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RESEARCH ARTICLE

Physiological mechanisms of exogenous calcium on alleviating salinity-induced stress in rice (*Oryza sativa* L.)

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Abstract Being more sensitive to salt stress among the cereals, growth of rice (*Oryza sativa* L.) has been habitually affected by salinity. Although, several practices have evolved to sustain the growth of rice under salinity, the enormous role of calcium (Ca^{2+}) as a signalling molecule in salt stress mitigation is still arcane. Considering this fact, an experiment was performed aiming to explicate the mechanism of salt-induced growth inhibition in rice and its alleviation by exogenous Ca^{2+}. At germination stage, 10 mM and 15 mM $CaCl_2$ primed rice (cv. *Binadhan-10* & *Binadhan-7*) seeds were grown in petri dishes for 9 days under 100 mM NaCl stress. At seedling stage, 9-day-old rice seedlings grown on sand were exposed to 100 mM NaCl alone and combined with 10 mM and 15 mM $CaCl_2$ for 15 days. This research revealed that salinity radically slowed down growth of rice seedlings and Ca^{2+} treatment noticeably improved growth performances. At germination stage, 10 mM $CaCl_2$ treatment significantly increased the final germination percentage, germination rate index (in *Binadhan-7*), shoot, root length (89.20, 67.58% in *Bindhan-10* & 84.72, 31.15% in *Bindhan-7*) and biomass production under salinity. Similarly, at seedling stage, 10 mM $CaCl_2$ supplementation in salt-stressed plants enhanced shoot length (42.17, 28.76%) and shoot dry weight (339.52, 396.20%) significantly in *Binadhan-10* & *Binadhan-7,* respectively, but enhanced root dry weight (36.76%) only in *Binadhan-10*. In addition, 10 mM $CaCl_2$ supplementation on salt-stressed seedlings increased the chlorophyll and proline content, and oppressed the accretion of reactive oxygen species thus protecting from oxidative damage more pronouncedly in *Binadhan-10* than *Binadhan-7* as reflected by the elevated levels of catalase and ascorbate peroxidase activity. The 15 mM $CaCl_2$ somehow also enhanced some growth parameters but overall was less effective than 10 mM $CaCl_2$ to alleviate salt stress, and sometimes showed negative effect. Therefore, supplementary application of calcium-rich fertilizers in saline prone soils can be an effective approach to acclimatize salt stress and cultivate rice successfully.

Keywords NaCl stress · Sandponic culture · Calcium · Photosynthetic pigments · Antioxidant enzymatic activity

Introduction

Environmental adversity exerts substantial amount of stresses on plants' existence (Mahmood-ur-Rahman et al. 2019), among which salinity is one of the dominant abiotic stresses that abruptly cease off the plant growth as well as developmental processes (Mbarki et al. 2018b; Safdar et al. 2019), resulting a huge annual yield loss all over the earth (Munns and Tester 2008). Next to drought, salinity is the prevalent soil problem in rice (*Oryza sativa* L.) and is contemplated as a powerful constraint in the way to amplify rice production worldwide (Ghosh et al. 2016). Currently, salinity affects nearly 6% of the entire world's land (Munns and Tester 2008) and has become an

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escalating problem in shoreline areas. Globally, no less than 10 million hectares of agricultural land are abandoned annually because of salinity. Salt-induced land degradation is a very important issue affecting the status of food productivity worldwide (Geist 2017; FAO 2016).

Salinity impairs the plant growth more than any other noxious materials in the earth (Xiong and Zhu 2002) and modulates several physiological and biochemical processes in plants (Goyal et al. 2018). Surplus salts in growth media affects plant growth in two ways. Firstly, high concentration of salt in the soil solution results in stunted root growth and causes the root to uptake water (osmotic stress) that also impedes ion flux to the shoot and leaves, and stimulates stomatal closure (Yang and Guo 2018; Reddy et al. 2017). Secondly, high concentrations of salts, particularly Na^+ in the transpiration stream of plants can be injurious to cells (salt-specific effect), resulting in an inhibition of several physiological and biochemical routes such as nutrient uptake (like potassium and calcium) and CO_2 assimilation (Tang et al. 2015; Parihar et al. 2015; Safdar et al. 2019). Salinity negatively regulates stomatal conductance, plant water relations and degrades photosynthetic pigments which triggers lower transpiration rate, photosynthesis, growth and biomass production (Tang et al. 2015; Morton et al. 2019). In chloroplasts, inadequacy of CO_2 assimilation due to excessive Na^+ accumulation in plants resulting in hyper-reduction of electron transport complex (ETC) is the key reason of reactive oxygen species (ROS) generation. Hyper-reduction of mitochondrial ETC is also a vital means of ROS production under stress condition (Miller et al. 2010; Saini et al. 2018). The superoxide radical, hydroxyl radical, singlet oxygen and hydrogen peroxide are the major ROS which causes oxidative stress to plants (Waszczak et al. 2018). The ROS can impair the function and structure of DNA, RNA and protein, thereby intensifying oxidative damage (Hossain et al. 2012; Kordrostami and Rabiei 2019). Moreover, higher Na^+ concentration, impairs the function of the Calcium-Dependent Protein Kinase, OsCPK12 that leads to early senescence in plants (Asano et al. 2012; Campo et al. 2014). Therefore, exogenous calcium supplementation might help to overexpress the OsCPK12 gene and confer salt stress tolerance.

Among all cereals, rice is more vulnerable to salt stress and shows variability in sensitivity to salinity at different developmental phases throughout its lifespan. However, germination phase is considered fairly tolerant but the early seedling stage is the most salinity sensitive growth stage that directly affects the yield (Zeng et al. 2001; Mickky et al. 2019). Besides, salt stress can cause nutritional discrepancy, especially K^+ insufficiency by impairing its uptake and initiating its leakage from the cell (Munns and Tester 2008; Parihar et al. 2015). Recently, several research works have revealed the salt stress response and tolerance mechanisms in crop plants (Gao et al. 2007; Mbarki et al. 2018b; Safdar et al. 2019) that includes a diversity of defense mechanisms such as osmotic, ionic and ROS homeostasis (Gupta and Huang 2014; Tang et al. 2015). The accumulation of high concentration of salt triggers the amplification of the cytosolic osmotic force. Under these circumstances, plants maintain cellular homeostasis by osmotic regulation processes, mostly by synthesizing osmolytes and depositing toxic ions in the cell-vacuoles (Reddy et al. 2017). In many plant species, production of different compatible osmoprotectants such as sucrose (Sami et al. 2016), glycine betaine (Kurepin et al. 2017), mannitol (Patel and Williamson 2016), trehalose (Kosar et al. 2018) and proline (Slama et al. 2015) helps to continue water relation, stabilize enzyme, protein complex and membrane under saline condition (Iqbal et al. 2015). Specifically, higher deposition of proline is considered to be interlinked with tolerance to osmotic stresses (Per et al. 2017). Plant defense against increased ROS under stressful condition is coupled with the maintenance of cellular redox equilibrium, which is mostly conferred by some non-enzymatic and enzymatic antioxidants such as ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione-S-transferase (GST), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Yan et al. 2013; Tang et al. 2015). The synchronized function of these enzymes strengthen a steadiness between the formation of and detoxification of ROS (Hanin et al. 2016).

Efforts are ongoing to improve plants ability to survive under diverse environmental stresses. In this prospect, the probable role of small biological molecules such as phytohormones and signalling molecules could be considered influential in amending plants adaptability against adverse environment conditions. Calcium (Ca^{2+}) is an essential plant element and acts as signalling molecules associated with adaptive responses against environmental stresses (Mahmood-ur-Rahman et al. 2019). Scientist have shown that exogenous Ca^{2+} supplements to irrigation water has caused mitigation of salt stress (Sohan et al. 1999; Jaleel et al. 2008; Rahman et al. 2016). The affirmative effect of Ca^{2+} in stress alleviation has been associated with some defence mechanisms. Ca^{2+} is the crucial element that helps in keeping the structural and functional integrity of membranes with stabilization of cell wall as well as regulation of ion homeostasis (Arshi et al. 2010; Morgan et al. 2014) consequently, playing a significant role in plant growth and development. Besides, an exogenous addition of Ca^{2+} reduces the effects of NaCl toxicity by facilitating a high K^+/Na^+ selectivity. It also intensifies the antioxidant enzymatic system and ROS detoxification system (Rahman et al. 2016).

In Bangladesh context, research works are hardly found on the ameliorative roles of exogenous Ca^{2+} on salt-stressed rice. Therefore, in the current study, we aimed to examine the protective roles of exogenous Ca^{2+} in alleviating the harmful consequences of salinity on the salt-tolerant and susceptible rice cultivars both at germination and seedling stage. For this purpose, we have examined the germination indices, morpho-physiological and biochemical parameters to assess salt tolerance in rice under Ca^{2+} treatments with specific highlight on compatible solute production and ROS detoxification system. As far as we know, very few experiments were performed on Ca-mediated salinity stress tolerance in two contrasting rice cultivars both at germination and seedling stage.

Materials and methods

Germination stage experiment

Two rice cultivars viz. *Binadhan-10* (high yielding, salt-tolerant) (Kibria et al. 2017) and *Binadhan-7* (high yielding, salt-susceptible) (Tahjib-Ul-Arif et al. 2018a) were collected from Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. The rice seeds were surface sterilized with 2.5% sodium hypochlorite for 15 min and washed thoroughly with distilled water several times. These seeds were soaked in 10 mM and 15 mM $CaCl_2$ solution for 24 h. Followed by washing excess $CaCl_2$, the 100 rice seeds were placed on each petri dishes with or without 100 mM NaCl. Therefore, the treatment combinations were: control (C), only tap water; 100 mM NaCl (S); 10 and 15 mM $CaCl_2$ with control (C + Ca10 and C + Ca15); 10 and 15 mM $CaCl_2$ with 100 mM NaCl (S + Ca10 and S + Ca15). All treatments were replicated for three times. The seeds were grown for 9 days under 100 mM NaCl stress condition and different morphological assessments were performed at germination stage.

Seedling stage experiment

For seedling stage experiment, twenty germinated rice seeds were sown in a 4.0 L plastic tray containing sterilized sands. The seedlings were grown for 3 days in tap water, following which 0.5 × Hoagland Nutrient solution (Hoagland and Arnon 1950) was added. The pH of the nutrient solution was maintained at 6.5. One group of 6-day old seedlings were treated with 10 and 15 mM $CaCl_2$ for 72 h while another group was kept untreated. The both pretreated and non-pretreated seedlings were exposed to 100 mM NaCl stress with a nutrient solution in two steps by increasing the dosage by 50 mM NaCl per day to avoid osmotic shock. The treatment combinations were similar as that of germination stage experiments and also all the treatments replicated for three times. Seedlings were grown under 100 mM NaCl stress for 15 days and meanwhile, the nutrient solutions were renewed at the 4-day interval. Visual toxic symptoms of salt injury were evaluated by using modified standard evaluation score (SES) of IRRI (International Rice Research Institute (IRRI) 2002) at 10th day after salinization. Different morphological parameters were assessed at 15th day after salinization and the third leaf of each rice seedlings was taken for determining several physiological and biochemical parameters.

Assessment of germination indices

The number of germinated seeds were counted every day and final germination percentage (FGP) and germination rate index (GRI) on the 7th day of germination stage experiment were calculated by the following formulae (Afrin et al. 2019).

$$\text{FGP} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds taken for germination}} \times 100$$

$$\text{GRI} = \sum \frac{\text{Number of germinated seeds at nth day}}{\text{n}}$$

Here, n = 1, 2, 3, 4.

Assessment of growth parameters

At 9th day, different growth parameters were recorded at germination stage. The growth parameters were assessed by measuring shoot and root length, fresh weight (FW) and dry weight (DW). Shoot length was measured from shootlet base to the leaf tip and root length was measured from rootlet base to the root tip. Five seedlings from each treatment were weighed for FW determination and DW were determined by drying the sample in an oven at 70 °C for 4 days till it attained a constant weight. Also, the growth parameters (shoot and root length and fresh weight) at the seedling stage after 15 days salt exposure were recorded similarly as germination stage.

Measurement of relative water content (RWC)

RWC of rice seedlings was measured as described previously by Tahjib-Ul-Arif et al. 2018b using following the formula:

$$\text{RWC} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

Proline content determination

Proline content was determined according to methods of Bates et al. 1973. 50 mg of fresh leaf sample was homogenized using 10 mL of 3.0% sulfosalicylic acid. After centrifuging at 11,500×*g*, the supernatant (2.0 mL) was mixed with 2 mL acid ninhydrin and 2 mL glacial acetic acid in a test tube. The test tubes were incubated for 1 h at 100 °C in a hot water bath and then transferred to an ice bath to terminate the reaction. 4.0 mL of toluene was added to each of the test tubes and then stirred vigorously for 15–20 s. The absorbance of the collected toluene was measured at 520 nm in a VIS/UV spectrophotometer (Shimadzu, UV-1201) against reagent blank. A standard curve was prepared with analytical grade proline for calculating proline content expressed as mg 100 g^{-1} fresh weight of leaves.

Determination of photosynthesis pigments

Chlorophyll (Chl) extraction was done by taking 0.05 g fresh leaf with 10 mL of 80% acetone. The absorbance of the supernatant was recorded at 645 nm and 663 nm wavelengths to determine the chlorophyll-a and chlorophyll-b according to the method developed by Roy et al. 2016 and results were expressed as mg g^{-1} FW. The formula for computing chlorophyll-a and chlorophyll-b were as follows:

$$\text{Chlorophyll a} = (13.19A_{663} - 2.57A_{645}) \times \text{dilution factor}$$
$$\text{Chlorophyll b} = (22.10A_{645} - 5.26A_{663}) \times \text{dilution factor}$$

where A_{663} = Absorbance at 663 nm wavelength and A_{645} = Absorbance at 645 nm wavelength.

Extraction and determination of antioxidant enzymatic activity

The 50 mg of the fresh leaf was used for extraction with 3 mL of 50 mM potassium phosphate buffer (pH 8.0). Catalase (CAT, EC: 1.11.1.6) activity was determined by following the method of Aebi 1984. The activity of catalase was calculated from the decrease in absorbance at 240 nm min^{-1} when the extinction coefficient of H_2O_2 was 40 M^{-1} cm^{-1}. Peroxidase (POD, EC: 1.11.1.7) activity was determined by following the method of Nakano and Asada 1981. The activity of peroxidase was calculated from the increase in absorbance at 470 nm min^{-1} when the extinction coefficient was 26.6 mM^{-1} cm^{-1}. Ascorbate peroxidase (APX, EC: 1.11.1.11) activity was determined by following the method of Nakano and Asada 1981. The activity of ascorbate peroxidase was calculated from the decrease in absorbance at 290 nm min^{-1} when the extinction coefficient was 2.8 mM^{-1} cm^{-1}. The concentration of these antioxidant enzymes was expressed as mM min^{-1} g^{-1} FW.

Statistical analysis

Two-way analysis of variance (ANOVA) was performed following the General Linear Model (GLM) procedure of Minitab 17.0 software where the two factor were treatment and cultivars. Fisher's least significant difference (LSD) method was employed to perform statistical comparisons among treatments considering 5% level of probability ($P < 0.5$).

Results

Exogenous Ca^{2+} enhances germination indices

Salt stress negatively affected the FGP of two tested rice cultivars. FGP of salt-stressed plants was reduced by 5.3% in *Binadhan-10* while 6.6% in *Binadhan-7* compared with that of control plants. Exogenous application of Ca^{2+} (10 and 15 mM $CaCl_2$) significantly increased the FGP of salt-susceptible *Binadhan-7* whereas non-significant change was observed in salt-tolerant *Binadhan-10* over only salt-stressed plants. Moreover, GRI of salt-tolerant *Binadhan-10* and salt-susceptible *Binadhan-7* showed a significant reduction under salt stress condition in comparison with control condition. Exogenous application of 10 mM $CaCl_2$ restored the GRI in both salt-stressed and non-stressed plants of both *Binadhan-10* and *Binadhan-7* (Table 1).

Exogenous Ca^{2+} synchronizes plantlet growth and biomass production at germination stage

Salt stress reduced the plantlet growth and biomass accumulation of both salt-tolerant *Binadhan-10* and salt-susceptible *Binadhan-7* rice cultivars. Shoot and root length, FW and DW of salt-stressed *Binadhan-10* reduced by 57, 30, 47.4, 25.9, 48.6 and 27.3%, respectively, whilst by 61, 35.5, 46.1, 53.6, 62.5 and 37.5%, respectively, in *Binadhan-7* over control plants. The reduction of these growth parameters due to salt stress was recovered after applying 10 mM exogenous $CaCl_2$ by 89.20, 67.58, 65.09, 38.25, 26.98 and 25.35% respectively in *Binadhan-10* where *Binadhan-7* showed 84.72, 31.15, 33.73, 36.56, 66.67 and 80.45% accretion, respectively compared to salt-stressed without Ca^{2+} treated plants. On the other hand, application of 15 mM $CaCl_2$ also enhanced shoot and root length, FW and DW more pronouncedly in salt-stressed *Binadhan-10* than *Binadhan-7*, compared with only stressed plants, but it

Table 1 Effects of exogenous calcium on final germination percentage (FGP) and germination rate index (GRI) of two rice cultivars under 100 mM NaCl stress at germination stage

Cultivars	Treatments	FGP (%)	GRI (day^{-1})
Binadhan-10	Control	100 ± 0.00a	24.38 ± 0.256a
	10 mM $CaCl_2$	100 ± 0.00a	24.09 ± 0.344a
	15 mM $CaCl_2$	100 ± 0.00a	23.92 ± 0.295a–c
	100 mM NaCl (S)	94.66 ± 1.33b	22.87 ± 0.307cd
	S + 10 mM $CaCl_2$	94.66 ± 1.33b	23.01 ± 0.301b–d
	S + 15 mM $CaCl_2$	92.00 ± 2.00b	22.49 ± 1.124d
Binadhan-7	Control	100 ± 0.00a	24.12 ± 0.255ab
	10 mM $CaCl_2$	100 ± 0.00a	24.31 ± 0.155a
	15 mM $CaCl_2$	100 ± 0.00a	24.06 ± 0.284ab
	100 mM NaCl (S)	93.33 ± 1.33b	21.86 ± 0.577d
	S + 10 mM $CaCl_2$	100 ± 0.00a	24.02 ± 0.275a–c
	S + 15 mM $CaCl_2$	100 ± 0.00a	23.72 ± 0.124a–c
Sig. level		*	*

Each value presents the means ± standard errors (*n = 3*) obtained from three independent replicates. Different letters in each column indicates statistically significant difference following Fisher's least significant difference method ($P < 0.05$, *)

was less effective than 10 mM $CaCl_2$. Under the non-stressed condition, Ca^{2+} application (both 10 mM and 15 mM $CaCl_2$) increased the shootlet and rootlet growth and biomass production compared with that of control condition (Table 2).

Exogenous Ca^{2+} amends the RWC

RWC of leaves showed an inverse trend with salinity. Under salt stress, salt-tolerant *Binadhan-10* retained RWC but significant reduction of RWC (3.6%) was noticed in salt-susceptible *Binadhan-7* in comparison with the control plants. A non-significant increase of RWC was observed both in *Binadhan-10* (1.63%) and *Binadhan-7* (2.54%) when the salt-stressed plants were treated with 10 mM $CaCl_2$ and application of 15 mM $CaCl_2$ in salt-stressed plants increased RWC significantly only in *Binadhan-10*, compared with only salt-stressed plants. On the other hand, under the non-stress condition, application of 10 mM $CaCl_2$ only increased RWC in *Binadhan-10* compared with the control condition. Overall, 10 mM $CaCl_2$ performed better than the 15 mM $CaCl_2$ (Table 2).

Exogenous Ca^{2+} improves the phenotypic response of rice seedlings

Several salt injuries including shoot growth reduction, root growth reduction, stunted growth and seedling drying were observed in the salinized conditions. In salt-stressed condition, *Binadhan-10* showed moderate tolerance (SES was 5.0) while *Binadhan-7* showed susceptible (SES was 7.0) characteristics based on SES score. But when the salt-stressed plants were treated with 10 mM $CaCl_2$, *Binadhan-10* showed tolerance (SES was 3.0) and *Binadhan-7* showed moderate tolerance (SES was 5.0) characters. However, application of 15 mM $CaCl_2$ in salt-stressed *Binadhan-10* (SES was 5.0) and *Binadhan-7* (SES was 7.0) did not enhance salt tolerance level compared with control (Table 3).

Retrieval of salinity induced growth retardation of rice seedlings by exogenous Ca^{2+}

To appraise the effects of salinity on rice seedling growth, we determined the shoot and root length and DW. Shoot and root length, shoot and root DW of salt-stressed *Binadhan-10* were significantly reduced by 42.69, 21.74, 83.35 and 58.36%, respectively, whereas *Binadhan-7* showed a reduction by 45.75, 0.62, 83.07 and 71.28%, respectively, in relative to control plants. When salt-stressed plants were subjected to 10 mM $CaCl_2$, shoot and root length, shoot and root DW of *Binadhan-10* were increased by 42.17, 10.63, 339.52 and 63.79%, respectively, while *Binadhan-7* showed accretion at 28.76, 0.13, 396.23 and 10.97%, respectively compared to only salt-stressed plants. On the other hand, application of 15 mM $CaCl_2$ on salt-stressed plants did not recover growth and biomass retardation caused by salinity. Under the non-stressed condition, application of 10 mM $CaCl_2$ did not affect plant growth and biomass production but 15 mM $CaCl_2$ treatment negatively affected plant growth parameters. 10 mM $CaCl_2$ performed better over 15 mM $CaCl_2$ in both salt-tolerant *Binadhan-10* and salt-susceptible *Binadhan-7* (Table 3).

Table 2 Effects of exogenous calcium on shoot and root length, fresh weight (FW) and dry weight (DW), and relative water content (RWC) of two rice cultivars under 100 mM NaCl stress at germination stage

Cultivars	Treatments	Shoot length (cm)	Root length (cm)	Shootlet FW (mg $plant^{-1}$)	Shootlet DW (mg $plant^{-1}$)	Rootlet FW (mg $plant^{-1}$)	Rootlet DW (mg $plant^{-1}$)	RWC (%)
Binadhan-10	Control	4.11 ± 0.02ab	4.03 ± 0.19cd	21.06 ± 0.13bc	2.46 ± 0.35bc	17.2 ± 1.14a–d	2.93 ± 0.17a	90.55 ± 1.74c
	10 mM $CaCl_2$	4.36 ± 0.17a	5.16 ± 0.52b	26.33 ± 2.76a	2.46 ± 0.73bc	24.00 ± 4.98a	2.93 ± 0.33a	96.21 ± 0.83a
	15 mM $CaCl_2$	3.80 ± 0.07b	6.32 ± 0.22a	22.46 ± 0.71b	2.13 ± 0.30bc	17.53 ± 0.88a–d	2.6 ± 0.11ab	93.79 ± 0.93a–c
	100 mM NaCl (S)	1.76 ± 0.16gh	2.82 ± 0.33e	11.06 ± 0.86f–g	1.26 ± 0.11e	12.73 ± 1.33cd	2.13 ± 0.13ab	90.44 ± 1.86c–e
	S + 10 mM $CaCl_2$	3.33 ± 0.21cd	4.72 ± 0.73bc	18.26 ± 0.43cd	1.6 ± 0.41de	17.6 ± 1.96a–d	2.67 ± 0.24ab	91.91 ± 0.81b–d
	S + 15 mM $CaCl_2$	2.81 ± 0.18e	4.98 ± 0.32b	15.00 ± 0.91de	1.26 ± 0.53e	18.6 ± 1.25a–c	2.53 ± 0.06ab	95.50 ± 1.05a
Binadhan-7	Control	3.70 ± 0.06bc	4.04 ± 0.08 cd	14.00 ± 1.27ef	3.20 ± 0.11a	24.00 ± 3.49a	2.13 ± 0.26ab	92.59 ± 0.22b–d
	10 mM $CaCl_2$	4.10 ± 0.08ab	4.85 ± 0.15bc	12.07 ± .60e–g	3.53 ± 0.06a	19.20 ± 1.03a–c	2.53 ± 0.17ab	94.30 ± 0.44ab
	15 mM $CaCl_2$	2.98 ± 0.25de	3.79 ± 0.15d	9.53 ± 1.83gh	2.60 ± 0.11b	21.07 ± 0.76ab	2.40 ± 0.06ab	94.06 ± 1.01a–c
	100 mM NaCl (S)	1.44 ± 0.05h	2.60 ± 0.03e	7.53 ± 0.78 h	1.20 ± 0.11e	11.13 ± 0.06d	1.33 ± 0.26c	89.25 ± 2.01e
	S + 10 mM $CaCl_2$	2.66 ± 0.02ef	3.41 ± 0.05de	10.07 ± 0.40gh	2.00 ± 0.11cd	15.20 ± 0.75b–d	2.27 ± 0.11ab	91.52 ± 1.43c–e
	S + 15 mM $CaCl_2$	2.23 ± 0.03fg	2.74 ± 0.04e	7.60 ± 0.60h	1.40 ± 0.11e	14.40 ± 0.46b–d	2.00 ± 0.20ab	89.14 ± 0.54e
Sig. level		*	*	*	*	*	*	*

Each value presents the means ± standard errors ($n = 3$) obtained from three independent replicates. Different letters in each column indicates statistically significant difference following Fisher's least significant difference method ($P < 0.05$, *)

Table 3 Effects of exogenous calcium on shoot length, root length, shoot dry weight, root dry weight and SES score of two rice cultivars under 100 mM NaCl stress at seedling stage

Cultivars	Treatments	Shoot length (cm)	Root length (cm)	Shoot dry weight (mg $plant^{-1}$)	Root dry weight (mg $plant^{-1}$)	SES score
Binadhan-10	Control	20.4 ± 0.373b	8.05 ± 0.167a	188.00 ± 0.002b	37.87 ± 0.001c	1.0
	10 mM $CaCl_2$	21.89 ± 0.430a	8.00 ± 0.352a	198.00 ± 0.007a	63.47 ± 0.003a	1.0
	15 mM $CaCl_2$	16.99 ± 0.137d	7.30 ± 0.593a–c	167.80 ± 0.002c	38.40 ± 0.001c	1.0
	100 mM NaCl (S)	11.69 ± 0.784h	6.30 ± 0.562c	31.30 ± 0.001 h	15.77 ± 0.0003f	5.0
	S + 10 mM $CaCl_2$	16.62 ± 0.456de	6.97 ± 0.218bc	137.57 ± 0.002e	25.83 ± 0.0006e	3.0
	S + 15 mM $CaCl_2$	14.46 ± 0.289f	6.95 ± 0.338c	118.67 ± 0.002f	17.50 ± 0.0004f	5.0
Binadhan-7	Control	18.58 ± 0.435c	8.03 ± 0.419a	156.50 ± 0.003d	37.50 ± 00c	1.0
	10 mM $CaCl_2$	18.33 ± 0.214c	8.02 ± 0.628a	168.30 ± 0.002c	48.77 ± 00b	1.0
	15 mM $CaCl_2$	15.81 ± 0.479e	6.33 ± 0.251c	152.33 ± 0.002d	32.13 ± 00d	1.0
	100 mM NaCl (S)	10.08 ± 0.686i	7.98 ± 0.577ab	26.50 ± 0.0007h	10.77 ± 0.09g	7.0
	S + 10 mM $CaCl_2$	12.98 ± 0.275g	7.99 ± 0.333a	131.50 ± 0.0002e	14.73 ± 00fg	5.0
	S + 15 mM $CaCl_2$	11.09 ± 0.145hi	6.76 ± 0.187c	109.67 ± 0.001g	10.97 ± 00g	7.0
Sig. level		*	*	*	*	

Each value presents the means ± standard errors (*n = 3*) obtained from three independent replicates. Different letters in each column indicates statistically significant difference following Fisher's least significant difference method ($P < 0.05$, *)

Effects of exogenous Ca^{2+} on photosynthetic pigments

Salinity impaired the photosynthetic pigments of rice seedlings. Salt stress resulted in significant decrease of chl-a, chl-b and total chls by 68.62, 81.03 and 71.62%, respectively in *Binadhan-10* and 83.4, 78.71 and 82.5%, respectively in *Binadhan-7* compared with control seedlings. Exogenous Ca^{2+} (10 mM $CaCl_2$) application to the salt-stressed plants improved chl-a, chl-b and total chls content by 56.6, 73.5 and 60.0%, respectively in *Binadhan-10* and 46.58, 39.0 and 45.02%, respectively in *Binadhan-7*, compared with only salt-stressed seedlings. In contrast, 15 mM $CaCl_2$ treatment on salt-stressed *Binadhan-10* and *Binadhan-7* seedlings did not improve chls content compared with only salt-stressed seedlings. Application of 10 mM $CaCl_2$ on non-stressed rice seedlings did show any significant effects on chls content whereas 15 mM $CaCl_2$ significantly decreased chls content compared with control seedlings. The increment of chls content due to 10 mM $CaCl_2$ treatment was more prominent in salt-tolerant *Binadhan-10* than salt-susceptible *Binadhan-7* (Fig. 1).

Effects of Ca^{2+} on proline content in rice seedlings

Salt stress significantly increased the proline content by 84.3 and 105.7%, respectively both in salt-tolerant *Binadhan-10* and salt-susceptible *Binadhan-7* cultivars compared with control seedlings. Application of Ca^{2+} accelerated the proline accumulation in both stressed and non-stressed seedlings. The proline content was increased in salt-stressed *Binadhan-10* (78.83%) and *Binadhan-7* (6.07%) when treated by 10 mM $CaCl_2$ in comparison with only salt-stressed seedlings. On the other hand, 15 mM $CaCl_2$ treatment on salt-stressed seedlings of *Binadhan-10* showed an increase in proline content by 65.27% whereas *Binadhan-7* showed a decrease by 35.52% over only salt-stressed seedlings (Fig. 2a).

Exogenous Ca^{2+} ameliorates the antioxidant enzymatic activities

CAT activity in *Binadhan-10* and *Binadhan-7* increased by 53.9 and 27.8%, respectively compared with control seedlings. The 10 mM $CaCl_2$ treatment on salt-stressed seedlings did not affect CAT activity in *Binadhan-10* but decreased it in *Binadhan-7* in relative to only salt-stressed seedlings. Compared with the control condition, application of either 10 mM or 15 mM $CaCl_2$ on non-stressed seedlings enhanced CAT activity both in *Binadhan-10* and *Binadhan-7*, but the increment was more prominent in 10 mM $CaCl_2$ treatment than 15 mM $CaCl_2$ (Fig. 2b).

The seedlings of *Binadhan-10* and *Binadhan-7* treated with NaCl increased POD activity by 61.6 and 106%, respectively compared with control seedlings. Moreover, 10 mM and 15 mM $CaCl_2$ application in salt-stressed seedlings did not affect the POD activity in *Binadhan-10* but decreased it in *Binadhan-7* by 5.5% compared with salt-stressed control seedlings. Under the non-stressed condition, 10 mM and 15 mM $CaCl_2$ also enhanced POD activity both in salt-tolerant *Binadhan-10* and salt-

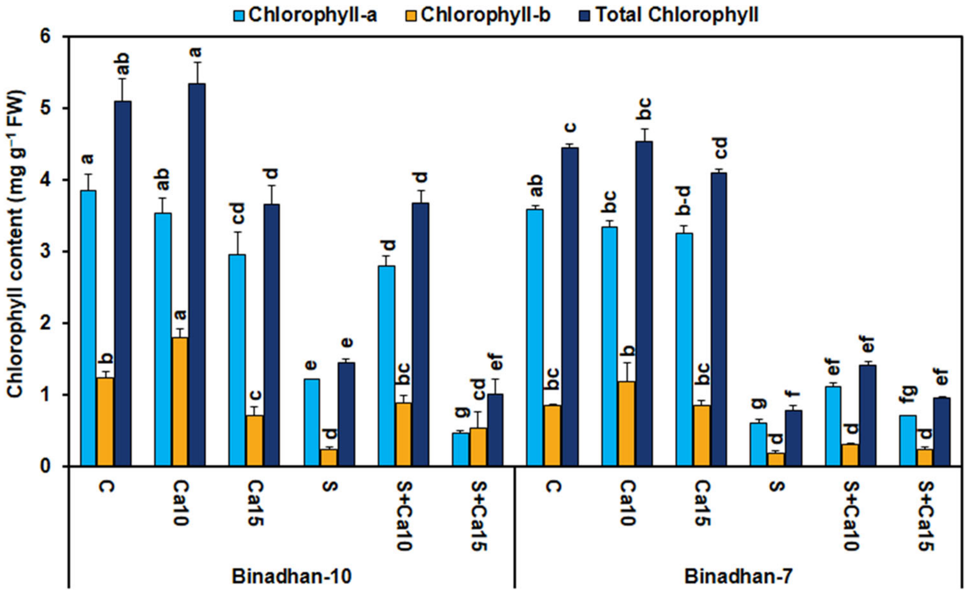

Fig. 1 Effects of exogenous calcium on chlorophyll content of two contrasting rice cultivars grown under 100 mM NaCl stress for 15 days at seedling stage. The vertical bar indicates means of three replicates (n = 3) and the error bar indicates standard errors. Statistically significant differences among the treatments and cultivars are indicated by different alphabetical letters at $P < 0.05$ according to Fisher's least significant difference test. FW, fresh weight; C, control (nutrient solution only); Ca10, Nutrient solution + 10 mM $CaCl_2$; Ca15, Nutrient solution + 15 mM $CaCl_2$; S, salt (100 mM NaCl); S + Ca10, 100 mM NaCl + 10 mM $CaCl_2$ and S + Ca15, 100 mM NaCl + 15 mM $CaCl_2$

susceptible *Binadhan-7* but the increment was intensified in salt-tolerant one (Fig. 2c).

Rice seedlings of *Binadhan-10* and *Binadhan-7* exposed to salt stress had no significant effect on APX activity compared with control seedlings. 10 mM and 15 mM $CaCl_2$ treatments on salt-stressed seedlings increased APX activity significantly in *Binadhan-10* but did not show an effect in *Binadhan-7*. Application of 10 mM and 15 mM $CaCl_2$ also in non-stressed seedlings enhanced APX activity significantly in *Binadhan-10*, but no change was found in *Binadhan-7* compared to control seedlings (Fig. 2d).

Discussion

Salinity is one of the most severe environmental stresses that causes a decline in growth rate, along with a suite of metabolic alterations in plants. Plants tolerance to salinity is a genetic trait that varies among species, as mirrored in their growth response under certain conditions (Goyal et al. 2018; Safdar et al. 2019). So a trivial accumulation of salt in the cell may badly affect the growth and development by ruining the usual physiological and biochemical processes (Parihar et al. 2015; Goyal et al. 2018; Yang and Guo 2018). In this context, plants have developed some rapid mechanisms to overcome salinity induced damage and acclimate themselves under saline condition (Tang et al. 2015). In addition to their escaping mechanisms, small biological molecules such as phytohormones and signalling molecules amend plants adaptability against adverse environment factors. Our research showed that exogenous application of Ca^{2+} amends growth and development under salt stress by regulating osmo-protection and ROS scavenging capability of two contrasting rice cultivars.

As a first indication, reduction of FGP and GRI is a common phenomenon under salt stress condition (Safdar et al. 2019). In the present study, we found the reduction of FGR and GRI both in *Binadhan-10* and *Binadhan-7* rice cultivars (Table 1) due to induced salinity which was consistent with the previous results (Shereen et al. 2011; Bahrani and Hagh Joo 2012; Vibhuti et al. 2015), which reported that rice lines showed more than 80% germination up to 75 mM NaCl at 72 h, while at higher salinity levels, these lines germinated after a delay of 3–6 days in 100 and 200 mM NaCl, respectively. Germination requires imbibition, hydration of protoplasm and restoration of enzymatic activity (Bewley 1997; Soundararajan et al. 2017). All these steps require sufficient supply of water. Salinity inhibits the normal water uptake (Coskun et al. 2016) that delays the imbibition and other physiological processes, entail less or delayed germination but exogenous application of calcium ameliorated the FGP and GRI in the present study (Table 1) which is consistent with the findings in *Phragmites karka* seeds (Zehra et al. 2012) and six forest tree seeds (Liu et al. 2011). The present study also revealed

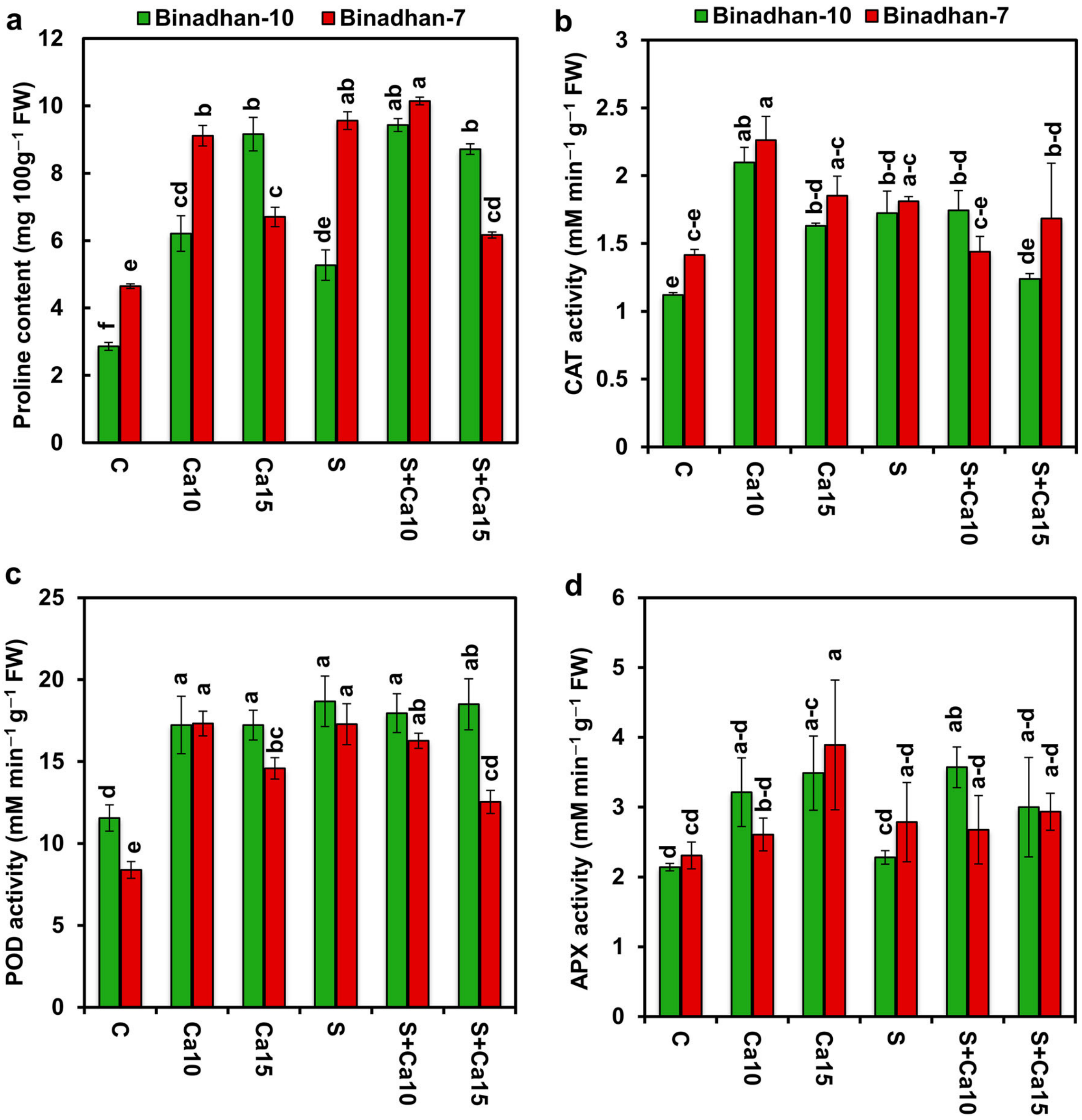

Fig. 2 Effect of exogenous calcium on **a** proline content, **b** catalase (CAT) activity, **c** peroxidase (POD) activity and **d** ascorbate peroxidase (APX) activity of two contrasting rice cultivars grown under 100 mM NaCl stress for 15 days. The vertical bar indicates means of three replicates (n = 3) and the error bar indicates standard errors. Statistically significant differences among the treatments and cultivars are indicated by different alphabetical letters at $P < 0.05$ according to Fisher's least significant difference test. FW, fresh weight; C, control (nutrient solution only); Ca10, Nutrient solution + 10 mM $CaCl_2$; Ca15, Nutrient solution + 15 mM $CaCl_2$; S, salt (100 mM NaCl); S + Ca10, 100 mM NaCl + 10 mM $CaCl_2$ and S + Ca15, 100 mM NaCl + 15 mM $CaCl_2$

that low concentration (10 mM $CaCl_2$) of exogenous calcium increased FGP and GRI over high concentration (15 mM $CaCl_2$), which is supported by previous reports in *Cichorium intybus* (Arshi et al. 2010), *Festuca ovina* (Salahshoor and Kazemi 2016) and *Melilotus officinalis* (Zhang et al. 2019).

Our present study showed the decline of RWC in induced saline condition (Table 2). It happened due to osmotic stress that makes the root rigid to uptake water (Goyal et al. 2018; Safdar et al. 2019). Exogenous supplementation of Ca^{2+} (10 mM $CaCl_2$) improved RWC in both rice cultivars whereas 15 mM $CaCl_2$ improved RWC only in salt-tolerant *Binadhan-10* under salt stress (Table 2). It might be due to, Ca^{2+} positively affecting stomatal functioning by keeping the guard cells turgid (Agurla et al. 2018) or by regulating stomatal conductance or by ensuring CO_2 availability (Sibole et al. 2003). Low RWC employed its adverse effect on all growth parameters measured in rice plants when exposed to 100 mM salt stress. In our study, we found salt stress significantly reduced the shoot and root length both at germination and seedling stage experiments (Tables 2, 3) which is consistent with the former findings on *Brassica campestris* (Keling and Zhujun 2010) and rice (Roy et al. 2016). Cells require turgor pressure for elongation (Guérin et al. 2016) but salinity abates the turgor pressure that might reduce the shoot and root length, thus leading to stunted growth. Exogenous application of Ca^{2+} ameliorated this detrimental effects of salinity, similar results were reported on *Calligonum mongolicum* (Xu et al. 2017), *Glycine max* (Yin et al. 2015) and rice (Tahjib-Ul-Arif et al. 2018c). Calcium helps in retaining RWC and turgor pressure and entail proper shoot and root growth (Tables 2, 3).

This mitigation of growth inhibition under NaCl-salt stress also might be due to the improved ion homeostasis with $CaCl_2$ supplementation. As a primary and an immediate response of plants exposed to salt stress, stimulation of K^+ leakage is occurred by Na^+ (Bose et al. 2014). In plant growth medium, excess Na^+ ion depolarizes root plasma membranes that activate guard cell outward rectifying potassium channels, decreasing cytosolic K^+ (Demidchik et al. 2002), displacing Ca^{2+} from membranes, and consequently disrupting the ion homeostasis (Wu and Wang 2012; Gu et al. 2016). This excess of cell Na^+ and shortage of Ca^{2+} and K^+ might also be cause of ROS overproduction (Demidchik and Maathuis 2007). However, it was reported that exogenously-applied Ca^{2+} promotes membrane stability, ameliorates salt toxicity by decreasing Na^+ content by reducing its uptake and transport and preventing its binding to cell wall, as well as increasing Ca^{2+} content in plants (Shabala and Pottosin 2014; Rahman et al. 2016). Therefore, treatment with Ca^{2+} might counter the adverse consequences by providing protection to the integrity and permeability of plasma membranes against Na^+ toxicity (Zehra et al. 2012).

Plant growth is intimately related with photosynthetic pigments and rate of photosynthesis (Tang et al. 2015). The photosynthetic pigments such as chlorophyll ‘a’, ‘b’ and total chlorophyll content were significantly decreased in salt-stressed rice seedlings (Fig. 2) and similar results were reported in rice (Zhen-hua et al. 2012) and *Pisum sativum* (Ozturk et al. 2012). Under higher salt concentrations, the accumulation of Na^+ and Cl^- ions increased which hinder the process of chlorophyll synthesis by influencing the activity of some chlorophyll synthesizing enzymes containing Fe^{3+} (Silva et al. 2011) or by increasing the activity of chlorophyll depredating enzyme, chlorophyllase and excess accumulation of ROS (Hasanuzzaman et al. 2014). Exogenous application of Ca^{2+} (10 mM $CaCl_2$) on salt-stressed rice cultivars increased the total chlorophyll content (Fig. 1). Previous study reported a similar trend of increment in total chlorophyll content by supplementary Ca^{2+} in *Trigonella foenum-graecum* (Oprica and Sandu 2014), *Festuca arundinacea* (Wang et al. 2017) and rice (Tahjib-Ul-Arif et al. 2018c). The addition of Ca^{2+} to the salt-stressed plants affects most of the physiological processes and our results showed that the exogenous addition of Ca^{2+} regulated the total content of photosynthetic pigments (Fig. 1). The increment of chlorophyll pigments under salt stress due to application of Ca^{2+} might be because of limited ROS production (Rahman et al. 2016).

Moreover, stunted shoot and root growth and physiological impairments (photosynthesis pigment degradation and impairment of electron flow) led to a reduction in shoot and root weight (biomass accumulation). In our study, we found a decreasing trend in fresh and dry weight of shoot and root under salt stress (Tables 2, 3). Salinity induces ABA-mediated stomatal closure that limits CO_2 fixation and disturbs the normal electron flow for carbon reduction in Calvin cycle which is associated with low fresh as well as dry matter production (Mbarki et al. 2018a, b; Acosta-Motos et al. 2017). These deleterious effects were quelled by exogenous application of calcium (Tables 2, 3). The effect of Ca^{2+} on increasing growth characters might be due to several reasons including that Ca^{2+} contributes to the composition of the cell wall and the cell membrane, therefore maintaining the balance and stability of membranes through the contacting different protein and lipids on the surface of the membrane (Davis et al. 2003). Calcium helps plants to withstand saline stress through several mechanisms including the stabilization of cell membranes and walls (Tuna et al. 2007) and organization of Na^+ and reduction of the toxic effects of NaCl through facilitating the transfer of Na^+/K^+ (Cuin et al. 2003). Furthermore, high rate of salinity causes an increase of Ca^{2+} in vacuole which is shifted from apoplast and the compartments within cells (Arshi et al. 2010). But it has been found that the lower concentration of Ca^{2+} improved the growth of rice plants and in the experiment, a lower concentration of Ca^{2+} performed better than higher concentration. Similar results were found in *Dolichos lablab* (D’Souza and Devaraj 2013) and *Atriplex halimus* (Soualem et al. 2014).

Production of osmolytes is a common phenomenon in stress condition to alleviate the physiological damage (Ghosh et al. 2016). In the present study, the proline content increased significantly both in salt-stress and combined treatment with salt and exogenous Ca^{2+} (Fig. 2a). Under salinity condition, the accumulation of proline showed increasing trend in rice (Polash et al. 2018), *Hordeum vulgare* (Reza et al. 2006), *Pisum sativum* (Ozturk et al. 2012) and *Trifolium alexandrinum* (Abdalla 2011). Proline may act as a non-toxic osmotic solute preferentially stabilizing the structure of macromolecules and organelles (Acosta-Motos et al. 2019). Increased proline in the stressed plants may be an adaptation to compensate the energy for growth and survival and thereby helps the plant to tolerate stress, as also observed in spinach leaves (Öztürk and Demir 2003) and in *Phyllanthus amarus* (Jaleel et al. 2008).

Salinity leads to other stresses. ROS production occurs when the plants are subjected to salt stress (Bose et al. 2014; Tang et al. 2015). In this circumstance, antioxidants are believed to play a very crucial role in the plant defence against ROS (Hanin et al. 2016). In the current study, we found increment in CAT activity with increasing the magnitude of NaCl in both tolerant and susceptible cultivars (Fig. 2b). Similar results are also found in rice (Nounjan et al. 2012) and *Vigna radiata* (Manivannan et al. 2007). CAT could convert hydrogen peroxide into oxygen and water to remove the peroxide in plants, and the higher action of CAT led to greater salt tolerance (Waszczak et al. 2018). The present study also revealed that the APX activity increased in all the rice cultivars with the increase of NaCl concentration (Fig. 2d) which was also supported by Weisany et al. (2012) on *Glycine max*, but also decrease in APX activity was also found in *Pisum sativum* (Ozturk et al. 2012). A major hydrogen peroxide detoxifying system in plant cells is the ascorbate–glutathione cycle, in which, APX enzymes play a key role catalyzing the conversion of H_2O_2 into H_2O, using ascorbate as a specific electron donor (Hossain and Fujita 2013). APX activity was higher in salt-tolerant cultivars than salt-sensitive (Yasar et al. 2008) which is consistent with our results (Fig. 2d). Salinity stress has a significant effect on the activity of POD, which was revealed in our present study (Fig. 2c). Almost all the plants showed a similar increasing response in POD activity under salinity stress, which was reported in *Glycine max* (Weisany et al. 2012) and *Solanum melongena* (Shaheen et al. 2013). It is believed that POD in cytosol and peroxisomes efficiently remove any H_2O_2 found outside the chloroplast (Asada 1992). Application of Ca^{2+} under control condition enhanced POD activity, which might be due to its dependency on Ca^{2+} ions and in absence of Ca^{2+}, POD is functionally inactive (Plieth and Vollbehr 2012). On the other hand, salt stress might have limited the Ca^{2+} uptake in combined treatment condition and somehow limited the further increment of POD activity.

Eventually, it can be concluded that Ca^{2+} participates in the regulatory mechanisms that activates the plants to adapt to adverse salt stress conditions by following reasons. First, exogenous Ca^{2+} improved the FGP and GRI as well as shoot length, root length, shoot weight, root weight, RWC and SES in both tolerant (*Binadhan-10*) and susceptible (*Binadhan-7*) rice plants. Second, Ca^{2+} augmented the photosynthetic capacity by restoring the photosynthetic pigments. Third, Ca^{2+} regulated the proline biosynthesis that compensates the energy for growth and survival and thereby helps the plant to tolerate stress. Fourth, Ca^{2+} reduced the oxidative damage through regulating the antioxidant defence and ROS detoxification system by boosting antioxidant enzymatic activity. Consequently, our outcomes offer a core foundation in salt stress mitigation by exogenous Ca^{2+} application and developing salt-tolerant rice genotypes by well tuning the level of endogenous Ca^{2+} through genetic manipulation of Ca^{2+} uptake mechanism. Finally, 10 mM $CaCl_2$ showed better performance than that of 15 mM $CaCl_2$ application to mitigate the adverse effect of salinity in rice.

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