et al. 2015).

Since salt stress causes both ionic toxicity and osmotic stress, creating physiological drought due to exosmosis and interruption of water uptake (Munns 2011) which causes an imbalance of cellular metabolic processes. 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). It is also claimed that the level of accumulated Pro reflects the intensity of stressed symptoms under various types of abiotic stresses (Loutfy et al. 2012; Metwally et al. 2003). Our results showed that proline content was increased significantly in rice seedlings subjected to NaCl-stress compared to that of control plants (Fig. 3d). Increased proline content in the salt-stressed plants may be due to increased activity of P5CS. The enzyme P5CS shows a positive correlation with NaCl stress (Murugan and Sathish 2005). 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* (Chandrasekar and Sandhyarani 1996) and in spinach leaves (Öztürk and Demir 2003). These results are also corroborated with the previous findings of Manivannan et al. (2007) and Al-Whaibi et al. (2012). However, under NaCl-salt stress, supplementation of 3 mM and 5 mM Ca^{2+} significantly decreased the proline content which suggested that Ca^{2+} ion somehow prevent water imbalance in plants thus reduced the need of excess proline synthesis. Similar results were found in salt-affected rice seedlings (Hasanuzzaman et al. 2014; Rahman et al. 2016).

Furthermore, rice seedlings exposed to salt stress showed lower relative water content (RWC) and higher proline accumulation (Table 3 and Fig. 3d). This result indicates a salt-induced water imbalance and osmotic stress (Rahman et al. 2016). Cellular dehydration by salt stress causes a decline in RWC that is a common phenomenon in plants grown under salt stress. Similar salt-induced water shortage and higher proline accumulation were observed in salt-affected rice seedlings (Hasanuzzaman et al. 2014; Rahman et al. 2016). However, exogenous Ca^{2+} neutralized the water loss (indicated by increased RWC) in the salt-affected rice seedlings (Table 3). Supplementation of rice seedlings with Ca^{2+} showed enhanced RWC, which may be due to the retention of water in their tissue.

The excess of cell Na^{+} and shortage of Ca^{2+} and K^{+} might also be caused by ROS (H_2O_2) and higher lipid peroxidation (as MDA) overproduction (Demidchik and Maathuis 2007). Up to a certain level, H_2O_2 production in cells can render a beneficial role to plants by a prompt triggering of tolerance mechanism against biotic and abiotic stress. But excess ROS generation under stressful conditions is considered as an indication of stress which can lead to lipid peroxidation, protein oxidation, enzymatic dysfunction, and ultimately directs to programmed cell death (Gill and Tuteja 2010). In the present study, rice seedlings subjected to NaCl stress caused higher H_2O_2 production and ultimately higher MDA content (Fig. 2) which, however, was declined by the exogenous application of Ca^{2+} to the salt-stressed rice seedlings, which might be due to Ca-mediated response that is disrupted under salt stress by decreasing Ca uptake. Our results are in line with those of Rahman et al. (2016) and Ahmad et al. (2018) who also demonstrated a lowered membrane damage by exogenously applied Ca^{2+} in rice and *Brassica juncea* plants.

In order to shield against oxidative stress, plants possess several antioxidant enzymes such as CAT, APX, and POD which work in an intimate coordination to scavenge the deleterious H_2O_2. CAT locates in peroxisome that converts H_2O_2 to H_2O (Das and Roychoudhury 2014). Unlike the CAT, the APX and POD requires electron donor in order to neutralize H_2O_2. APX uses ascorbic acid whereas POD requires guaiacol as a reducing agent (Das and Roychoudhury 2014; Gill and Tuteja 2010). In the present study, an increase in activities of APX and POD was noticed while decreased the activity of the efficient ROS scavenging enzyme, CAT (Figs. 3a-c). This result corroborate with the previous findings of Ahmad et al. (2018) who reported that

decreased CAT activities leads to accumulation of H_2O_2 which will not be efficiently neutralized only by this extent of increased activity of APX and POD. As a result, under salt stress conditions, accumulation of excess H_2O_2 (Fig. 2a) triggered to hamper the growth (Table 3) of rice seedlings in the current experiment. On the other hand, in present experiment, exogenously applied Ca^{2+} further boosted the activity of these antioxidant enzymes (Figs. 3a-c) which scavenged the deleterious H_2O_2 (Fig. 2a) efficiently and eventually reduced MDA content (Fig. 2b) and seedling growth (Table 3). This result is in agreement with previous findings in *Brassica juncea* and *Pennisetum* plants (Ahmad et al. 2018; Erinle et al. 2016). Stimulation of these antioxidant enzyme activities indicates that they are associated with oxidative stress tolerance (Mishra et al. 2013)

Conclusion

The results of the current study suggests that application of exogenous Ca^{2+} reversed salinity induced damage of germination indices and growth parameters by improving photosynthetic pigments and antioxidant enzymes. Coordinated activity of antioxidant enzymes in exogenous Ca^{2+} applied plants scavenged the deleterious ROS such as H_2O_2, thereby decreased lipid peroxidation (MDA). Maintaining water status is crucial under salt stress conditions and exogenous Ca^{2+} helped the rice plants to maintain water status without excess Pro accumulation. However, exogenously-applied Ca^{2+} level of 3 mM was more effective than the level of 5 mM. Thus, our findings profoundly support the ameliorative role of Ca^{2+} in salt stress mitigation in crop plants such as rice and support the efficacy of its use in crop cultivation. Therefore, it can be recommended that 3 mM Ca^{2+} supplementation can be an effective practice to enhance salt tolerance of rice and its cultivation in saline-prone areas of Bangladesh. Furthermore, it is necessary to conduct further experiments under field conditions for better tuning of our results.

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