

# Exogenous application of calcium and silica alleviates cadmium toxicity by suppressing oxidative damage in rice seedlings

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**Abstract** The present study was undertaken to examine the possible roles of calcium ( $\text{Ca}^{2+}$ ) and silica (Si) in protection against oxidative damage due to  $\text{Cd}^{2+}$  toxicity in rice (*Oryza sativa* L.) seedlings grown in hydroponics. Rice seedlings raised for 12 days in hydroponics containing  $\text{Cd}(\text{NO}_3)_2$  (75  $\mu\text{M}$ ) showed reduced growth; increase in the level of reactive oxygen species (ROS) ( $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$ ), thiobarbituric acid reactive substances (TBARSs) and protein carbonylation; and increase in the activity of antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX) compared to untreated controls. Exogenously added  $\text{Ca}^{2+}$  (2 mM) and Si (200  $\mu\text{M}$ ) significantly alleviated negative effect of  $\text{Cd}^{2+}$  by restoration of growth of the seedlings, suppression of  $\text{Cd}^{2+}$  uptake and restoration of root plasma membrane integrity. The levels of  $\text{O}_2^{\cdot-}$ ,  $\text{H}_2\text{O}_2$ , lipid peroxidation and protein carbonyls were much lower when  $\text{Ca}^{2+}$  and Si were added in the growth medium along with  $\text{Cd}^{2+}$  as compared to Cd-alone-treated seedlings.  $\text{Ca}^{2+}$  and Si lowered Cd-induced increase in SOD, GPX and APX activities while they elevated Cd-induced decline in CAT activity. Using histochemical staining of  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  in leaf tissues, it was further confirmed that added  $\text{Ca}^{2+}$  and Si suppressed Cd-induced accumulation of  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  in the leaves. The results suggest that exogenous application of  $\text{Ca}^{2+}$  and Si appears to be advantageous for rice plants in alleviating  $\text{Cd}^{2+}$  toxicity effects by reducing  $\text{Cd}^{2+}$  uptake, decreasing ROS production and suppressing oxidative damage. The observations indicate that  $\text{Ca}^{2+}$  and Si treatments can help in reducing  $\text{Cd}^{2+}$  toxicity in rice plants.

**Keywords** Cadmium toxicity · Calcium · Silica · Alleviation · Oxidative stress · Antioxidative defence

## Introduction

Cadmium ( $\text{Cd}^{2+}$ ) is a widespread long-range transported, potent heavy metal pollutant of the environment. It is toxic to all life forms, and at a very low concentration, it causes many morphological, physiological, biochemical and structural changes in growing plants ultimately leading to decline in productivity (Shah et al. 2001; Sharma and Dubey 2006). The sources primarily responsible for release and accumulation of  $\text{Cd}^{2+}$  in the environment include metallurgical industries, waste incinerators, urban traffic, cement factories, application of commercial fertilizers, etc. (Shah et al. 2001).  $\text{Cd}^{2+}$  is taken up by plants mainly by roots from the soil; however, some amount of  $\text{Cd}^{2+}$  also enters in the shoots via leaves (Gallego et al. 2012). In higher plants, uptake of  $\text{Cd}^{2+}$  mainly depends on its availability and concentration in the surrounding root zone (Clemens 2006). It is suggested that in a concentration more than 5–10  $\mu\text{g g}^{-1}$  leaf dry mass,  $\text{Cd}^{2+}$  is toxic to most of the plants (White and Brown 2010).

For its transport inside the plant body,  $\text{Cd}^{2+}$  uses the transporters of many essential elements, mainly the transporters involved in micronutrient uptake. These transporter proteins lack specificities for particular metal. Various metal transporters engaged in transport of calcium ( $\text{Ca}^{2+}$ ),  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  allow  $\text{Cd}^{2+}$  transport in the cells (Clemens 2006). High uptake of  $\text{Cd}^{2+}$  causes reduction in plant growth (Sandalio et al. 2009), decreased uptake of nutrients from the soil, decrease in photosynthesis, damage to root tips, inhibition of root metabolism, inhibition in activities of many enzymes and induction of oxidative stress in the tissues (Sandalio et al. 2001). Involvement of  $\text{Cd}^{2+}$  in increased production of reactive oxygen species (ROS) such as

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superoxide radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $\cdot OH$ ) and induction of oxidative stress has been documented in many crop species (Shah et al. 2001; Andresen and Küpper 2013).  $Cd^{2+}$  is a redox non-active metal and does not involve directly in cellular redox processes (Clemens 2006), but it has been shown that  $Cd^{2+}$  promotes ROS production that leads to oxidative damage to cellular biomolecules and causes impairment to redox homeostasis of the cell (Sandalo et al. 2001; Andresen and Küpper 2013).  $Cd^{2+}$  can indirectly activate NADPH oxidase present on membranes leading to enhanced production of  $O_2^{\cdot-}$  and  $H_2O_2$  (Romero-Puertas et al. 2004). Oxidative stress is regarded as important event in expression of  $Cd^{2+}$  toxicity in rice plants (Shah et al. 2001). To quench increasingly produced ROS under  $Cd^{2+}$  toxicity and other abiotic stresses and to prevent the cells against oxidative damage caused by ROS, plants possess antioxidative defence system comprising the non-enzymic components such as ascorbate (AsA), glutathione (GSH) and the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and the enzymes of Halliwell–Asada pathway such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Mittler et al. 2004; Moller et al. 2007; Gill and Tuteja 2010; Sharma et al. 2012; Andresen and Küpper 2013).

Attempts have been made by various groups of workers to alleviate  $Cd^{2+}$  toxicity effects in plants by application of mineral nutrients and compounds such as salicylic acid and nitric acid (Hayat et al. 2010; Xu et al. 2013).  $Ca^{2+}$  is an important micronutrient for plants and is actively involved in the regulation of plant cell metabolism and in signal transduction (Hall 2002).  $Ca^{2+}$  has high physical resemblance with  $Cd^{2+}$  including charge and radius, and at the same pH,  $Cd^{2+}$  competes with  $Ca^{2+}$  for its accumulation in the plants through root cells (White 2000). Many workers have reported reversing of  $Cd^{2+}$ -induced damage by exogenous application of  $Ca^{2+}$  in plant species such as *Arabidopsis thaliana*, *Sedum alfredii* and leguminous plants (Talukdar 2012). Tian et al. (2011) showed that exogenous  $Ca^{2+}$  protected *S. alfredii* plants from  $Cd^{2+}$ -induced damage by decreasing the accumulation of  $Cd^{2+}$  in the shoots. In *Lens culinaris* plants, exogenous  $Ca^{2+}$  alleviated the impact of  $Cd^{2+}$ -induced oxidative stress by modulating the activity of antioxidative enzymes (Talukdar 2012). It has been observed that exogenous application of moderate  $Ca^{2+}$  promotes membrane stability and re-stabilizes  $Ca^{2+}$ -mediated signalling inside the plants (Cramer 1985). Silica (Si) is the second most abundant element on earth surface and in the soil (Zeng et al. 2011). Though Si is a non-essential metal, it is considered beneficial for the growth of higher plants and it also protects plants against various abiotic stresses (Liang et al. 2005). Si has been shown to promote resistance in plants against the toxicity of the metals

$Cd^{2+}$  and  $Mn^{2+}$  (Liang et al. 2005; Shi et al. 2005a, b, 2010; Song et al. 2009). Rice seedlings grown in presence of  $Cd^{2+}$  + Si in nutrient medium showed significant increase in biomass in comparison with seedlings grown in presence of  $Cd^{2+}$  alone (Shi et al. 2005b).

As oxidative stress is an important component in the expression of  $Cd^{2+}$  toxicity in rice plants (Shah et al. 2001), in order to explore the possibilities to minimize the toxic effects of  $Cd^{2+}$  in growing rice plants by using minerals and other chemical agents such as  $Ca^{2+}$  and  $Si^{2+}$ , the present study was undertaken to examine the effects of toxic level of  $Cd^{2+}$  on growth, production of ROS, induction of oxidative stress and status of antioxidative defence components in growing indica rice seedlings and to explore the possible role of exogenously applied  $Ca^{2+}$  and Si in alleviation of  $Cd^{2+}$  toxicity-induced oxidative stress effects in the seedlings.

## Materials and methods

### Plant material and metal treatments

Seeds of rice (*Oryza sativa* L.) cv. Malviya-36, commonly grown in India and sensitive to  $Cd^{2+}$ , were used. Sensitivity of this cultivar towards  $Cd^{2+}$  was evaluated on the basis of root/shoot growth inhibition and other morphological parameters (data not reported here). Seeds were surface sterilized with 0.1 % sodium hypochlorite solution for 10 min, rinsed with glass-distilled water and imbibed for 24 h in water. Seedlings were then raised in plastic pots in hydroponics containing either Yoshida nutrient solution (Yoshida et al. 1976) which served as control or nutrient solutions supplemented with 75  $\mu M$   $Cd(NO_3)_2$ , 75  $\mu M$   $Cd(NO_3)_2$  + 2 mM  $CaCl_2$  and 75  $\mu M$   $Cd(NO_3)_2$  + 200  $\mu M$   $K_2SiO_3$  which served as treatment solutions. Treatment concentration of  $Cd^{2+}$  was standardized following similar studies conducted by us earlier (Shah et al. 2001). Pots were kept for the growth of seedlings for 12 days in a green house at  $28 \pm 1$  °C under 80 % relative humidity and 12-h light/dark cycle with 190–200  $\mu mol\ m^{-2}\ s^{-1}$  irradiance as described earlier (Shah et al. 2001). Seedlings were uprooted at 12 days of growth, and all the measurements, physiological analyses and biochemical determinations were done in triplicate.

### Rice seedling vigour, relative water content and metal uptake

At 12-day growth of seedlings, the lengths of roots and shoots as well as fresh biomass were determined. Root/shoot samples were dried in an oven at 70 °C for 3 days, and dry mass was determined on the basis of ten random samplings in triplicate. Relative water content (RWC) was determined in roots and shoots of control and metal-treated seedlings according to

Weatherley (1950). RWC was calculated using the formula:  $RWC = (FW - DW) / (TW - DW) \times 100$ , where FW = fresh weight, DW = dry weight and TW = turgid weight. Concentration of absorbed  $Cd^{2+}$  in roots/shoots was determined using inductively coupled plasma–optical emission spectrometer (ICP-OES, Optima 7000 DV, PerkinElmer) following the protocol of Allen et al. (1986). Plant samples were washed thoroughly with ion-free water (Milli-Q) and oven dried at 70 °C for 3 days. Dried samples weighing 150 mg were used for quantification of intracellular  $Cd^{2+}$ . Samples were ground to fine powder and digested in diacid mixture (nitric acid/perchloric acid). Digested samples were diluted to 25 ml with ion-free water and filtered with 0.45- $\mu$ M membrane filter. Filtrates were then subjected to analysis of  $Cd^{2+}$  using ICP-OES, and the data obtained were compared with standards taken from PerkinElmer (USA).

#### Measurement of the level of ROS, lipid peroxidation and membrane damage

The rate of  $O_2^{\cdot -}$  production was measured by observing the oxidation of epinephrine to adrenochrome following the method of Mishra and Fridovich (1972). Results were expressed as change in absorbance at 480 nm  $min^{-1} g^{-1}$  tissue fresh weight. The level of  $H_2O_2$  was measured in the tissues as described by Jana and Choudhuri (1981). Fresh root/shoot samples weighing 150 mg were homogenized in 3 ml of 50 mM sodium phosphate buffer (pH 6.5). After centrifugation at 10,000g for 10 min, the supernatant was used to determine  $H_2O_2$  content by reaction with titanium sulphate. The intensity of yellow colour developed was recorded at 410 nm, and the amount of  $H_2O_2$  was calculated using extinction coefficient 0.28  $\mu M^{-1} cm^{-1}$  and expressed as nmol  $g^{-1}$  tissue fresh weight. The level of lipid peroxidation products was measured in terms of thiobarbituric acid reactive substances (TBARSs) according to the method of Heath and Packer (1968). The concentrations of lipid peroxides were expressed as nmol TBARS  $g^{-1}$  fresh weight of the tissues using an extinction coefficient of 155  $mM^{-1} cm^{-1}$ . The extent of plasma membrane injury due to  $Cd^{2+}$  treatment was determined in roots of the plants by a spectrophotometric assay of Evans blue stain retained by cells as described by Schützendübel et al. (2001). For this, roots from  $Cd^{2+}$ -,  $Cd^{2+} + Ca^{2+}$ - and  $Cd^{2+} + Si$ -treated seedlings were stained with 0.025 % (w/v) Evans blue solution and washed with 100  $\mu$ M  $CaCl_2$ . The stained portions from the root tips were removed with a razor blade. Ten root pieces from the identical positions were placed together, and the trapped Evans blue was released by homogenizing the root portions in 1 ml of 1 % (w/v) aqueous sodium dodecyl sulphate (SDS). The homogenate was centrifuged at 13,500 $\times$ g for 10 min. The optical density of supernatant was measured spectrophotometrically at 600 nm.

#### Histochemical localization of ROS, lipid peroxidation and membrane damage

Localization of  $O_2^{\cdot -}$  in situ was detected in the leaves of the control and treated seedlings using the dye nitroblue tetrazolium (NBT) as substrate following the method of Frahy and Schopfer (2001). Leaf discs (3–5 mm) were immersed in 6 mM NBT (prepared in 10 mM Na-citrate buffer, pH 6.0) at 25 °C for 8 h under light. The leaves were then immersed in boiling ethanol (95 %) for 10 min to decolourize the leaves except the dark blue insoluble formazan deposits produced by the reaction of NBT with  $O_2^{\cdot -}$ . After cooling, leaf discs were photographed. Histochemical detection of  $H_2O_2$  in situ was done according to Thordal-Christensen et al. (1997). Leaf discs, 3–5 mm, were incubated in a solution containing 1 mg  $ml^{-1}$  3,3'-diaminobenzidine (DAB) prepared in HCl-acidified (pH 3.8) water. After 8 h of incubation at 25 °C, leaf discs were kept in boiling 95 % ethanol for 10 min and were then cleared in saturated chloral hydrate. Leaf discs were then examined under a light microscope. Reddish brown-coloured spots appeared to be characteristic of the reaction of DAB with  $H_2O_2$ . Histochemical detection of lipid peroxides was done in roots as described by Pompella et al. (1987). Excised root tips (5–10 mm) were stained with Schiff's reagent for 20 min, which detects aldehydes that originate from lipid peroxidation. Stained root tips were rinsed with sulphite solution (0.5 % [w/v]  $K_2S_2O_5$  in 0.05 M HCl) and then placed in this solution for 10 min to retain the staining colour. The loss of plasma membrane integrity was evaluated in root tips of the seedlings microscopically as described by Schützendübel et al. (2001) by examining the Evans blue stain retained by cells. Roots from control and  $Cd^{2+}$ -,  $Cd^{2+} + Ca^{2+}$ - and  $Cd^{2+} + Si^{2+}$ -treated intact seedlings were stained with 0.25 % (w/v) aqueous Evans blue solution, for 30 min, and after rinsing with water, root tips were visualized under light microscope.

#### Determination of protein thiol, protein carbonylation and proteolytic activity

To assess the extent of protein oxidation, protein thiol content was determined in roots and shoots. Fresh samples weighing 150 mg were homogenized in 0.15 % (w/v) sodium ascorbate solution, and the homogenate was centrifuged at 22,000 $\times$ g for 15 min at 4 °C. Total thiol and non-protein thiol contents were determined in the supernatant using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) following the method of de Kok and Kuiper (1986). The content of protein thiol was calculated by subtracting the content of non-protein thiol from total thiol and expressed as nmol  $g^{-1}$  fresh weight of tissues. The content of protein-bound carbonyls was determined following the method of Levine et al. (1994). Root and shoot samples weighing 1 g were homogenized in 2.0 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA, 2 mM

dithiothreitol, 0.2 % (v/v) Triton X-100 and 1 mM phenylmethane sulphonyl fluoride (PMSF). After centrifugation at 25,000×g for 30 min at 4 °C, supernatants containing 500 µg protein were mixed with 1 % (w/v) streptomycin sulphate for 20 min to remove the nucleic acids. After centrifugation at 2000×g, supernatants in 200-µl volumes were mixed with 300 µl of 10 mM 2,4-dinitrophenyl hydrazine (DNPH) (freshly prepared) in 2 M HCl. Individual blank samples were incubated in 2 M HCl. After 1-h incubation at 25 °C, proteins were precipitated with pre-cooled 10 % (w/v) trichloroacetic acid (TCA) and the pellets were washed three times with 500 µl of ethanol/ethyl acetate (1:1). The pellets were then dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer (pH 2.3), and the absorbance was measured at 370 nm using UV-vis spectrophotometer (PerkinElmer, LAMBDA EZ 201, USA). Carbonyl content was calculated using a molar absorption coefficient for aliphatic hydrazones as 22,000 M<sup>-1</sup> cm<sup>-1</sup> and expressed in terms of nmol carbonyl mg<sup>-1</sup> protein. Proteolytic activity was measured in enzyme extracts prepared from root and shoot samples as described by Polge et al. (2009). Tissues weighing 300 mg were homogenized using chilled mortar and pestle in 2 ml of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 15,000×g for 15 min at 4 °C, and supernatants were dialyzed in cellophane membrane. Proteolytic activity was assayed in dialyzed enzyme (0.25 ml) by reaction with 0.25 ml of 2 % azocasein prepared in 0.1 M Tris-HCl (pH 8.4). After 6 h of incubation at 28 °C, the reaction was stopped by adding 1.2 ml of 10 % TCA. After placing on ice for 10 min, the contents were centrifuged at 3000×g for 20 min. Then, 1.0 ml of the resulting supernatant was mixed with 1.0 ml of 1.0 M NaOH. After 30 min, absorbance was recorded at 440 nm using spectrophotometer. The extinction coefficient of azocasein 1 % in 1 M NaOH was taken as 37 L cm<sup>-1</sup> g<sup>-1</sup>. One unit of proteolytic activity is expressed as mg of azocasein degraded min<sup>-1</sup> mg<sup>-1</sup> protein.

#### Determination of ascorbate and glutathione pool

The contents of reduced AsA, oxidized ascorbate (dehydroascorbate (DHA)) and total ascorbate (AsA + DHA) were determined following the method of Law et al. (1983). Fresh root/shoot samples weighing 200 mg were homogenized using a chilled mortar and pestle in 5 ml of 5 % (w/v) m-phosphoric acid and centrifuged at 22,000×g for 10 min at 4 °C. For estimation of total ascorbate, 100 µl of the supernatant was incubated with 110 mM KH<sub>2</sub>PO<sub>4</sub>, 3.6 mM EDTA and 1.5 mM dithiothreitol (DTT) in a total volume of 700 µl to reduce all DHA to AsA. One hundred microlitres of 0.5 % *N*-ethylmaleimide (NEM) was added to remove excess DTT. AsA was analyzed in a similar manner except that 200 µl of deionized water was substituted for DTT and NEM. After the addition of 400 µl 10 % (w/v) TCA, 400 µl

44 % *O*-phosphoric acid, 400 µl 65 mM α,α'-bipyridyl in 70 % ethanol and 200 µl 100 mM FeCl<sub>3</sub> were added. After incubation at 40 °C for 1 h in a shaking water bath, colour was developed. Absorbance was measured at 525 nm. Standards for AsA and DHA were prepared between 0 and 5 mM in 5 % (w/v) m-phosphoric acid. For each sample, DHA was estimated from the difference between total ascorbate and AsA. Total glutathione (GSH + GSSG), oxidized glutathione (GSSG) and reduced glutathione (GSH) contents were determined following the method of Griffith (1980). Samples weighing 200 mg were homogenized in cold in 5 % (w/v) sulphosalicylic acid and centrifuged at 22,000×g for 10 min at 4 °C. Two solutions, namely, solution A (pH 7.2) consisting of 100 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 40 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 15 mM EDTA, 1.8 mM DTNB and 0.04 % bovine serum albumin (BSA, Sigma) and solution B (pH 7.2) consisting of 1 mM EDTA, 50 mM imidazole, 0.2 % BSA and 2 U ml<sup>-1</sup> GR, were prepared. Total glutathione was measured in a reaction mixture consisting of 400 µl of solution A, 320 µl of solution B, 400 µl of a 1:5 dilution of supernatant in 0.5 M KH<sub>2</sub>PO<sub>4</sub> (pH 7.0) and 80 µl of 3.0 mM NADPH. The reaction rate was measured following change in absorbance at 412 nm for 5 min. To estimate GSSG, a similar method was followed except that 1 ml of 1:2 diluted supernatant in 0.5 M KH<sub>2</sub>PO<sub>4</sub> (pH 6.0) was prior incubated with 20 µl of 2-vinylpyridine at 25 °C for 1 h to derivatize GSH. GSH and GSSG standards were between 0 and 18 µM in 5 % (w/v) sulphosalicylic acid diluted appropriately with 0.5 M KH<sub>2</sub>PO<sub>4</sub> (pH 7.0). For each sample, GSH was estimated from the difference between total glutathione and GSSG.

#### Assays of antioxidative enzymes

The activity of SOD was determined using the method of Beauchamp and Fridovich (1971) based on the inhibition of p-nitro blue tetrazolium chloride (NBT) reduction by O<sub>2</sub><sup>-</sup> under light. Excised leaves weighing 200 mg were homogenized using a chilled mortar and pestle in 5 ml of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1 % (v/v) Triton X-100 and 2 % (w/v) polyvinyl pyrrolidone (PVP). After centrifugation at 22,000×g for 10 min at 4 °C, supernatants were dialyzed in cellophane membrane tubing for 6 h against the extraction buffer in cold with three to four changes of the buffer. In the supernatant, total SOD activity was determined. One unit of SOD activity is expressed as the amount of enzyme required to cause 50 % inhibition of the rate of NBT reduction measured at 560 nm. CAT activity was assayed according to Beers and Sizer (1952) by measuring the decomposition of H<sub>2</sub>O<sub>2</sub> at 240 nm (extinction coefficient of 0.036 mM<sup>-1</sup> cm<sup>-1</sup>) by observing the decrease in absorbance using a UV-vis spectrophotometer (PerkinElmer, LAMBDA EZ 201, CA, USA). The activity of GPX was assayed in enzyme extracts according to Eglely et al. (1983). Fresh tissue (±200 mg) was homogenized in 3 ml of chilled 50 mM Na-



phosphate buffer (pH 7.0). After centrifugation and dialysis, enzyme activity was assayed by measuring increase in absorbance due to formation of tetraguaiacohinone at 420 nm (extinction coefficient of  $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ) at 30-s intervals up to 2 min using spectrophotometer (Bausch and Lomb, Spectronic 20, USA). Enzyme specific activity is expressed as  $\mu\text{mol H}_2\text{O}_2$  reduced  $\text{mg}^{-1} \text{ protein min}^{-1}$ . The activity of APX was assayed according to Nakano and Asada (1981).  $\text{H}_2\text{O}_2$ -dependent oxidation of AsA was followed by measuring the decrease in absorbance at 290 nm (extinction coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Enzyme specific activity is expressed as  $\mu\text{mol AsA oxidized mg}^{-1} \text{ protein min}^{-1}$ . MDHAR activity was assayed following the method of Hossain et al. (1984). Samples weighing 200 mg were homogenized using a chilled mortar and pestle in 3 ml of extraction medium containing 100 mM K-phosphate buffer (pH 7.5), 1 mM EDTA, 2 % (w/v) PVP and 1 mM AsA (added prior to use). Homogenate was centrifuged at  $22,000 \times g$  for 10 min at  $4^\circ\text{C}$  and dialyzed. The assay mixture in 1-ml reaction medium contained 90 mM potassium phosphate buffer (pH 7.5), 0.01 mM EDTA, 0.0125 % Triton X-100, 0.25 U ascorbate oxidase (units as described by Sigma Chemicals Co., USA), 0.2 mM NADH and enzyme extract. The reaction was followed by measuring the decrease in absorbance at 340 nm due to NADH oxidation. Enzyme specific activity is expressed as nmol NADH oxidized  $\text{mg}^{-1} \text{ protein min}^{-1}$ . The activity of DHAR was assayed by measuring the reduction of DHA in the presence of GSH at 265 nm according to Hossain et al. (1984) using a UV–vis spectrophotometer after accounting for the non-enzymic reduction of DHA by GSH. Enzyme specific activity is expressed as nmol DHA reduced  $\text{mg}^{-1} \text{ protein min}^{-1}$ . GR activity was assayed using the method of Schaedle and Bassham (1977) following the oxidation of NADPH at 340 nm. Enzyme specific activity is expressed as nmol NADPH oxidized  $\text{mg}^{-1} \text{ protein min}^{-1}$ .

#### Protein estimation

In all preparations, protein content was estimated according to the method of Bradford (1976) using bovine serum albumin (BSA, Sigma) as standard.

#### Statistical analysis

All experiments were performed in triplicate. Values are mean  $\pm$ SD based on three independent determinations, and bars indicate standard deviations. Differences among control and treatments were analyzed by one-way ANOVA multiple comparison analysis using GraphPad Prism. Asterisks (\*), (\*\*), and (\*\*\*) indicate values that differ significantly from controls, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, whereas (#), (##) and (###) indicate values that differ significantly from  $\text{Cd}^{2+}$  treatment to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}^{2+}$  treatments, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively.

## Results

### Effect of exogenous $\text{Ca}^{2+}$ and Si on growth of $\text{Cd}^{2+}$ -treated rice seedlings

When growth of rice seedlings was recorded for 12 days, it was observed that  $75 \mu\text{M Cd}(\text{NO}_3)_2$  in the growth medium significantly inhibited length and fresh weights of roots and shoots (Table 1). Rice seedlings grown for 12 days in presence of  $75 \mu\text{M Cd}(\text{NO}_3)_2$  showed 40 % ( $p \leq 0.01$ ) reduction in length of roots and 56 % ( $p \leq 0.001$ ) reduction in shoots compared to the control-grown seedlings. Presence of  $\text{Ca}^{2+}$  and Si resulted in significant alleviation of  $\text{Cd}^{2+}$ -induced retardation in the growth of the seedlings. Presence of  $\text{Ca}^{2+}$  resulted in 29 % ( $p \leq 0.01$ ) increase in root length and 40 % ( $p \leq 0.01$ ) increase in shoot length compared to  $\text{Cd}^{2+}$ -treated seedlings, whereas presence of Si led to 17 % increase in length of roots and 30 % ( $p \leq 0.05$ ) increase in the length shoots compared to  $\text{Cd}^{2+}$ -treated seedlings. Similar to the decline in length,  $\text{Cd}^{2+}$  treatment for 12 days caused 41 % ( $p \leq 0.01$ ) decline in fresh weight of roots and 42 % ( $p \leq 0.01$ ) decline in fresh weight of shoots in the seedlings (Table 1). Exogenous  $\text{Ca}^{2+}$  treatment resulted in 60 % ( $p \leq 0.01$ ) increase in fresh weight of roots and 36 % ( $p \leq 0.05$ ) increase in fresh weight of shoots compared to  $\text{Cd}^{2+}$ -treated seedlings. Similarly,  $200\text{-}\mu\text{M Si}$  treatment along with  $\text{Cd}^{2+}$  resulted in 45 % ( $p \leq 0.01$ ) increase in fresh weight of roots and 25 % ( $p \leq 0.05$ ) increase in fresh weight of shoots compared to  $\text{Cd}^{2+}$ -alone-treated seedlings. With Cd treatment, an insignificant decline in RWC of roots and shoots was observed. Treatments with  $\text{Ca}^{2+}$  and Si along with  $\text{Cd}^{2+}$  resulted in higher level of RWC in the seedlings compared to Cd-treated seedlings (Table 1).

**Table 1** Effect of  $\text{Cd}^{2+}$  ( $75 \mu\text{M}$ ),  $\text{Cd}^{2+} + \text{Ca}^{2+}$  ( $2 \text{ mM}$ ) and  $\text{Cd}^{2+} + \text{Si}$  ( $200 \mu\text{M}$ ) in the growth medium on (A) length, (B) fresh weight and (C) relative water content of roots and shoots of rice seedlings at 12 days of growth

Samples	Treatments	Length (cm plant <sup>-1</sup> )	Fresh weight (g plant <sup>-1</sup> )	Relative water content (%)
Root	Control	7.1 $\pm$ 0.6	0.091 $\pm$ 0.010	97.72 $\pm$ 9.94
	$\text{Cd}^{2+}$	3.0 $\pm$ 0.4**	0.053 $\pm$ 0.004**	83.91 $\pm$ 7.55
	$\text{Cd}^{2+} + \text{Ca}^{2+}$	6.1 $\pm$ 0.5	0.086 $\pm$ 0.007###	91.72 $\pm$ 9.62
	$\text{Cd}^{2+} + \text{Si}$	5.2 $\pm$ 0.5*	0.077 $\pm$ 0.08#	89.28 $\pm$ 9.24
Shoot	Control	16.5 $\pm$ 1.5	0.342 $\pm$ 0.041	93.29 $\pm$ 9.65
	$\text{Cd}^{2+}$	7.3 $\pm$ 0.6***	0.197 $\pm$ 0.016**	79.84 $\pm$ 8.58
	$\text{Cd}^{2+} + \text{Ca}^{2+}$	12.3 $\pm$ 1.1***	0.268 $\pm$ 0.024###	88.72 $\pm$ 9.25
	$\text{Cd}^{2+} + \text{Si}$	10.5 $\pm$ 1.0***	0.238 $\pm$ 0.021#	86.91 $\pm$ 8.25

Control represents untreated seedlings. Values are mean $\pm$ SD based on three independent determinations, and bars indicate standard deviations. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , values that differ significantly from control; # $p \leq 0.05$ , ## $p \leq 0.01$ , values significantly differing from  $\text{Cd}^{2+}$  to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , according to one-way ANOVA multiple comparison analysis using GraphPad Prism

### Effect of Ca<sup>2+</sup> and Si treatments on Cd<sup>2+</sup> content in the tissues

Treatment of rice seedlings with Cd(NO<sub>3</sub>)<sub>2</sub> resulted in uptake of Cd<sup>2+</sup> in roots as well as shoots (Table 2). ICP-OES analysis of Cd<sup>2+</sup> revealed that the concentration of absorbed Cd<sup>2+</sup> was greater in roots than in shoots. Rice seedlings exposed to 75 μM Cd(NO<sub>3</sub>)<sub>2</sub> for 12 days showed 1.421 μmol Cd<sup>2+</sup> g<sup>-1</sup> dry wt in roots and 0.898 μmol Cd<sup>2+</sup> g<sup>-1</sup> dry wt in shoots. We also analyzed the seedlings which were co-treated with Ca<sup>2+</sup> or Si along with Cd<sup>2+</sup> for the same timescale. Results indicated that Ca<sup>2+</sup> and Si treatments reduced Cd<sup>2+</sup> accumulation in the roots and shoots. Ca<sup>2+</sup> treatment resulted in lesser Cd<sup>2+</sup> accumulation in the seedlings compared to Si. Ca<sup>2+</sup> treatment resulted into decreased accumulation of Cd in roots and shoots of the seedlings. Seedlings treated with 75 μM Cd(NO<sub>3</sub>)<sub>2</sub> + 2 mM CaCl<sub>2</sub> for 12 days showed 0.831 μmol Cd<sup>2+</sup> g<sup>-1</sup> dry wt in roots and 0.507 μmol Cd<sup>2+</sup> g<sup>-1</sup> dry wt in shoots. This showed that exogenous Ca<sup>2+</sup> treatment of seedlings along with Cd<sup>2+</sup> resulted in 41–43 % decreased uptake of Cd<sup>2+</sup>, compared to Cd<sup>2+</sup>-alone-treated seedlings. Similar to Ca<sup>2+</sup>, treatment of seedlings with Si along with Cd<sup>2+</sup> also resulted in decreased uptake of Cd<sup>2+</sup> by the seedlings. With 200-μM Si treatment, uptake of Cd<sup>2+</sup> was reduced by 20 % in roots and by 25 % in shoots compared to Cd<sup>2+</sup>-alone-treated seedlings.

### Effect of Ca<sup>2+</sup> and Si on Cd-induced ROS generation, lipid peroxidation and membrane damage

Cd<sup>2+</sup> treatment for 12 days caused 73 % ( $p \leq 0.001$ ) increase in O<sub>2</sub><sup>-</sup> level in roots and 68 % ( $p \leq 0.001$ ) increase in shoots of the seedlings (Fig. 1a). Presence of Ca<sup>2+</sup> and Si in the growth

**Table 2** Cadmium content in roots and shoots of rice seedlings grown for 12 days in Yoshida nutrient medium containing 75 μM Cd(NO<sub>3</sub>)<sub>2</sub>, 75 μM Cd(NO<sub>3</sub>)<sub>2</sub> + 2 mM CaCl<sub>2</sub> and 75 μM Cd(NO<sub>3</sub>)<sub>2</sub> + 200 μM K<sub>2</sub>SiO<sub>3</sub>

Samples	Treatments	Cd content (μmol g <sup>-1</sup> dry wt)	Evans blue (fold uptake in root)
Root	Control	–	1.00±0.00
	Cd <sup>2+</sup>	1.421±1.249	5.3218±0.5791***
	Cd <sup>2+</sup> + Ca <sup>2+</sup>	0.831±0.0824###	2.8172±0.3007****
	Cd <sup>2+</sup> + Si <sup>2</sup>	0.126±1.106	3.0815±0.2958****
Shoot	Control	–	–
	Cd <sup>2+</sup>	0.898±0.0818	–
	Cd <sup>2+</sup> + Ca <sup>2</sup>	0.507±0.0698###	–
	Cd <sup>2+</sup> + Si	0.672±0.0597	–

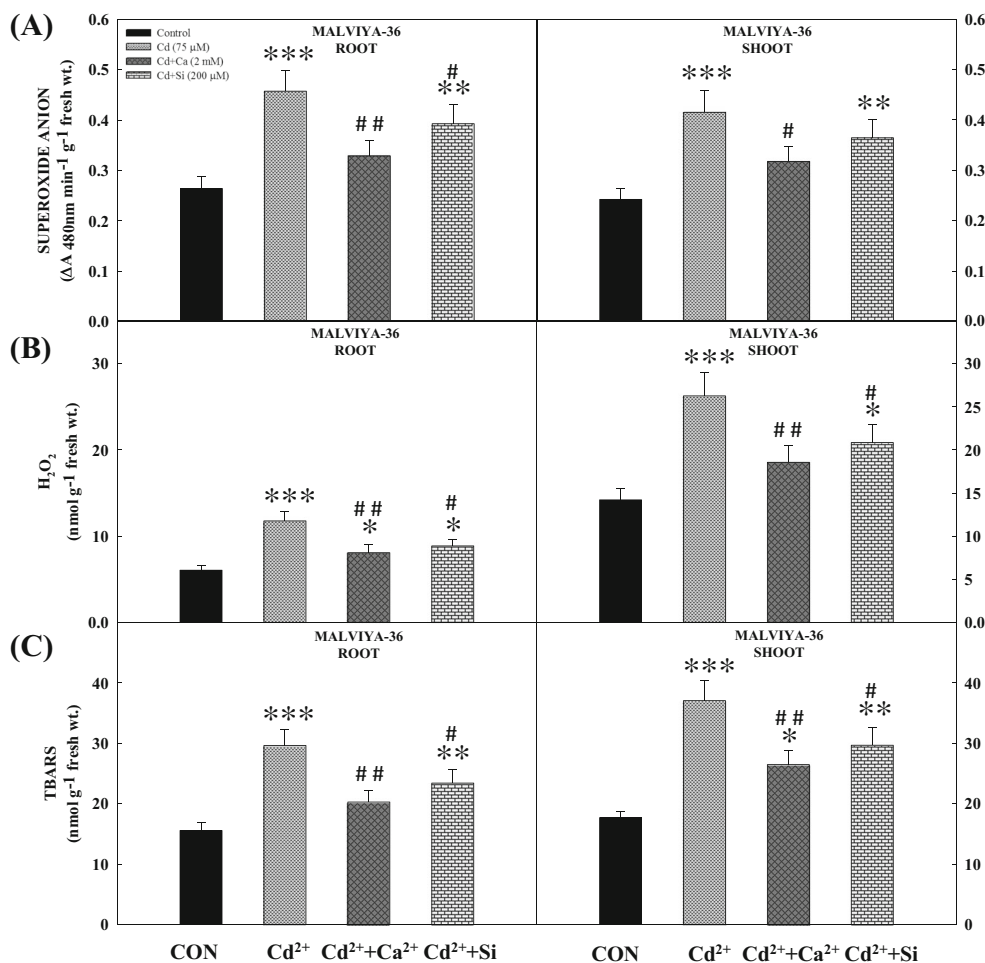
Control represents nutrient medium without metal treatment. Values are mean±SD based on three independent determinations, and bars indicate standard deviations

\*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , values that differ significantly from control; ### $p \leq 0.01$ , values significantly differing from Cd<sup>2+</sup> to Cd<sup>2+</sup> + Ca<sup>2+</sup> and Cd<sup>2+</sup> + Si, according to one-way ANOVA multiple comparison analysis using GraphPad Prism

medium suppressed Cd-induced O<sub>2</sub><sup>-</sup> generation in both roots and shoots. Similarly, Cd<sup>2+</sup> treatment caused increased production of H<sub>2</sub>O<sub>2</sub> in rice seedlings compared to controls (Fig. 1b). With Cd<sup>2+</sup> treatment of the seedlings for 12 days, H<sub>2</sub>O<sub>2</sub> level increased by 94 % ( $p \leq 0.001$ ) in roots and 83 % ( $p \leq 0.001$ ) in shoots. A decline in Cd-induced H<sub>2</sub>O<sub>2</sub> production was observed in roots and shoots when Ca<sup>2+</sup> or Si was present in the medium. In Cd<sup>2+</sup>-treated seedlings, increased ROS content in the cells resulted in oxidative damage of lipids leading to increased level of lipid peroxides, expressed in terms of TBARS content. Lipid peroxidation is a direct measure of oxidative stress in the tissues, and after 12 days of Cd<sup>2+</sup> treatment, we observed 90 % ( $p \leq 0.001$ ) increase in TBARS content in roots and 109 % ( $p \leq 0.001$ ) increased content in shoots compared to controls (Fig. 1c). In the present investigation, we observed that exogenous application of Ca<sup>2+</sup> and Si ameliorated Cd-induced oxidative damage in rice seedlings by reducing the level of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and lipid peroxides. Ca<sup>2+</sup> appeared to be a better ameliorator than Si in protecting the cells against oxidative damage. Uptake of the dye Evans blue has been widely used as an indicator of loss of plasma membrane integrity as well as an indicator of cell death. In our experiments, nearly 5.32-fold ( $p \leq 0.001$ ) higher uptake of dye was observed in the root tips of Cd<sup>2+</sup>-treated seedlings compared to controls (Table 2). This indicated a significant loss of root plasma membrane integrity due to Cd<sup>2+</sup> treatment. Presence of Ca<sup>2+</sup> or Si in the treatment medium resulted in considerable protection of membrane integrity marked by less uptake of dye in the roots, compared to the uptake of dye in Cd-treated roots. Treatment with Ca<sup>2+</sup> and Si caused 47 % ( $p \leq 0.01$ ) and 42 % ( $p \leq 0.01$ ), respectively, less absorption of Evans blue through root membrane compared to Cd<sup>2+</sup>-treated seedlings, showing that the presence of Ca<sup>2+</sup> and Si protected integrity of cell membrane against Cd<sup>2+</sup>-induced damage.

### Localization of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> in leaves of seedlings

To examine histochemical localization of O<sub>2</sub><sup>-</sup> in situ in the cells, the dye NBT was used. This dye reacts with O<sub>2</sub><sup>-</sup> and forms blue-coloured formazan spots in the cells. More intense dark-blue-coloured patches were observed in Cd<sup>2+</sup>-treated rice leaves compared to untreated controls, suggesting that Cd<sup>2+</sup> treatment of seedlings enhanced O<sub>2</sub><sup>-</sup> generation in the leaves (Fig. 2a). Intensity and number of formazan spots decreased in the leaves of the seedlings grown in presence of Cd<sup>2+</sup> + Ca<sup>2+</sup> or Cd<sup>2+</sup> + Si in comparison with Cd<sup>2+</sup>-treated seedlings. This suggests that treatment with Ca<sup>2+</sup> and Si suppressed Cd-induced O<sub>2</sub><sup>-</sup> generation in rice leaves. The generation of H<sub>2</sub>O<sub>2</sub> was histochemically detected in the leaves of seedlings using DAB, which reacts with H<sub>2</sub>O<sub>2</sub>, yielding dark brown precipitates. We observed very few spots with very less intensity on leaf surface of control grown seedlings, whereas in leaves of Cd<sup>2+</sup>-treated seedlings, more intensely stained spots were seen, showing greater accumulation of H<sub>2</sub>O<sub>2</sub> due to Cd<sup>2+</sup> treatment



**Fig. 1** Effect of  $\text{Cd}^{2+}$ ,  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  in the growth medium on contents of **a** superoxide anion, **b** hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and **c** thiobarbituric acid reactive substances (TBARS, a measure of lipid peroxidation) in roots and shoots of rice seedlings at 12 days of growth. Values are mean  $\pm$  SD based on three independent determinations, and bars indicate standard deviations. Asterisks (\*), (\*\*) and (\*\*\*) indicate

values that differ significantly from control, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, whereas number signs (#) and (##) indicate values significantly differing from  $\text{Cd}^{2+}$  to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively, according to one-way ANOVA multiple comparison analysis using GraphPad Prism

(Fig. 2b). Intensity and number of formazan spots decreased in the leaves of the seedlings grown under  $\text{Cd}^{2+} + \text{Ca}^{2+}$  or  $\text{Cd}^{2+} + \text{Si}^{2+}$  in comparison with  $\text{Cd}^{2+}$ -treated seedlings, though they were higher than control grown seedlings. These visual results reflect the protective role of  $\text{Ca}^{2+}$  as well as Si over  $\text{Cd}^{2+}$ -induced generation of ROS and oxidative damages.

#### Histochemical detection of lipid peroxidation and membrane damage in root tips

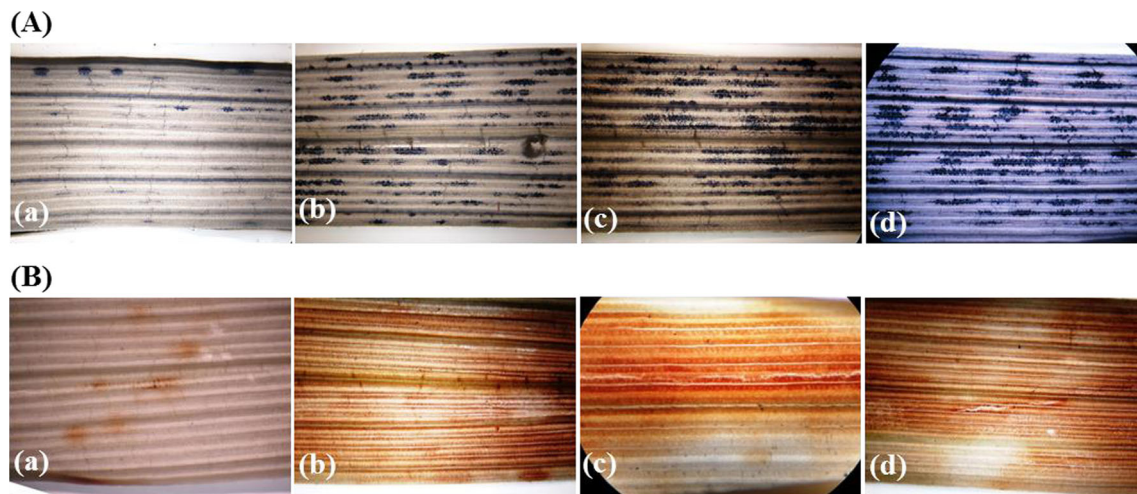
Lipid peroxidation was examined histochemically in the roots using Schiff's reagent. Root surfaces got stained with this reagent depicting formation of lipid peroxides (Fig. 3a). Increased formation of lipid peroxides marked by intensely coloured root surfaces was observed in roots of  $\text{Cd}^{2+}$ -treated seedlings compared to controls. Presence of  $\text{Ca}^{2+}$  and Si reduced colour formation on root surfaces compared to  $\text{Cd}^{2+}$ -treated seedlings which reflects decreased lipid

peroxidation in the seedlings due to  $\text{Ca}^{2+}$  and Si treatments compared to  $\text{Cd}^{2+}$ -treated seedlings. Similar results were obtained when root tips of the seedlings were stained with Evans blue and examined microscopically for histochemical localization of the dye (Fig. 3b). Seedlings exposed to  $\text{Cd}(\text{NO}_3)_2$  (75  $\mu\text{M}$ ) showed intense staining of Evans blue in root tips, suggesting that  $\text{Cd}^{2+}$  treatment resulted in alteration of cell membrane integrity in roots. Comparatively lesser staining with Evans blue in  $\text{Ca}^{2+}$ - or Si-treated root tips compared to root tips of Cd-treated seedlings indicates that both  $\text{Ca}^{2+}$  and Si protect root tips against  $\text{Cd}^{2+}$ -induced plasma membrane damage.

#### Effect of Ca and Si on Cd-induced alterations in protein and non-protein thiols

In response to  $\text{Cd}^{2+}$  treatment, a significant decline in protein thiol level was observed in the seedlings (Fig. 4a). Rice





**Fig. 2** **a** Microscopic view of localization of superoxide anion ( $O_2^{\cdot -}$ ) in situ in rice leaves using NBT staining. Leaves of the seedlings grown for 12 days either in nutrient solution (control, *a*) or in nutrient solution containing  $Cd^{2+}$  (*b*),  $Cd^{2+} + Ca^{2+}$  (*c*) and  $Cd^{2+} + Si$  (*d*) were used. Dark-stained patches represent  $O_2^{\cdot -}$  produced in mesophyll cells. **b**

Histochemical detection of hydrogen peroxide in situ by 3,3'-diaminobenzidine (DAB) uptake method in rice leaves. Seedlings grown for 16 days either in nutrient solution (control, *a*) or in nutrient solution containing  $Cd^{2+}$  (*b*),  $Cd^{2+} + Ca^{2+}$  (*c*) and  $Cd^{2+} + Si$  (*d*) were used. Dark spots represent presence of  $H_2O_2$

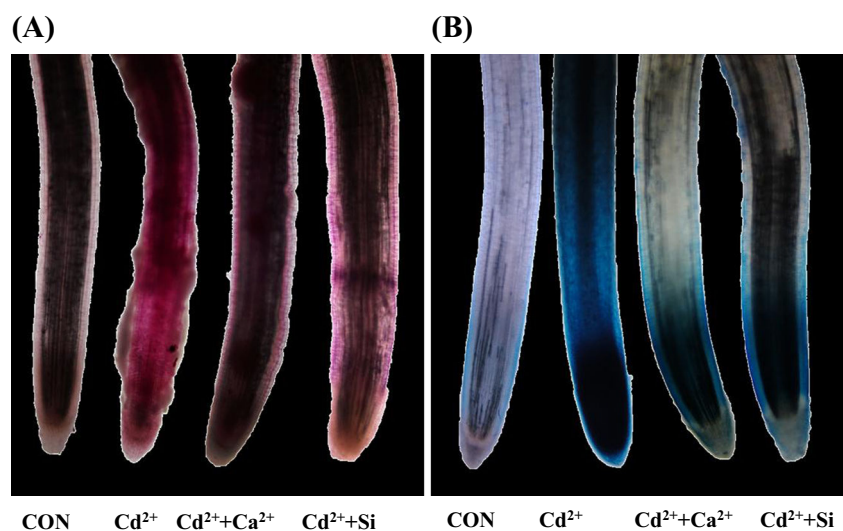
seedlings grown for 12 days in presence of  $75 \mu M Cd(NO_3)_2$  showed 58 % ( $p \leq 0.01$ ) decline in protein thiol level in roots and 25 % ( $p \leq 0.05$ ) decline in the level in shoots compared to controls. The level of protein thiol was always higher in shoots than in roots in both control as well as  $Cd^{2+}$  treatments. Treatment with  $Ca^{2+}$  or Si considerably alleviated Cd-induced decline in protein thiol level. Both  $Ca^{2+}$  and Si appeared to be equally effective in restoring Cd-induced decline in protein thiol level in the seedlings. Non-protein thiol level was elevated in both roots and shoots of  $Cd^{2+}$ -treated seedlings compared to controls (Fig. 4b). With  $Cd^{2+}$  treatment nearly 70 % ( $p \leq 0.01$ ), increase in non-protein thiol level was observed in roots and 43 % ( $p \leq 0.01$ ) increase in shoots compared to controls.  $Ca^{2+}$  and Si promoted a further increase in the level of non-protein thiol in both roots

and shoots of the Cd-stressed seedlings. These results reflect the protective role of  $Ca^{2+}$  and Si by promoting increased synthesis of thiol-containing compounds in Cd-stressed seedlings and in maintaining a high reduction pool in the cells.

Effect of  $Ca^{2+}$  and Si on  $Cd^{2+}$ -induced protein carbonylation and proteolytic activity

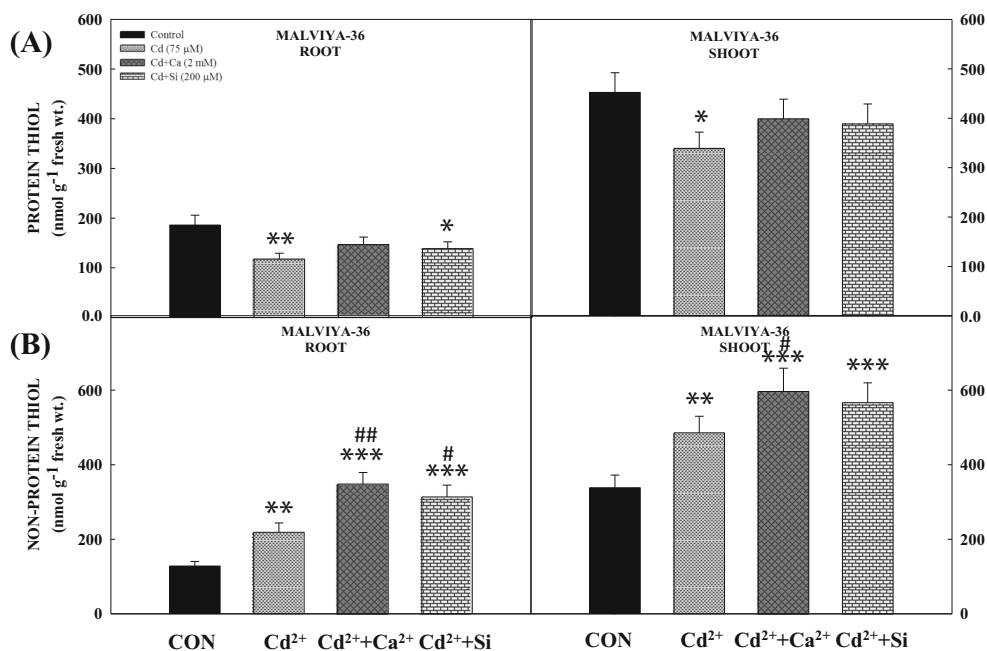
Exposure of rice seedlings to  $Cd^{2+}$  led to increased level of protein carbonyls (Fig. 5a). Seedlings treated with  $Cd^{2+}$  for 12 days showed 88 % ( $p \leq 0.001$ ) increase in protein carbonyls in roots and 78 % ( $p \leq 0.001$ ) increase in shoots compared to controls. Exogenous treatment of rice seedlings with  $Ca^{2+}$  and Si along with  $Cd^{2+}$  led to considerable decline in the level of

**Fig. 3** **a** Histochemical detection of lipid peroxidation in root tips of rice seedlings. Pink-red-stained root portions represent extent of lipid peroxidation in the cells. Control represents untreated seedlings. **b** Histochemical detection of loss of plasma membrane integrity in root tips of rice seedlings treated with  $Cd^{2+}$ ,  $Cd^{2+} + Ca^{2+}$  or  $Cd^{2+} + Si$ . Intense blue-stained root portions represent increased Evans blue uptake by the cells, showing loss of plasma membrane integrity



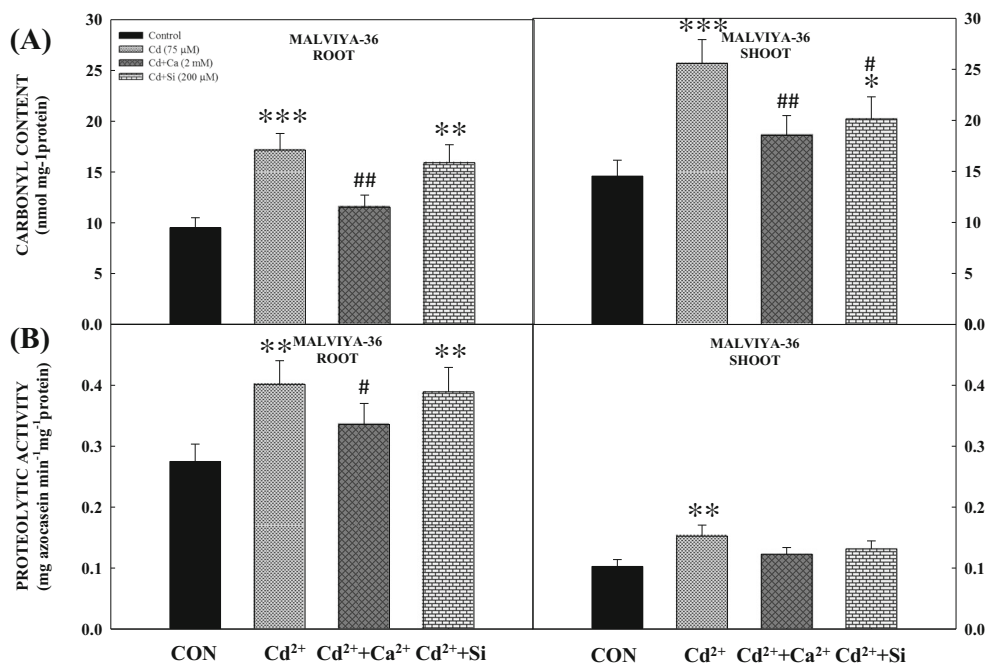


**Fig. 4** Effect of  $\text{Cd}^{2+}$ ,  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  in the growth medium on **a** protein thiol and **b** non-protein thiol in roots and shoots of rice seedlings at 12 days of growth. Values are mean  $\pm$  SD based on three independent determinations, and *bars* indicate standard deviations. *Asterisks* (\*), (\*\*), and (\*\*\*) indicate values that differ significantly from control, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, whereas *number signs* (#) and (##) indicate values significantly differing from  $\text{Cd}^{2+}$  to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively, according to one-way ANOVA multiple comparison analysis using GraphPad Prism



protein carbonyls compared to the level in  $\text{Cd}^{2+}$ -treated seedlings. Parallel to increase in the content of protein-bound carbonyls, the proteolytic activity also increased in the seedlings with  $\text{Cd}^{2+}$  treatment (Fig. 5b). Rice seedlings subjected to  $\text{Cd}^{2+}$  treatment for 12 days showed 46 % ( $p \leq 0.01$ ) increased proteolytic activity in roots and 48 % ( $p \leq 0.01$ ) increased activity in shoots compared to controls. With the presence of  $\text{Ca}^{2+}$  and Si in the medium, a considerable decline in total protease activity was observed in the seedlings compared to the activity in Cd-stressed seedlings.

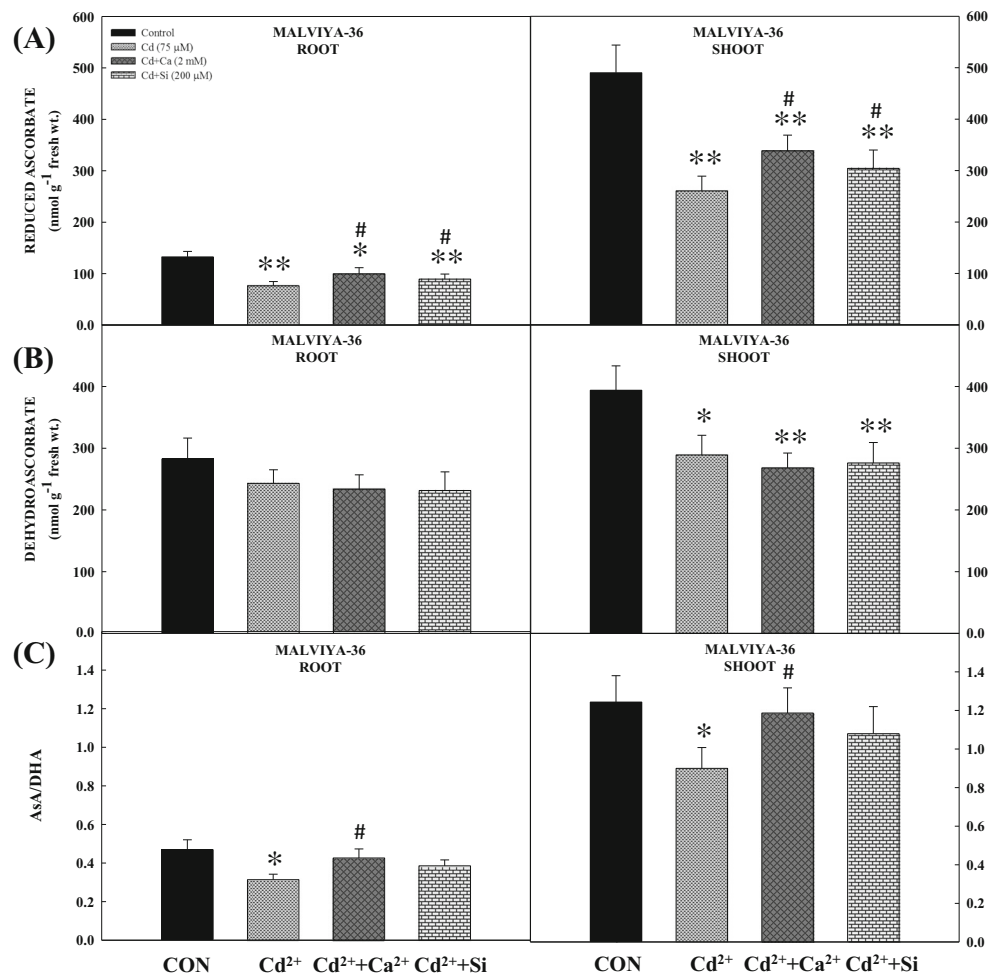
**Fig. 5** Effect of  $\text{Cd}^{2+}$ ,  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  in the growth medium on **a** carbonyl content and **b** proteolytic activity in roots and shoots of rice seedlings at 12 days of growth. Values are mean  $\pm$  SD based on three independent determinations, and *bars* indicate standard deviations. *Asterisks* (\*), (\*\*), and (\*\*\*) indicate values that differ significantly from control, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, whereas *number signs* (#) and (##) indicate values significantly differing from  $\text{Cd}^{2+}$  to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively, according to one-way ANOVA multiple comparison analysis using GraphPad Prism



Effect of Ca and Si on Cd-induced changes in non-enzymic antioxidants

The content of reduced form of ascorbate (AsA) and its oxidized form (DHA) as well as the ratio of AsA/DHA declined in  $\text{Cd}^{2+}$ -treated seedlings compared to controls (Fig. 6). We observed 42 % ( $p \leq 0.01$ ) decline in AsA level in roots and 46 % ( $p \leq 0.01$ ) decline in shoots in rice seedling exposed to 75 μM  $\text{Cd}^{2+}$  for 12 days (Fig. 6a), whereas under similar conditions, DHA level declined by 14 % in roots and by 26 % ( $p \leq 0.05$ ) in

**Fig. 6** Effect of  $\text{Cd}^{2+}$ ,  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  in the growth medium on the levels of **a** reduced ascorbate (*AsA*), **b** dehydroascorbate (*DHA*) and **c** ratio of *AsA/DHA* in roots and shoots of rice seedlings at 12 days of growth. Values are mean  $\pm$  SD based on three independent determinations, and bars indicate standard deviations. Asterisks (\*), (\*\*), (\*\*\*) indicate values that differ significantly from control, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, whereas number sign (#) indicates values significantly differing from  $\text{Cd}^{2+}$  to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , at  $p \leq 0.05$  according to one-way ANOVA multiple comparison analysis using GraphPad Prism



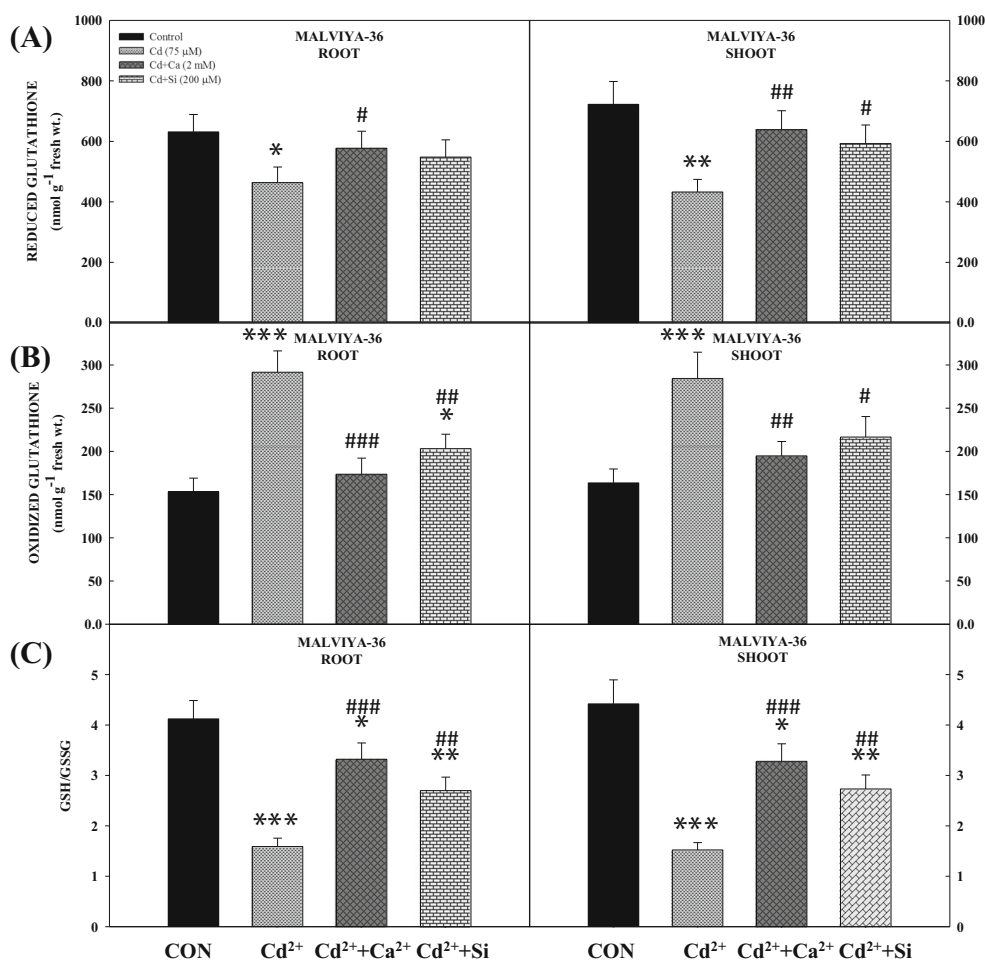
shoots compared to controls (Fig. 6b). Changes in the reduced and oxidized forms of ascorbate under  $\text{Cd}^{2+}$  toxicity also resulted into drop in *AsA/DHA* ratio in the cell. In rice seedlings grown for 12 days under  $\text{Cd}^{2+}$  treatment, 32 % ( $p \leq 0.05$ ) decline in *AsA/DHA* ratio was observed in roots and 25 % ( $p \leq 0.05$ ) decline in shoots (Fig. 6c).  $\text{Ca}^{2+}$  and Si treatments resulted in increased levels of *AsA* in the seedlings compared to Cd-treated seedlings. Increase in *AsA* level increased *AsA/DHA* ratio in  $\text{Cd}^{2+} + \text{Ca}^{2+}$ - and  $\text{Cd}^{2+} + \text{Si}$ -treated seedlings compared to  $\text{Cd}^{2+}$ -alone-treated seedlings. The level of another antioxidant GSH declined significantly in rice seedlings due to  $\text{Cd}^{2+}$  treatment, whereas the level of its oxidized form GSSG increased (Fig. 7). In seedlings exposed to  $75 \mu\text{M}$   $\text{Cd}^{2+}$  for 12 days, 26 % ( $p \leq 0.05$ ) decline in GSH level was observed in roots and 40 % ( $p \leq 0.01$ ) decline in shoots compared to the level in controls (Fig. 7a). The ratio GSH/GSSG declined in the seedlings with  $\text{Cd}^{2+}$  treatment (Fig. 7c).  $\text{Ca}^{2+}$  and Si application resulted in a positive change in total glutathione pool in roots and shoots of  $\text{Cd}^{2+}$  stressed seedlings. GSH level increased in  $\text{Cd}^{2+}$ -treated

seedlings compared to controls, whereas GSSG level was regulated towards control. This increased GSH and regulated GSSG level resulted in increase in GSH/GSSG ratio in both roots and shoots of  $\text{Cd}^{2+} + \text{Ca}^{2+}$ - and  $\text{Cd}^{2+} + \text{Si}$ -treated rice seedlings as compared to Cd-treated seedlings.

Effect of Ca and Si on Cd-induced alterations in the activities of antioxidative enzymes

$\text{Cd}^{2+}$  treatment resulted in increase in the activities of almost all antioxidative enzymes studied except CAT.  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  treatment for 12 days resulted in 85 % ( $p \leq 0.001$ ) increase in SOD activity in roots and 54 % ( $p \leq 0.01$ ) increased activity in shoots (Fig. 8a). With the presence of  $2 \text{ mM}$   $\text{CaCl}_2$  or  $200 \mu\text{M}$  Si in the medium along with  $75 \mu\text{M}$   $\text{Cd}^{2+}$ , apparently distinct patterns of antioxidative enzyme activities were observed in the seedlings (Fig. 8).  $\text{Ca}^{2+}$  treatment resulted in suppression of Cd-induced increase in SOD activity, whereas exogenous application of Si resulted in further increase in SOD activity in both roots and shoots. The activity of CAT showed distinct behaviour compared to other antioxidative enzymes under various treatments. The activity

**Fig. 7** Effect of  $\text{Cd}^{2+}$ ,  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  in the growth medium on the contents of **a** reduced glutathione (*GSH*), **b** oxidized glutathione (*GSSG*) and **c** ratio of *GSH/GSSG* in roots and shoots of rice seedlings at 12 days of growth. Values are mean  $\pm$  SD based on three independent determinations, and *bars* indicate standard deviations. *Asterisks* (\*), (\*\*), (\*\*\*) indicate values that differ significantly from control, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, whereas *number signs* (#), (##) and (###) indicate values significantly differing from  $\text{Cd}^{2+}$  to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, according to one-way ANOVA multiple comparison analysis using GraphPad Prism



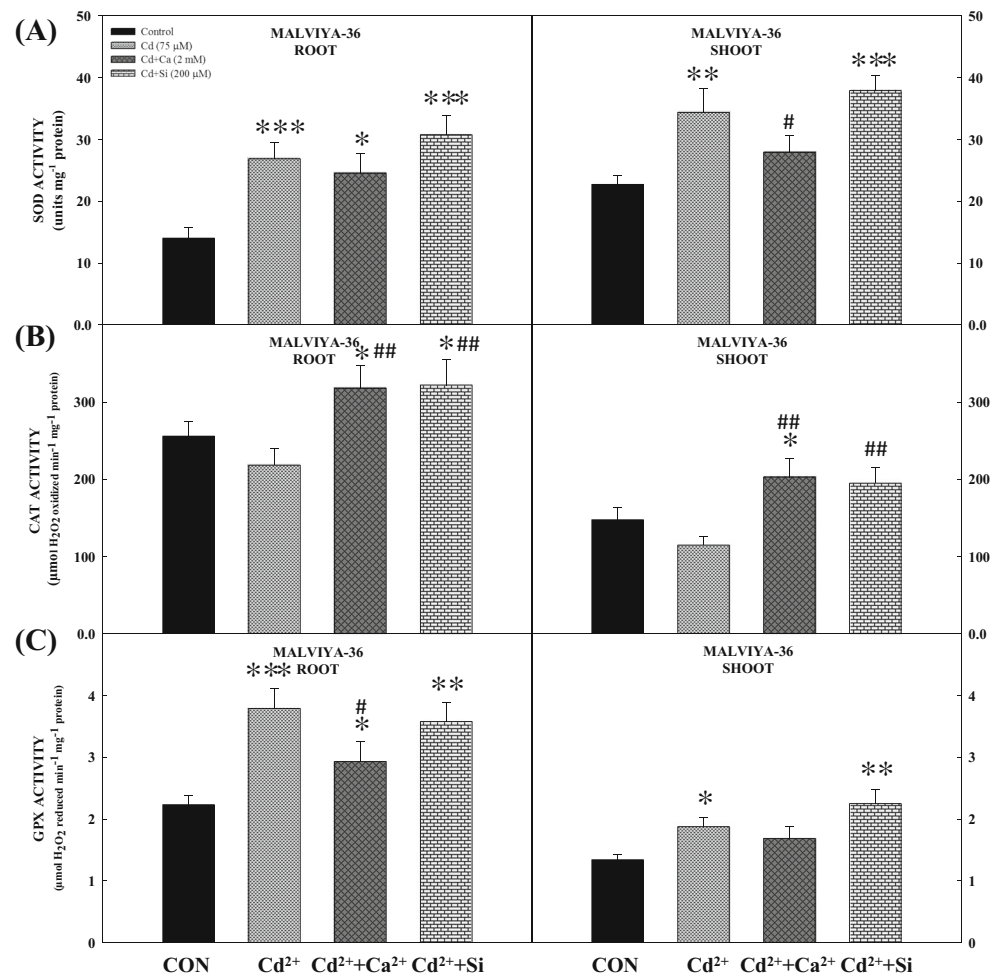
of CAT decreased in both roots and shoots due to  $\text{Cd}^{2+}$  treatment. Rice seedlings grown for 12 days under  $\text{Cd}^{2+}$  treatment showed 15 % decline in CAT activity in roots and 20 % decline in shoots compared to controls (Fig. 8b). Significant increase in CAT activity was observed in the seedlings grown in presence of  $\text{Ca}^{2+} + \text{Cd}^{2+}$  or  $\text{Si} + \text{Cd}^{2+}$  compared to Cd-alone-treated or control-grown seedlings. With the presence of  $\text{Ca}^{2+}$  in the medium, we observed 30 % ( $p \leq 0.05$ ) and 45 % ( $p \leq 0.01$ ) increase in CAT activity in roots and 35 % ( $p \leq 0.05$ ) and 50 % ( $p \leq 0.01$ ) increase in shoots compared to the activity in control and  $\text{Cd}^{2+}$ -treated plants. Whereas the presence of Si in the medium resulted in 30 % ( $p \leq 0.05$ ) and 51 % ( $p \leq 0.01$ ) increase in root CAT activity and 23 % and 45 % ( $p \leq 0.01$ ) increase in shoot activity in the seedlings as compared to control-grown and  $\text{Cd}^{2+}$ -treated seedlings. Unlike CAT, the activity of GPX increased in both roots as well as shoots of the seedlings with  $\text{Cd}^{2+}$  treatment. Cd-induced increase in GPX activity was much higher in roots than in shoots. Seedlings exposed to 75  $\mu\text{M}$   $\text{Cd}^{2+}$  for 12 days showed 70 % ( $p \leq 0.001$ ) increase in GPX activity in roots and 39 % ( $p \leq 0.01$ ) increase in activity in shoots compared to controls (Fig. 8c). In presence of  $\text{Ca}^{2+}$  or

Si in the treatment medium, there was suppression in Cd-induced increase in GPX activity.  $\text{Ca}^{2+}$  treatment resulted into a marked and conspicuous suppression in Cd-induced increase in root GPX activity compared to Si.

To elucidate the mechanism of the maintenance of redox balance under  $\text{Cd}^{2+}$  toxicity, we examined the activities of the enzymes of ascorbate–glutathione cycle. A marked increase in the activity of APX was observed in both roots and shoots of Cd-treated seedlings compared to controls (Fig. 9). Roots always maintained higher APX activity than shoots under both control and  $\text{Cd}^{2+}$  treatments. Seedlings grown under 75  $\mu\text{M}$   $\text{Cd}^{2+}$  for 12 days showed 56 % ( $p \leq 0.01$ ) increased APX activity in roots and 47 % ( $p \leq 0.01$ ) increased activity in shoots compared to controls.  $\text{Ca}^{2+}$  treatment resulted in decline in APX activity in the seedlings compared to  $\text{Cd}^{2+}$ -alone-treated seedlings; however, the activity was higher than control-grown seedlings (Fig. 9a). Treatment with Si promoted APX activity to a significantly higher level compared to control 69 % ( $p \leq 0.001$ ) as well as  $\text{Cd}^{2+}$ -alone-treated seedlings.  $\text{Cd}^{2+}$  treatment resulted in significant increase in MDHAR activity in the seedlings (Fig. 9b). Seedlings grown under 75  $\mu\text{M}$   $\text{Cd}^{2+}$  for 12 days showed 60 % ( $p \leq 0.01$ )



**Fig. 8** Effect of  $\text{Cd}^{2+}$ ,  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  in the growth medium on activities of **a** superoxide dismutase (*SOD*), **b** catalase (*CAT*) and **c** guaiacol peroxidase (*GPX*) in roots and shoots of rice seedlings at 12 days of growth. Values are mean  $\pm$  SD based on three independent determinations, and bars indicate standard deviations. Asterisks (\*), (\*\*), (\*\*\*) indicate values that differ significantly from control, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, whereas number signs (#) and (##) indicate values significantly differing from  $\text{Cd}^{2+}$  to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively, according to one-way ANOVA multiple comparison analysis using GraphPad Prism



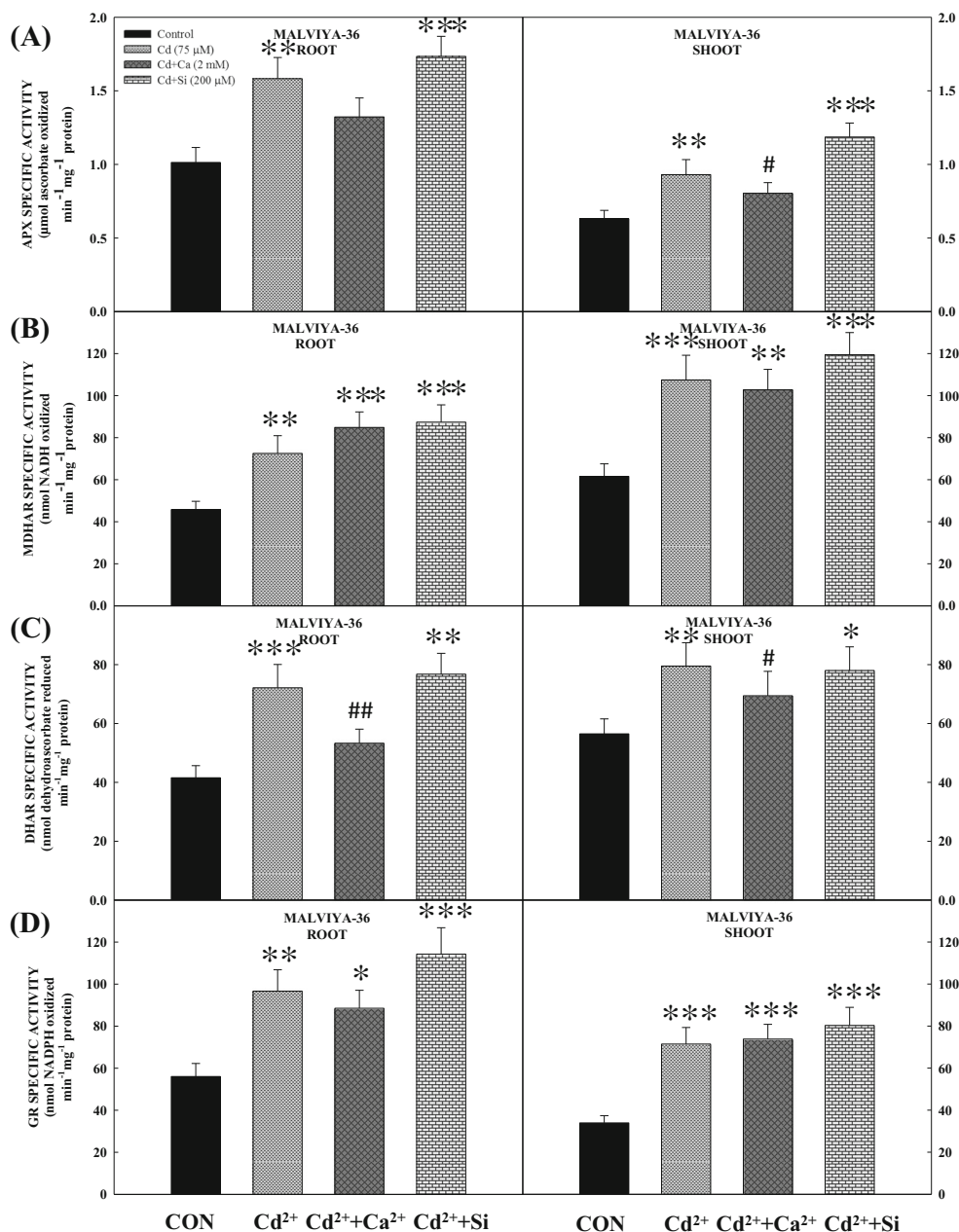
increase in MDHAR activity in roots and 76 % ( $p \leq 0.001$ ) increased activity in shoots compared to the controls. MDHAR activity was greater in shoots than in roots in controls as well as under different treatments. Similar to MDHAR, the activity of DHAR also increased in seedlings from  $\text{Cd}^{2+}$  treatment. Seedlings grown under  $75 \mu\text{M}$   $\text{Cd}^{2+}$  for 12 days showed 77 % ( $p \leq 0.001$ ) increased DHAR activity in roots and 41 % ( $p \leq 0.01$ ) increased activity in shoots (Fig. 9c). GR, an important enzyme needed for reduction of GSSG using NADPH, showed increased activity in the rice seedlings with  $\text{Cd}^{2+}$  treatment (Fig. 9d). Rice seedlings raised under  $75 \mu\text{M}$   $\text{Cd}^{2+}$  for 12 days showed 71 % ( $p \leq 0.01$ ) increased GR activity in roots and 115 % ( $p \leq 0.001$ ) increased activity in shoots compared to the activity in controls (Fig. 9d).  $\text{Ca}^{2+}$  treatment resulted in little increase in MDHAR activity in roots and almost similar level of activity in shoots compared to  $\text{Cd}^{2+}$ -treated seedlings, whereas DHAR activity was lower in  $\text{Ca}^{2+} + \text{Cd}^{2+}$ -treated seedlings compared to  $\text{Cd}^{2+}$ -alone-treated seedlings. GR activity was also lower in the roots of  $\text{Ca}^{2+} + \text{Cd}^{2+}$ -treated seedlings compared to  $\text{Cd}^{2+}$ -alone-treated seedlings. With the presence of Si, however in the medium, the activities of all the three enzymes MDHAR, DHAR and

GR increased further as compared to the activities in  $\text{Cd}^{2+}$ -treated seedlings.

## Discussion

Increasing concentrations of various metals like  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{+}$ ,  $\text{Cr}^{5+}$  and  $\text{Ni}^{2+}$  in the agricultural land cause either directly or indirectly increased production of ROS and thereby intense oxidative damage to growing plants. Being a redox-inactive metal,  $\text{Cd}^{2+}$  does not participate in Fenton-type ROS-producing reactions; it indirectly activates NADPH oxidases in membranes giving rise to ROS generation and also promotes Fenton-type reactions by increasing free  $\text{Fe}^{++}$  ion by replacing it from its active sites (Romero-Puertas et al. 2004). Increasing pieces of evidence suggest that oxidative damage is an important factor in the expression of toxicity caused due to many metals in plants (Sandalo et al. 2009; Sharma et al. 2012). It has been shown that exogenous application of  $\text{Ca}^{2+}$  and Si alleviates the  $\text{Cd}^{2+}$ -induced growth inhibition in many plant species (Liang et al. 2005; Tian et al. 2011). In our

**Fig. 9** Effect of  $\text{Cd}^{2+}$ ,  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  in the growth medium on activities of **a** ascorbate peroxidase (APX), **b** monodehydroascorbate reductase (MDHAR), **c** dehydroascorbate reductase (DHAR) and **d** glutathione reductase (GR) in roots and shoots of rice seedlings at 12 days of growth. Values are mean  $\pm$  SD based on three independent determinations, and bars indicate standard deviations. Asterisks (\*), (\*\*), and (\*\*\*) indicate values that differ significantly from control, at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively, whereas number signs (#) and (##) indicate values significantly differing from  $\text{Cd}^{2+}$  to  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , at  $p \leq 0.05$  and  $p \leq 0.01$ , respectively, according to one-way ANOVA multiple comparison analysis using GraphPad Prism



experiments performed in hydroponics, 75  $\mu\text{M}$   $\text{Cd}^{2+}$  significantly inhibited the growth of rice seedlings. It has been observed that  $\text{Cd}^{2+}$  irreversibly inhibits proton pump responsible for elongation of cells (Aidid and Okamoto 1993). Whereas the presence of  $\text{Ca}^{2+}$  and Si in the medium protected the seedlings against  $\text{Cd}^{2+}$ -induced growth inhibition. Restoration of growth of the seedlings due to presence of  $\text{Ca}^{2+}$  or Si paralleled with decreased accumulation of  $\text{Cd}^{2+}$  by the seedlings. In *S. alfredii* plants, exogenous treatment of  $\text{Ca}^{2+}$  significantly restored root growth inhibited due to  $\text{Cd}^{2+}$  (Tian et al. 2011). It is suggested that addition of  $\text{Ca}^{2+}$  to the growth medium reduces  $\text{Cd}^{2+}$  activity at plasma membrane surface of root cells (Tian et al. 2011). Supplying exogenous

Si has been shown to alleviate  $\text{Cr}^{5+}$ -induced reduction in root and shoot growth and metabolism in rice (Zeng et al. 2011). Our knowledge related to the mechanism of Si-induced growth promotion in plants is insufficient; however, it is believed that Si increases cell wall extensibility (Hossain et al. 2002). Our results indicate that the presence of  $\text{Ca}^{2+}$  and Si in nutrient medium plays important role in restoring plant growth due to  $\text{Cd}^{2+}$  toxicity in rice plants.

$\text{Cd}^{2+}$  treatment resulted in significant decline in plant biomass in comparison with control, whereas significant restoration in biomass was observed with presence of  $\text{Ca}^{2+}$  and Si. It has been shown that  $\text{Ca}^{2+}$  or Si reduces  $\text{Cd}^{2+}$ -induced decline in biomass in different plant species (Sandalo et al. 2009;

Farzadfar et al. 2013). In our experiments, we observed that treatment of rice seedlings with 75  $\mu\text{M}$   $\text{Cd}(\text{NO}_3)_2$  promoted accumulation of  $\text{Cd}^{2+}$  within the plants, whereas with the presence of  $\text{Ca}^{2+}$  or Si in the nutrient medium, the accumulation of  $\text{Cd}^{2+}$  was significantly reduced by the roots. Due to high resemblance in chemical character,  $\text{Cd}^{2+}$  uses  $\text{Ca}^{2+}$  transporters for its uptake inside the cells; therefore, increased  $\text{Ca}^{2+}$  in the nutrient medium might compete with  $\text{Cd}^{2+}$  for its uptake and internalization in the cells. It has been observed that exogenous application of  $\text{Ca}^{2+}$  alleviates  $\text{Cd}^{2+}$ -induced damage in plants by decreasing the accumulation of  $\text{Cd}^{2+}$  in plant parts promoting plant growth (Tian et al. 2011) and membrane stability and re-stabilizing  $\text{Ca}^{2+}$ -mediated signalling inside the plants (Cramer 1985). Presence of Si inside the cell has been shown to reduce accumulation as well as translocation of available heavy metals in the cell. In cowpea plants, it has been reported that Si modifies cation-binding capacity of cell wall and lowers soluble symplastic  $\text{Mn}^{2+}$  concentration (Horst et al. 1999). Si has been shown to detoxify the toxic Mn present in symplast (Iwasaki et al. 2002). In peanut plants, Si pretreatment reduced  $\text{Cd}^{2+}$  accumulation in the tissues (Shi et al. 2010). Si in its secondary action enhances the uptake of  $\text{Ca}^{2+}$  in plant roots, which competes with  $\text{Cd}^{2+}$  for uptake and lowers its transport from roots to shoots (Song et al. 2009). Though there is a possibility of competition of  $\text{Cd}^{2+}$  with  $\text{Ca}^{2+}$  and Si for decreased  $\text{Cd}^{2+}$  accumulation in growing rice plants as observed in our studies, the possibility of reduced uptake of free ionic species of  $\text{Cd}^{2+}$  due to ionic interactions with  $\text{Ca}^{2+}$  and Si also cannot be ruled out.

Increased production of the ROS ( $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$ ) is the key contributor in  $\text{Cd}^{2+}$ -induced oxidative damage in plants (Shah et al. 2001; Romero-Puertas et al. 2004). Though  $\text{Cd}^{2+}$  is not involved in redox reactions in cells, it causes production of ROS in plants.  $\text{Cd}^{2+}$  indirectly activates NADPH oxidase present at membrane surface and subsequently promotes formation of ROS leading to oxidative damage in cells (Romero-Puertas et al. 2004). Though increased production of ROS in the cell poses threat to cellular biomolecules, ROS also act as signal molecules and activate stress-responsive and defence-related genes via signalling pathways (Sharma et al. 2012). In our studies, we observed that exogenous application of  $\text{Ca}^{2+}$  or Si along with  $\text{Cd}^{2+}$  reduced ROS production in the tissues in comparison of Cd-stressed seedlings.  $\text{Ca}^{2+}$  supplementation has been shown to reduce Cd-induced oxidative damage by lowering formation of ROS ( $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$ ) in *S. alfredii* and *Matricaria chamomilla* L. plants (Tian et al. 2011; Farzadfar et al. 2013). Many workers have shown that Si reduces generation of ROS in cells under toxicities due to the metals  $\text{Mn}^{2+}$  (Shi et al. 2005b) and  $\text{Cd}^{2+}$  (Song et al. 2009).

The overproduced ROS, if not properly scavenged, causes oxidative damage to membrane lipids, proteins, chloroplastic pigments, enzymes, nucleic acids, etc. Lipid peroxidation is regarded as an effective indicator of cellular oxidative damage

(Srivastava and Dubey 2011); therefore, we examined the level of TBARS in roots and shoots of rice seedlings under different treatments. Our results showed significant increase in TBARS level in the seedlings grown in the presence of  $\text{Cd}^{2+}$ . Interestingly in seedlings grown under  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$ , we observed remarkable decline in TBARS content compared to  $\text{Cd}^{2+}$ -treated seedlings. This shows that  $\text{Ca}^{2+}$  and Si considerably protect Cd-induced oxidative injury in rice seedlings, and this protection might be related to the avoidance of  $\text{H}_2\text{O}_2$  generation and the reduction of  $\text{Cd}^{2+}$  uptake (Song et al. 2009; Farzadfar et al. 2013). Besides increased lipid peroxidation, we observed significant increase in membrane damage in root tips of Cd-exposed seedlings. Permeability and integrity of the membrane change due to peroxidation of membrane lipids which damages cell membranes irreversibly. Evans blue uptake assay both histochemically and spectrophotometrically confirmed increased permeability of cell membranes due to  $\text{Cd}^{2+}$  treatment. Further,  $\text{Ca}^{2+}$  and Si significantly reduced the internalization of Evans blue inside the root cells by promoting membrane integrity and decreasing lipid peroxidation.

A decline in the level of protein thiol and an increase in the level of non-protein thiol were observed in the seedlings grown in presence of  $\text{Cd}^{2+}$ . A decline in protein thiol level indicates oxidation of  $-\text{SH}$  groups of proteins under  $\text{Cd}^{2+}$  toxicity. Non-protein thiols in rice mainly compose of cysteine,  $\gamma$ -glutamyl cysteine, GSH, hydroxymethyl GSH, phytochelatins [PCs; ( $\gamma$ -Glu-Cys) $n$ -Gly] and hydroxymethyl phytochelatins (hm-PCs) (Klapheck et al. 1994). In our studies, a decline in the level of GSH and an increase in the level of non-protein thiol indicate that  $\text{Cd}^{2+}$  toxicity might favour induction of the synthesis of PCs and hm-PCs and other low-molecular-weight thiol-rich compounds which are known to play a role in the detoxification mechanism against heavy metals in plants (Zenk 1996). Rice seedlings grown under  $\text{Ca}^{2+}$  and Si treatments showed restoration in protein thiol level. Whereas non-protein thiol content also increased in seedling grown under  $\text{Cd}^{2+} + \text{Ca}^{2+}$  and  $\text{Cd}^{2+} + \text{Si}$  treatments compared to control and Cd-treated seedlings, showing limiting effect of  $\text{Ca}^{2+}$  and Si over  $\text{Cd}^{2+}$  accumulation, translocation and induced oxidative stress. Klapheck et al. (1994) showed that  $\text{Cd}^{2+}$ -treated rice plants had lower level of GSH content and increased levels of  $\gamma$ -Glu-Cys peptides and PCs compared to non-treated plants.

Protein carbonylation is another consequence of oxidative damage to proteins, and it is widely used as a marker of protein oxidation (Moller et al. 2007). The oxidation of a number of protein amino acids particularly Arg, His, Lys, Pro, Thr and Trp may lead to inhibition or alteration in protein functioning and increase its susceptibility towards proteolytic attack (Moller et al. 2007). Our studies showed a significant increase in carbonyl content as well as increase in proteolytic activity in roots and shoots of Cd-treated seedlings compared



to controls. Srivastava and Dubey (2011) reported that excess  $Mn^{2+}$  led to carbonylation in rice seedlings in dose- and time-dependent manner. In our studies with application of  $Ca^{2+}$  and Si along with  $Cd^{2+}$ , a significant decline in carbonyl content as well as proteolytic activity was noticed as compared to the carbonylation and proteolysis in Cd-treated seedlings. A direct correlation was noticed between increase in the level of carbonylation of proteins and proteolytic activity under different treatments. It has been observed that carbonylated proteins when accumulate in the cells become toxic and also become more susceptible to proteolysis due to unfolding of target protein domains (Moller et al. 2007). In  $Cd^{2+}$ -exposed pea and *Arabidopsis* plants, enhanced proteolytic activity leads to more efficient degradation of oxidized proteins (Romero-Puertas et al. 2002). In our studies, increased production of ROS might cause increased oxidation of proteins which in turn would trigger enhanced protease activity under different treatments. As  $Ca^{2+}$  and Si in the treatment medium lowered generation of ROS, compared to  $Cd^{2+}$ -treated seedlings,  $Ca^{2+}$  and Si appear to safeguard cellular proteins against oxidative injury and thus indirectly restrict protease activity compared to the activity in  $Cd^{2+}$ -treated seedlings.

Increased level of ROS in the cells alters cellular redox status due to oxidative damage resulting in disruption of redox signalling and redox control (Noctor 2006). The cellular redox state in the cells maintains homeostasis and enables the plants to deal with redox events such as oxidative stress (Srivastava and Dubey 2011). Our results showed a decline in the ratio of both AsA/DHA and GSH/GSSG in  $Cd^{2+}$ -treated rice seedlings. AsA is a potent ROS scavenger and it provides protection to the cell organelles and biomolecules from oxidative damage by directly scavenging  $O_2^{\cdot-}$  and  $\cdot OH$  (Gill and Tuteja 2010). Transgenic plants with higher AsA contents showed improved tolerance to oxidative stress (Wang et al. 2010). In our studies, a high level of AsA and a higher AsA/DHA ratio in  $Cd^{2+} + Ca^{2+}$ - and  $Cd^{2+} + Si$ -treated seedlings compared to Cd-alone-treated seedlings suggest that  $Cd^{2+}$  tolerance in rice is associated with maintenance of a higher level of reduced form of ascorbate in the tissues. Similarly, the reduced glutathione (GSH) is also a potent scavenger of the ROS ( $O_2^{\cdot-}$ ,  $H_2O_2$  and  $\cdot OH$ ) and plays an important role in antioxidative defence system by regenerating AsA via the AsA–GSH cycle (Gill and Tuteja 2010). GSH has an important role in avoiding ROS-induced oxidative damage and in maintaining normal reduced state of the cell (Sharma et al. 2012). A delicate balance between the GSH and GSSG is essential to maintain cellular redox state (Sharma et al. 2012). Due to its crucial role in antioxidative defence, GSH level is used as a stress marker and an elevated GSH level is correlated with the ability of plants to withstand abiotic stress-induced oxidative damage (Gill and Tuteja 2010). Many workers have reported decline in reduction pool of cell under metal toxicity (Shi et al. 2005b; Song et al. 2009; Srivastava and Dubey 2011). Our results

indicate that similar to AsA, a high level of GSH and a higher GSH/GSSG ratio under  $Ca^{2+}$  and Si treatments are associated with Cd toxicity tolerance in rice seedlings and that exogenous  $Ca^{2+}$  and Si help in maintaining adequate reduced levels of ascorbate and glutathione in the cells under  $Cd^{2+}$  toxicity to withstand Cd-induced oxidative damage.

Under various abiotic stresses including metal toxicity, the antioxidative defence system of plants scavenges overproduced ROS depending on their concentration in the cell (Gill and Tuteja 2010). A delicate balance always exists between ROS produced and cellular antioxidative defence machinery, in order to minimize cellular damage. Among the antioxidative enzymes, SOD catalyzes the conversion of  $O_2^{\cdot-}$  to less toxic  $H_2O_2$  and  $O_2$  and it is considered as the first line of defence against ROS in plants. We observed a marked increase in SOD activity in  $Cd^{2+}$ -exposed rice seedlings. Increased SOD activity in plants is directly related with generation of  $O_2^{\cdot-}$  in the cells and is considered as induction of protection mechanism against abiotic stresses (Srivastava and Dubey 2011). In rice plants grown under  $Cd^{2+} + Ca^{2+}$  and  $Cd^{2+} + Si$  treatments, we observed two distinct patterns in SOD activity; presence of  $Ca^{2+}$  resulted in considerable decline in SOD activity whereas Si caused increase in SOD activity in comparison with  $Cd^{2+}$ -alone-treated seedlings. Similar to our studies, Farzadfar et al. (2013) reported that  $Ca^{2+}$  treatment resulted in decline in SOD activity in Cd-treated *M. chamomilla* plants. It has been reported that exogenous Si led to an increase in SOD activity in *Cucumis sativus* plants grown under Mn stress (Shi et al. 2005b). The intracellular level of  $H_2O_2$  is regulated by a wide range of enzymes; two most importantly are CATs and peroxidases, both of which take care of overproduced  $H_2O_2$  due to high SOD activity under oxidative stress. We observed decline in CAT activity in roots and shoots of  $Cd^{2+}$ -treated seedlings, suggesting that the  $H_2O_2$  scavenging mechanism by CAT is possibly less effective when seedlings are subjected to prolonged  $Cd^{2+}$  treatment of nearly 12 days. Srivastava and Dubey (2011) reported an increase in CAT activity in rice seedlings exposed to 5–10 days with Mn excess, and the activity declined when Mn stress persisted for longer duration. Decline in CAT activity in metal-exposed plants could be attributed to either inactivation of enzyme due to its direct interaction with metal ions or ROS (Dat et al. 2000) or due to its decreased synthesis or impaired protein assembly (Ushimaru et al. 1999).  $Ca^{2+}$  and Si treatment to rice seedlings significantly increased CAT expression when present along with Cd in growth medium. According to Shi et al. (2005a), Si treatment resulted in increase in CAT activity in *C. sativus* grown under  $Mn^{2+}$  toxicity. Pyngrope et al. (2013) showed that higher CAT level in drought-tolerant seedlings protects the seedlings from oxidative damages by warding them from overproduced ROS. Peroxidases play important role in scavenging  $H_2O_2$  in plants. We observed significant increase in APX and GPX activities

in Cd<sup>2+</sup>-treated seedlings compared to controls, and presence of Ca<sup>2+</sup> and Si in the medium further regulated this increase. Farzadfar et al. (2013) also observed that Cd<sup>2+</sup> treatment resulted in exponential increase in peroxidase activity in *M. chamomilla* plants, which was significantly regulated by Ca<sup>2+</sup> treatment. Shi et al. (2005a), however, reported that exogenous application of Si promoted APX activity but GPX activity declined. The enzymes MDHAR and DHAR together with GR participate in regeneration of antioxidants AsA and GSH and help in maintaining redox cellular balance. Optimum activity of GR is necessary in the cells to maintain an optimum pool of GSH. The activity of these enzymes increased in rice seedlings with Cd<sup>2+</sup> treatment. Srivastava and Dubey (2011) reported increased activities of the enzymes MDHAR, DHAR and GR in rice seedlings under Mn<sup>2+</sup> exposure and suggested that Mn<sup>2+</sup> induced GSH and AsA regenerating system in the seedlings in order to maintain their requisite levels in the tissues to cope up with the adverse conditions of oxidative damage due to Mn<sup>2+</sup>. Other studies have also revealed enhanced activity of the enzymes of AsA–GSH cycle in different plant species under toxicity of the metals Mn<sup>2+</sup> and Cd<sup>2+</sup> (Shah et al. 2001; Shi et al. 2005b; Srivastava and Dubey 2011). Presence of Cd<sup>2+</sup> and Si, in our studies, helped in maintaining sufficiently high activity levels of the enzymes of AsA–GSH cycle, suggesting further that Ca<sup>2+</sup> and Si help the cells in generating sufficiently reduced levels of ascorbate and glutathione under Cd<sup>2+</sup> toxicity.

In conclusion, results of the present study suggest that Cd<sup>2+</sup> toxicity in rice seedlings is associated with induction of oxidative stress in the seedlings due to overproduced O<sub>2</sub><sup>·-</sup> and H<sub>2</sub>O<sub>2</sub>. Among the antioxidative enzymes, SOD, GPX APX, MDHAR, DHAR and GR appear to play a key role in scavenging O<sub>2</sub><sup>·-</sup> and H<sub>2</sub>O<sub>2</sub> under Cd<sup>2+</sup> toxicity. Ca<sup>2+</sup> and Si appear to protect rice plants from Cd<sup>2+</sup> toxicity by adequately maintaining pool of antioxidants AsA and GSH and higher activity levels of key antioxidant enzymes. Nearly 50 % less Cd<sup>2+</sup> was detected in roots as well as shoots when Ca<sup>2+</sup> was present in the growth medium. Whereas Si, which was not so efficient as Ca<sup>2+</sup> to prevent Cd<sup>2+</sup> entry in plant roots, restricted Cd<sup>2+</sup> uptake to a considerable extent. But, Si significantly promoted plant's antioxidative defence system so that plants were able to cope up with overproduced ROS to diminish the effects of oxidative stress. The results suggest that exogenous application of Ca<sup>2+</sup> and Si in the growth medium ameliorates Cd-induced oxidative stress in rice seedlings. The observations indicate that Ca<sup>2+</sup> and Si treatments can help in reducing Cd<sup>2+</sup> toxicity in rice plants.

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**Conflict of interest** The authors hereby declare that there is no conflict of interest.

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