Manganese-excess induces oxidative stress, lowers the pool of antioxidants and elevates activities of key antioxidative enzymes in rice seedlings

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Abstract Manganese (Mn) is an essential element for plant growth but in excess, specially in acidic soils, it can become phytotoxic. In order to investigate whether oxidative stress is associated with the expression of Mn toxicity during early seedling establishment of rice plants, we examined the changes in the level of reactive oxygen species (ROS), oxidative stress induced an alteration in the level of non-enzymic antioxidants and activities of antioxidative enzymes in rice seedlings grown in sand cultures containing 3 and 6 mM $MnCl_2$. Mn treatment inhibited growth of rice seedlings, the metal increasingly accumulated in roots and shoots and caused damage to membranes. Mn treated plants showed increased generation of superoxide anion ($O_2^{.-}$), elevated levels of H_2O_2 and thiobarbituric acid reactive substances (TBARS) and decline in protein thiol. The level of nonprotein thiol, however, increased due to Mn treatment. A decline in contents of reduced ascorbate (AsA) and glutathione (GSH) as well as decline in ratios of their reduced to oxidize forms was observed in Mn-treated seedlings. The activities of antioxidative enzymes superoxide dismutase (SOD) and its isoforms Mn SOD, Cu/Zn SOD, Fe SOD as well as guaiacol peroxidase (GPX) increased in the seedlings due to Mn treatment however, catalase (CAT) activity increased in 10 days old seedlings but it declined by 20 days under Mn treatment. The enzymes of Halliwell-Asada cycle, ascorbate peroxidase (APX) monodehydoascorbate reductase (MDHAR), dehyroascorbate reductase (DHAR) and glutathione reductase (GR) increased significantly in Mn treated seedlings over controls. Results suggest that in rice seedlings excess Mn induces oxidative stress, imbalances the levels of antioxidants and the antioxidative enzymes SOD, GPX, APX and GR appear to play an important role in scavenging ROS and withstanding oxidative stress induced by Mn.

Introduction

Manganese (Mn) is an essential element for plant growth and development and plays key role in activation of several enzymes of various metabolic pathways associated with photosynthesis, respiration and synthesis of proteins, carbohydrates, etc. (Boojar and Goodarzi 2008). Excess Mn is stored in vacuoles, cell walls and chloroplast thylakoids (Gonzales and Lynch 1999) and Mn can become phytotoxic at high foliar concentrations (El-jaoual and Cox 1998). The bioavailability of Mn increases as soil gets acidified and the pH decreases below 5.5 (Lucas and Davis 1961). Under such conditions Mn is easily taken up by plant roots and causes retardation in plant growth and interferes with various metabolic processes (Lidon and Teixeira 2000; Hauck et al. 2003). Excess Mn inhibits chlorophyll biosynthesis and causes decline in photosynthetic rate (Hauck et al. 2002). The symptoms of Mn toxicity vary widely among plant species. Necrotic brown spotting on leaves, petioles and stem is a common symptom of Mn toxicity (Horst and Marschner 1978; Wu 1994). Another common symptom 'crincle-leaf' that occurs in the youngest leaf, stem and petiole tissues, is associated with chlorosis and browning of these tissues (Wu 1994;

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Bachman and Miller 1995). Roots exhibiting Mn toxicity are commonly brown in colour and sometimes show cracks within (Le Bot et al. 1990).

The toxic effects of many essential and nonessential elements have been linked to the increased production of ROS, such as O_2^-, hydroxyl radical ($^\cdot OH$), H_2O_2, etc. (Stohs and Bagchi 1995). These ROS cause oxidative damage to the vital cellular components such as membrane lipids, proteins, enzymes, pigments and nucleic acids (Dat et al. 2000; Shah et al. 2001; Verma and Dubey 2003; Sharma and Dubey 2007). To control cellular level of ROS and to protect cells under stressful conditions, plant tissues possess antioxidative defense system comprising of non-enzymic antioxidants ascorbate (AsA), glutathione (GSH), carotenoids, α-tocopherol and antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and enzymes of Halliwell-Asada pathway ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), de-hydroascorbate reductase (DHAR) and glutathione reductase (GR). These antioxidant compounds remove, neutralize and scavange ROS (Noctor and Foyer 1998; Verma and Dubey 2003). Under normal conditions, production and removal of ROS are well regulated, but toxicity due to metals causes overproduction of ROS, leading to oxidative stress and imbalance in the levels of cellular antioxidants (Shah et al. 2001; Sharma and Dubey 2007). It has been shown that excess Mn induces oxidative stress in many plant species and alters the activity levels of antioxidative enzymes (Demirevska-kepova et al. 2004; Boozar and Goodarzi 2008). Though several varieties of rice are highly Mn tolerant, the extent of such tolerance is highly variable in the early stages of vegetative growth (Nelson 1983). Soil conditions often present in acid and volcanic soils can lead to Mn toxicity in many natural and agricultural systems (Carver and Ownby 1995). Rice is staple food crop for the world population and its productivity is limited due to soil acidity. Metals like Cd, Pb, Al and Ni have been shown to significantly impair the vegetative growth of rice plants during early seedling establishment stages and induce oxidative stress in the tissues (Shah et al. 2001; Verma and Dubey 2003; Sharma and Dubey 2007; Maheshwari and Dubey 2009). As Mn toxicity may serve as an important component affecting establishment of rice crop and its cultivation in acid soils, in order to test the hypothesis whether oxidative stress is involved in expression of Mn toxicity during establishment of rice seedlings in acid soils and to infer how antioxidative defense system of rice plants behaves under Mn-excess, the present study was undertaken to examine the effect of increasing concentration of Mn in sand cultures on its uptake by rice plants, production of ROS, possible induction of oxidative stress, redox status of the antioxidants ascorbate, glutathione and responses of antioxidant enzymes in growing Indica rice seedlings.

Materials and methods

Plant material and toxicity levels

Rice (*Oryza sativa* L.) cv. Pant-12, a commonly grown variety in India was used in this study. Seeds were surface sterilized with 0.1% sodium hypochlorite solution for 10 min, rinsed with distilled water and imbibed for 24 h in water. Seedlings were then raised in plastic pots containing purified sea sand saturated with either Yoshida nutrient solution (Yoshida et al. 1976) which served as control or nutrient solutions supplemented with 3 and 6 mM $MnCl_2$ which served as treatment solutions. The 3 and 6 mM concentrations represent moderately toxic and highly toxic concentrations of Mn for the growth of rice seedlings, based on our earlier studies (data not reported here). Sand cultures were maintained in pots, at pH 4.5, at field saturation capacity and received control and respective treatment solutions when needed to saturate the sand. Seedlings were raised for 20 days in a green house at $28 \pm 1°C$ under 80% relative humidity and 12 h light/dark cycle with 190–200 μmol m^{-2} s^{-1} irradiance. Seedlings were uprooted at 10 and 20th day and all analyses and enzyme assays were performed in triplicate.

Rice seedling vigour and Mn uptake

At each sampling time, root and shoot length as well as root and shoot fresh biomass were determined based on 10 random samplings in triplicate. To determine Mn concentration in the seedlings, harvested seedlings were washed with double-distilled water. Roots and shoots were separated, oven dried (70°C for 3 days), ground to a fine powder, and digested in an acid mixture (HNO_3:H_2SO_4: $HClO_4$; 5:1:1) (Allen et al. 1986). Digested samples were diluted with deionized double-distilled water and Mn concentrations were determined using an atomic absorption spectrometer (Model No.-2380, Perkin–Elmer, USA).

Measurement of O_2^- and H_2O_2

At 10 and 20 days growth of seedlings the rate of production of O_2^- was measured based on the oxidation of epinephrine to adrenochrome according to the method of Mishra and Fridovich (1972). Increase in absorbance was recorded at 1 min interval up to 10 min at 480 nm using a spectrophotometer (Model SL 177, ELICO Ltd, and India). The rate of H_2O_2 production was measured spectrophotometrically using titanium sulphate following the method of Jana and Choudhuri (1981). 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 μM^{-1} cm^{-1}.

Determination of membrane injury, lipid peroxidation and protein oxidation

The extent of plasma membrane injury due to Mn treatment was determined in roots of the plants by a spectrophotometric assay of Evans blue stain retained by cells as described by (Baker and Mock 1994 and Ikegawa et al. 1998). After Mn treatment, roots were stained with a 0.025% (w/v) Evans blue solution and washed with 100 μM 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) at room temperature. The homogenate was centrifuged at $13,500 \times g$ for 10 min. The optical density of supernatant was measured spectrophotometrically at 600 nm.

The level of lipid peroxidation products was measured in terms of thiobarbituric acid reactive substances (TBARS) 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}.

To assess the extent of protein oxidation, protein thiol content was determined in roots and shoots. Fresh tissue ($\pm$ 150 g) was homogenized in 0.15% (w/v) sodium ascorbate solution and the homogenate was centrifuged at $30,000 \times g$ for 15 min at 4°C. Total thiol and non-protein thiol contents were determined in the supernatant using DTNB (5,5′-dithiobis-2 nitrobenzoic acid) following the method of de Kok and Kuiper (1986). The content of protein thiols was calculated by subtracting the content of non-protein thiols from total thiols and expressed in nmol g^{-1} fresh weight of tissues.

Determination of ascorbate and glutathione pool

The contents of reduced ascorbate (AsA), oxidized ascorbate (dehydroascorbate, DHA) and of total ascorbate (AsA + DHA) were determined using the method of Law et al. (1983). Fresh root/shoot samples weighing about 200 mg were homogenized using a chilled mortar and pestle in 5 ml of 5% (w/v) m-phosphoric acid. Following centrifugation at $10,000 \times g$ for 15 min at 4°C, the supernatant was used for the determination of AsA + DHA as well as AsA. For each sample DHA content was deduced from the difference between total ascorbate and AsA. AsA and DHA standards were between 0 and 5 mM in 5% (w/v) m-phosphoric acid. The contents of reduced glutathione (GSH), oxidized glutathione (GSSG) and total glutathione (GSH + GSSG) were determined according to Griffith (1980). Fresh root/shoot samples weighing 200 mg were homogenized in 5% (w/v) sulphosalicylic acid using a chilled mortar and pestle. The homogenate was centrifuged at $10,000 \times g$ for 15 min at 4°C. The supernatant obtained was used to quantify GSH and GSSG. GSH and GSSG standards were between 0 and 18 μM in 5% (w/v) sulphosalicylic acid diluted appropriately with 0.5 M KH$_2$PO$_4$ (pH 7.0).

Determination of antioxidative enzyme activities

SOD activity was determined using the method of Beauchamp and Fridovich (1971) based on the inhibition of p-nitro blue tetrazolium chloride (NBT) reduction by O$_2^-$ under light. Freshly uprooted root/shoot samples 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 $22,000 \times g$ for 10 min at 4°C, supernatants were dialyzed in cellophane membrane tubings for 6 h against the extraction buffer in cold with 3–4 changes of the buffer. In the supernatant total SOD activity was determined. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction measured at 560 nm. Activities of different isoenzymes of SOD were determined by incubating the enzyme extracts separately with 12 mM KCN (for Mn SOD and Fe SOD) or 10 mM H$_2$O$_2$ (for Mn SOD) for 30 min at 4°C prior to assay.

Catalase activity was assayed according to Beers and Sizer (1952). Fresh tissue ($\pm$ 200 mg) was homogenized using chilled mortar and pestle in 5 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 0.5 mM EDTA, 0.5% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at $22,000 \times g$ for 10 min at 4°C. The supernatant after dialysis in cellophane membrane tubings was used for CAT assay by measuring the decomposition of H$_2$O$_2$ at 240 nm (extinction coefficient of 0.036 mM^{-1} cm^{-1}) by observing decrease in absorbance using a UV–VIS spectrophotometer (Perkin Elmer, LAMBDA EZ 201, USA). Enzyme specific activity is expressed as μmol H$_2$O$_2$ oxidized mg^{-1}protein min^{-1}.

The activity of guaiacol peroxidase (GPX) was assayed according to Egley et al. (1983). Fresh tissue ($\pm$ 200 mg) was homogenized in 3 ml of chilled 50 mM Na- phosphate buffer (pH 7.0). The homogenate was centrifuged at $22,000 \times g$ for 10 min at 4°C and the supernatant after dialysis was used for enzyme assay. Increase in absorbance due to formation of tetraguaiacohinone was measured at 420 nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}) at 30 s intervals up to 2 min using spectrophotometer (Bausch and Lomb, Spectronic 20, USA). Enzyme specific activity is expressed as μmol H$_2$O$_2$ reduced mg^{-1} protein min^{-1}.

Ascorbate peroxidase activity was assayed using the method of Nakano and Asada (1981). Fresh tissue (± 200 mg) was homogenized in 3 ml of 50 mM K-phosphate buffer (pH 7.8) containing 1% (w/v) PVP, 1 mM AsA and 1 mM PMSF. After centrifugation at $22,000 \times g$ for 10 min at 4°C, the supernatant was dialyzed and used for enzyme assay. H_2O_2-dependent oxidation of AsA was followed by measuring decrease in absorbance at 290 nm (extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) using UV–VIS spectrophotometer. Enzyme specific activity is expressed as μmol AsA oxidized mg^{-1} protein min^{-1}. The activity of MDHAR was assayed according to (Hossain et al. 1984). Freshly uprooted root/shoot 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, which was just added prior to use. The homogenate was centrifuged at $22,000 \times g$ for 10 min at 4°C and in the supernatant MDHAR was assayed by measuring the decrease in absorbance at 340 nm due to NADH oxidation using UV–VIS spectrophotometer. Enzyme specific activity is expressed as nmol NADH oxidized mg^{-1} protein min^{-1}. The enzyme extract for DHAR was prepared as described for MDHAR and the activity was determined according to Doulis et al. (1997) by measuring reduction of DHA in the presence of GSH at 265 nm using UV–VIS spectrophotometer after accounting for the non-enzymic reduction of DHA by GSH. Enzyme specific activity is expressed as nmol DHA reduced mg^{-1} protein min^{-1}. Glutathione reductase activity was assayed using the method of Schaedle and Bassham (1977). Fresh root/shoot samples weighing about 200 mg were homogenized using a chilled mortar and pestle in 5 ml of 50 mM Tris–HCl buffer (pH 7.6) containing 1.0 mM EDTA, 0.1 mM Triton X-100 and 2% PVP. The homogenate was centrifuged at $22,000 \times g$ for 30 min at 4°C and in the supernatant GR was assayed following the oxidation of NADPH at 340 nm. Enzyme specific activity is expressed as nmol NADPH oxidized mg^{-1} protein min^{-1}.

Protein estimation

In all enzyme 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 indicate mean ± S.D. based on three independent determinations. Differences among control and treatments were analyzed by one factorial ANOVA followed by Tukey's test. Asterisks (*) were used to identify the level of significance of the difference between control and Mn treatments as: $*P \leq 0.05$ and $**P \leq 0.01$.

Results

Effect of Mn on growth of rice seedlings and Mn content in the tissues

When growth of rice seedlings was recorded during 5–20 days period in sand cultures, it was observed that 3 and 6 mM $MnCl_2$ in the growth medium caused a significant inhibition in length and fresh weight of the seedlings (Fig. 1). The extent of inhibition increased with increase in concentration of $MnCl_2$ and exposure time. Rice seedlings grown for 20 days in presence of 6 mM $MnCl_2$ showed nearly 44% ($P \leq 0.01$) reduction in length of roots and 43% ($P \leq 0.01$) reduction in shoots compared to the control grown seedlings. Under similar conditions about 41% ($P \leq 0.01$) decline in fresh wt. of roots and 43% ($P \leq 0.01$) decline in shoots were noted compared to controls. Uptake of Mn increased in the seedlings with increasing concentration of $MnCl_2$ in the growth medium and increase in exposure time (Fig. 1). Concentration of absorbed Mn was greater in shoots than in roots. Rice seedlings exposed to 6 mM $MnCl_2$ for 20 days showed 352.24 μg Mn^{2+} g^{-1} dry wt. in roots and 365.13 μg g^{-1} dry wt. in shoots.

Effect of Mn on generation of reactive oxygen species

In comparison to controls generation of O_2^- increased in both roots as well as shoots of the rice seedlings with increasing concentration of Mn treatment and time of its exposure (Fig. 2). Rice seedlings exposed to 6 mM Mn^{2+} for 20 days showed nearly 197% ($P \leq 0.01$) increased O_2^- level in roots and 199% ($P \leq 0.01$) increased level in shoots in comparison to controls. Similarly the level of H_2O_2 was higher in Mn treated seedlings compared to controls (Fig. 2). With 6 mM Mn treatment for 20 days about 28% ($P \leq 0.05$) increased H_2O_2 level was observed in roots and 61% ($P \leq 0.01$) increased level in shoots. The level of H_2O_2 was always higher in shoots than in roots in both control as well as Mn treated seedlings.

Effect of Mn on lipid peroxidation and protein thiolation

The level of lipid peroxidation was measured in terms of TBARS production. TBARS content increased progressively in the seedlings with increase in the concentration of Mn treatment and duration of exposure (Fig. 3). The increase in TBARS content due to Mn treatment was
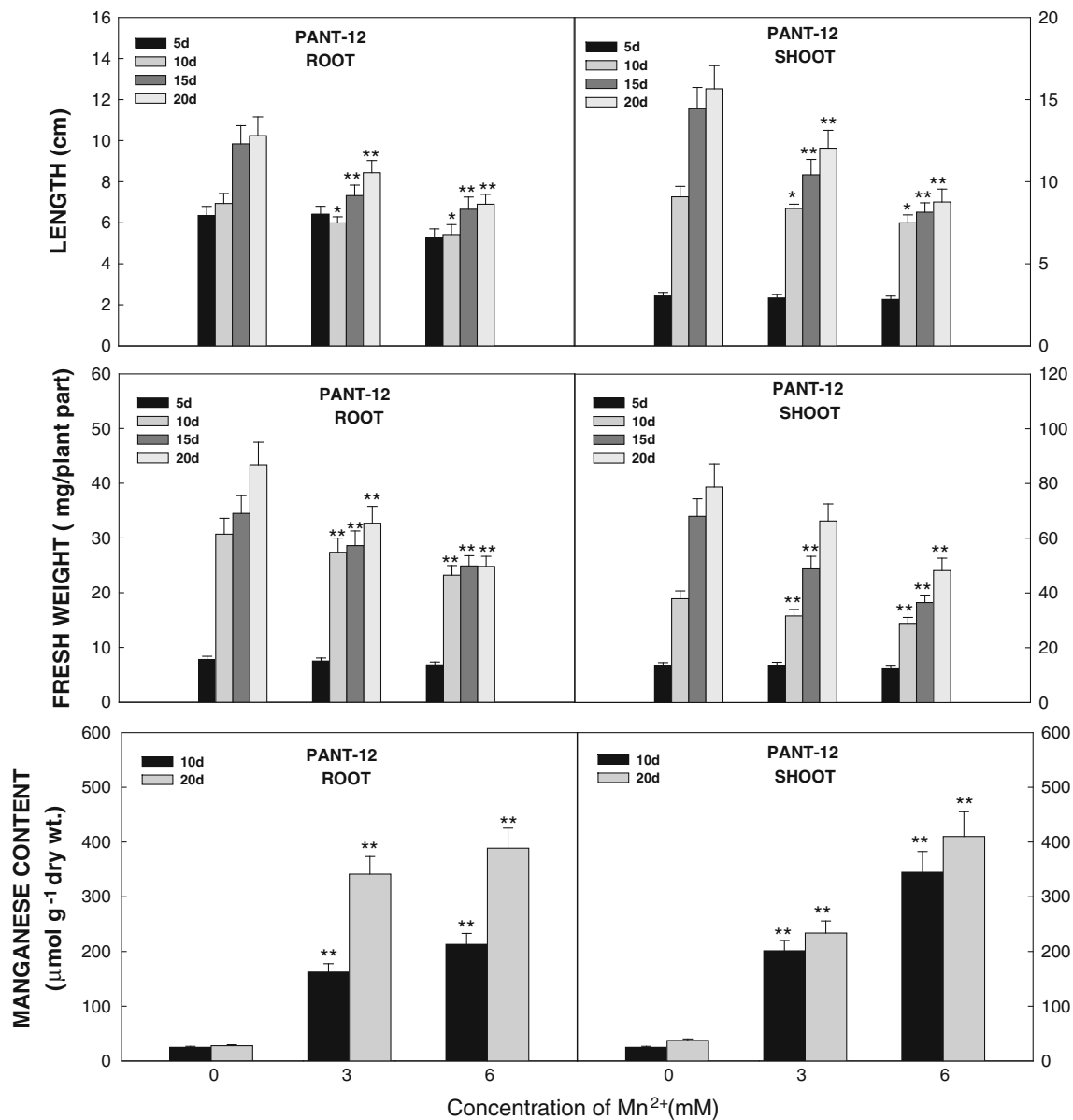
Fig. 1 Effect of increasing Mn concentration in the growth medium on the length, fresh weight and Mn content of roots and shoots of rice seedlings at 5–20 days of growth. Values are mean ± SD based on three independent determinations and *bars* indicate standard deviations. * and ** indicate values that differ significantly from controls at $P \leq 0.05$ and $P \leq 0.01$, respectively according to Tukey's multiple range test

greater in shoots than in roots. Rice seedlings grown in presence of 6 mM MnCl$_2$ for 20 days showed about 22% increased TBARS level in roots and 63% ($P \leq 0.01$) increased level in shoots compared to the levels in controls. In response to Mn treatment a significant decline in protein thiol level was observed in the seedlings (Fig. 3). Rice seedlings grown for 20 days in presence of 6 mM MnCl$_2$ showed about 38% ($P \leq 0.01$) decline in protein thiol level in roots and 52% ($P \leq 0.01$) 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 Mn treatments. The level of non protein thiol, however,

increased significantly ($P \leq 0.01$) due to Mn treatment in 10 days grown seedlings however, no definite trend could be observed after prolonged Mn exposure for 20 days. Seedlings exposed to 6 mM MnCl$_2$ for 10 days showed 58% ($P \leq 0.01$) increase in non protein thiol level in roots and 211% ($P \leq 0.01$) increased level in shoots compared to controls.

Effect of Mn treatment on membrane damage

Uptake of the dye Evans blue has been widely used as an indicator of loss of plasma membrane integrity as well as
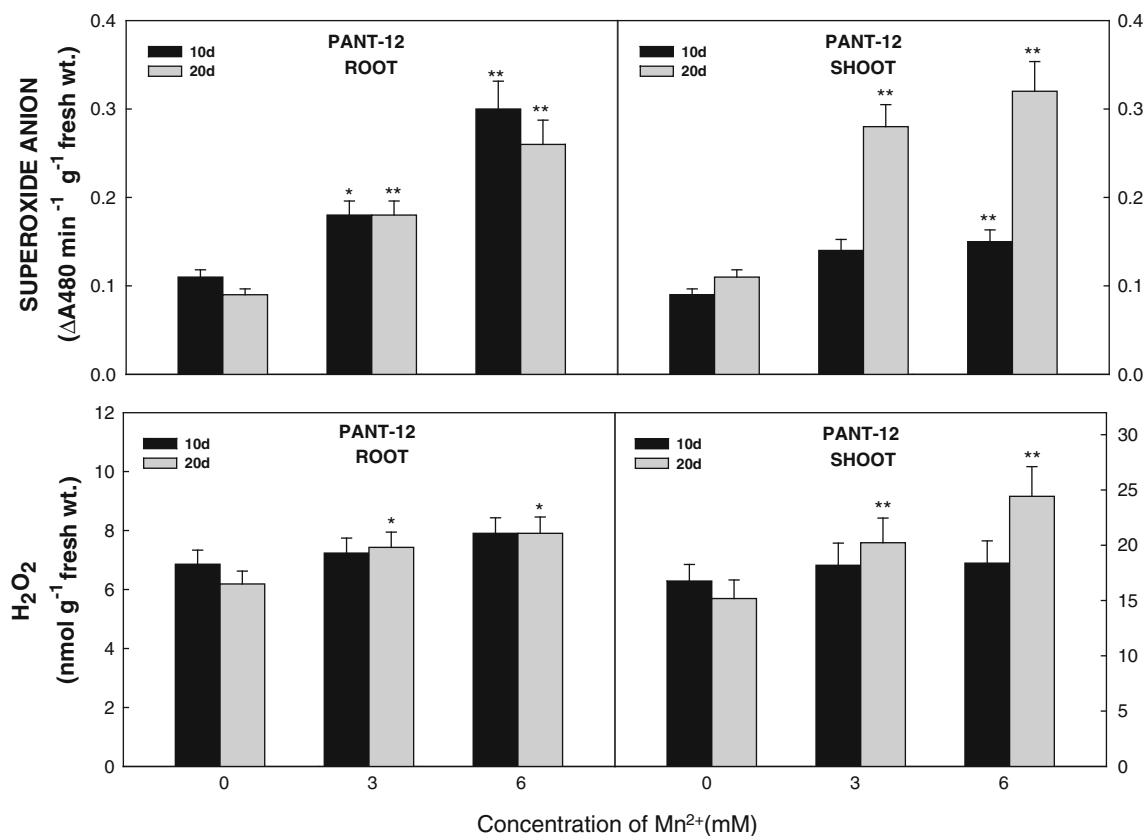
Fig. 2 Effect of increasing Mn concentration in the growth medium on the root and shoot contents of superoxide anion and hydrogen peroxide (H_2O_2) in rice seedlings at 10 and 20 days of growth. Values are mean ± SD based on three independent determinations and *bars* indicate standard deviations. * and ** indicate values that differ significantly from controls at $P \leq 0.05$ and $P \leq 0.01$, respectively according to Tukey's multiple range test

an indicator of cell death. In our experiments the uptake of Evans blue increased in the roots of Mn-treated rice seedlings compared to controls (Table 1). Mn treatment led to nearly 80% uptake of Evans blue in roots of 20 days grown rice seedlings. These observations indicate an increase in permeability of root plasma membrane due to Mn treatment.

Effect of Mn on nonenzymic antioxidants

The content of AsA and its oxidized form DHA as well as the ratio of AsA/DHA declined in rice seedlings with Mn treatment (Fig. 4). Seedlings exposed to 6 mM $MnCl_2$ for 20 days showed nearly 48% ($P \leq 0.01$) decline in AsA level in roots and 65% ($P \leq 0.01$) decline in shoots, whereas under similar conditions DHA level declined by 27% ($P \leq 0.05$) in roots and by 32% ($P \leq 0.01$) in shoots. Mn treatment caused greater decline in the level of AsA than DHA in the seedlings. The level of GSH declined significantly in rice seedlings due to Mn treatment, whereas its oxidized form GSSG showed increased level in moderately Mn (3 mM) stressed seedlings and it declined due

to higher level (6 mM) of Mn treatment (Fig. 5). In seedlings exposed to 6 mM $MnCl_2$ for 20 days about 40% ($P \leq 0.01$) decline in GSH level was observed in roots and 46% ($P \leq 0.01$) decline in shoots compared to the level in controls. The ratio GSH/GSSH showed a consistent decline in the seedlings with increase in the concentration and length of Mn treatment.

Effect of Mn on activity of antioxidative enzymes

The activity of total SOD as well as its isoforms Mn SOD, Cu/Zn SOD and Fe SOD increased consistently in both roots and shoots of rice seedlings with increasing concentration of Mn in the growth medium (Fig. 6). About 75% ($P \leq 0.01$) increase in total SOD activity was observed in roots and 102% ($P \leq 0.01$) increased activity in shoots of 6 mM Mn^{2+}- stressed 20 days grown rice seedlings. Among the isoforms of SOD, nearly 95% ($P \leq 0.01$) increase in activity of Mn SOD, 102% ($P \leq 0.01$) increase in Cu/Zn SOD and about 50% ($P \leq 0.01$) increase in Fe SOD activity were observed in the shoots of 6 mM Mn^{2+} stressed seedlings.
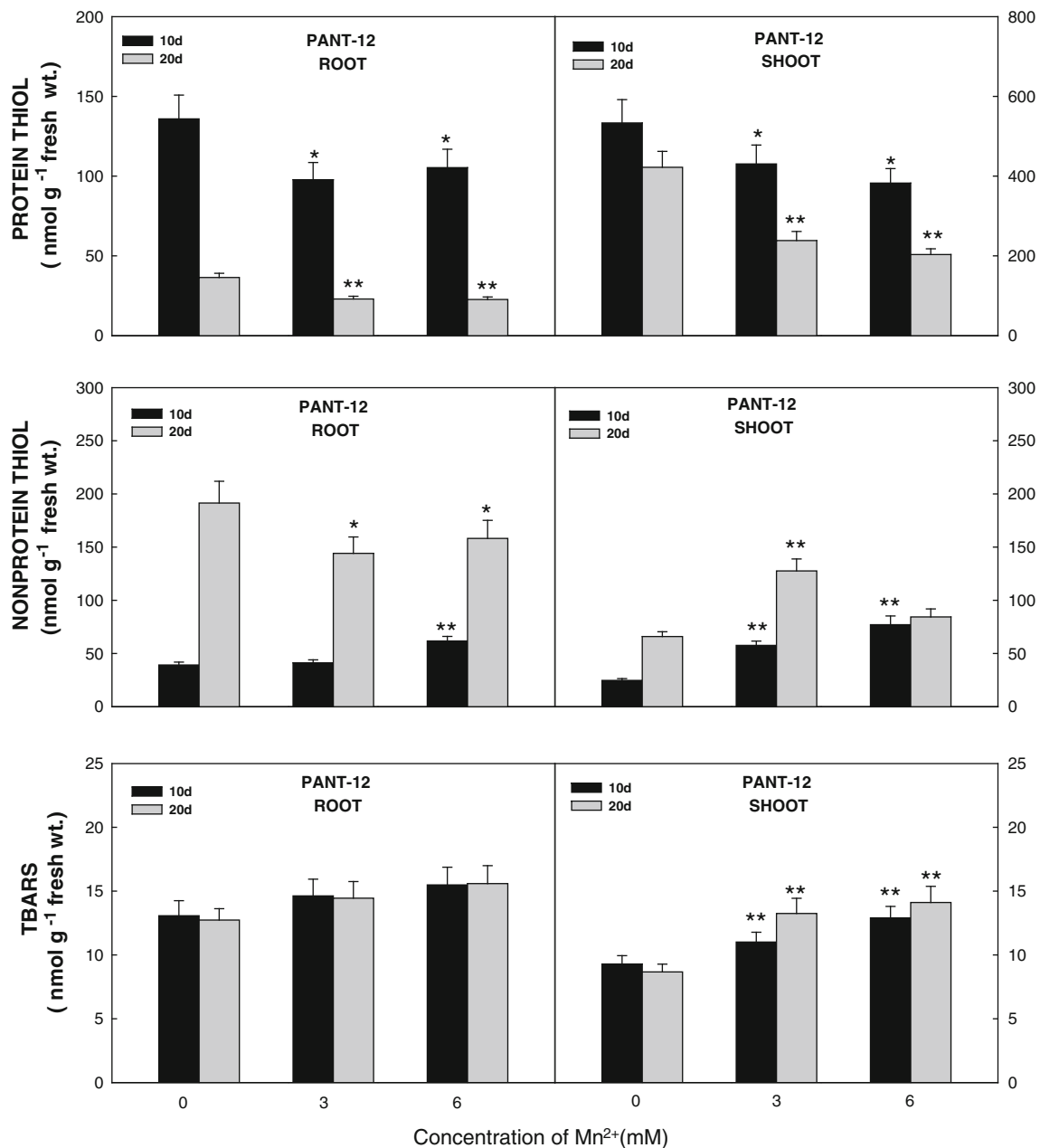
Fig. 3 Effect of increasing Mn concentration in the growth medium on the root and shoot contents of protein thiol, nonprotein thiol and thiobarbituric acid reactive substances (TBARS) in rice seedlings at 10 and 20 days of growth. Values are mean ± SD based on three independent determinations and *bars* indicate standard deviations. * and ** indicate values that differ significantly from controls at $P \leq 0.05$ and $P \leq 0.01$, respectively according to Tukey's multiple range test

The activity of CAT showed varying behaviour in the seedlings depending on the length of Mn treatment (Fig. 7). The activity of enzyme increased in both roots and shoots due to Mn treatment in 10 days grown seedlings, whereas under prolonged exposure of seedlings to Mn for 20 days, decline in CAT activity was observed.

The activity of GPX showed a concomitant increase in the seedlings with increase in concentration and length of Mn treatment (Fig. 7). Nearly 47% ($P \leq 0.01$) increase in

GPX activity was observed in roots and 99% ($P \leq 0.01$) increase in shoots of 20 days grown seedlings exposed to 6 mM $MnCl_2$.

To elucidate the mechanism of redox balance maintenance under Mn toxicity, we measured the activity of the enzymes involved in the ascorbate–glutathione cycle. A marked increase in the activity of APX was observed in both roots and shoots of the seedlings with increasing concentration and length of Mn treatment (Fig. 7).

Table.1 Effect of increasing concentrations of MnCl2 in the growth medium on membrane damage, measured in terms of percent of evans blue uptake, in roots of 10 and 20 days grown rice seedlings

Evans blue uptake (%)

Rice cultivar	Mn treatment	10 days	20 days
Pant-12	Control	0	0
	3 mM MnCl2	9.5 0.665**	67.8 4.74**
	6 mM MnCl2	26.1 2.34**	80.3 7.23**

Data represent mean values ± SD based on three independent determinations

** indicates values that differ significantly from controls at $P \leq 0.01$ according to Tukey's multiple range test

Seedlings exposed to 6 mM Mn^{2+} for 20 days showed about 119% ($P \leq 0.01$) increase in APX activity in roots and 91% ($P \leq 0.01$) increased activity in shoots compared to activity in controls.

Similarly, with Mn treatment a significant increase in MDHAR activity was observed in the seedlings (Fig. 8). The increase in enzyme activity due to Mn treatment was greater in shoots than in roots. Rice seedlings grown for 20 days in presence of 6 mM MnCl$_2$ showed about 58% ($P \leq 0.01$) increase in MDHAR activity in roots and about 201% ($P \leq 0.01$) increase in activity in shoots compared to the controls. Similar to MDHAR, the activity of DHAR also increased in seedlings due to Mn treatment (Fig. 8). Under 6 mM Mn treatment for 20 days, seedlings showed about 68% ($P \leq 0.01$) increased DHAR activity in roots and 91% ($P \leq 0.01$) increased activity in shoots. Glutathione reductase, which catalyses the NADPH-dependent reduction of oxidized glutathione showed increased activity in the rice seedlings with increasing concentration of Mn treatment (Fig. 8). Rice seedlings raised under 6 mM MnCl$_2$ for 20 days showed about 57% ($P \leq 0.01$) increased GR activity in roots and 219% ($P \leq 0.01$) increased activity in shoots compared to the activity in control grown seedlings.

Discussion

In acidic soils due to high availability of Mn, its uptake increases by plant roots (Shi et al. 2006). Mn excess retards plant growth by interfering with normal cellular metabolic events (Subrahmanyam and Rathore 2000; Fecht-Christoffers et al. 2003). Oxidative stress has been implicated as one of the major events leading to cellular damage due to toxicity of many metals (Srivastava et al. 2005; Shi et al. 2006; Sandalio et al. 2009). Therefore, the present study was undertaken to examine whether oxidative stress is involved in the expression of Mn toxicity during establishment of rice seedlings, in order to contribute to our understanding of a possible relationship between Mn

excess and oxidative stress in rice tissues as well as to observe the response of antioxidative defense system of rice seedlings, when exposed to Mn-excess.

In our experiments significant inhibition in growth of rice seedlings was observed with 3 and 6 mM MnCl$_2$ in the growth medium. With increasing Mn concentration and exposure time, root/shoot length and fresh weights of the seedlings declined and accumulation of Mn increased in the seedlings, with greater accumulation in shoots than in roots. Rice, in general, is a crop which is fairly tolerant to Mn, however, great genotypic variation exists among the rice varieties for their responses towards Mn toxicity and its accumulation in the tissues (Nelson 1983). The relative Mn tolerance could be attributed to greater oxidizing power of rice roots that could oxidize the toxic forms of Mn (Mn^{2+}) into less toxic forms (Mn^{4+}) (Wang et al. 2002).

Most of the stressful conditions of the environment activate a common response involving the overproduction of ROS such as O_2^- and H_2O_2 in plant cells. Our results showed increased levels of O_2^- and H_2O_2 in the rice seedlings exposed to Mn excess. Similar results showing increased production of ROS due to toxicity of various metals have been shown in many crop species (Wang et al. 2004; Shi et al. 2005; Maheshwari and Dubey 2009; Sandalio et al. 2009). These overproduced ROS can cause oxidative damage to the biomolecules such as membrane lipids, proteins, chloroplastic pigments, enzymes, nucleic acids, etc. Lipid peroxidation is regarded as an effective indicator of cellular oxidative damage (Verma and Dubey 2003). Our results showed increased TBARS level in rice seedlings growing in presence of excess Mn which suggests that Mn induces oxidative stress in rice seedlings. Many metals such as Cd, Pb, Al and Ni have been shown to cause increased production of ROS and induce lipid peroxidation in rice seedlings (Shah et al. 2001; Verma and Dubey 2003; Sharma and Dubey 2007; Maheshwari and Dubey 2009). Mn excess caused elevated level of H_2O_2 and induced oxidative stress in barley, *Cucumis sativus* and *Populus cathayana* plants (Demirevska-Kepova et al. 2004; Shi et al. 2006; Lei et al. 2007). In our studies Mn induced oxidative stress paralleled with increase in permeability of root plasma membrane.

Plants possess efficient antioxidative defense system comprising of non-enzymic and enzymic components that protect them from destructive oxidative reactions. An enhanced level of antioxidative components is often correlated with increased stress tolerance of plants (Fecht-Christoffers et al. 2003; Shi et al. 2005). In our experiments reduced forms of non-enzymic antioxidants AsA and GSH as well as their redox ratios declined in the seedlings with increasing concentration and length of Mn exposure. This suggests that Mn toxicity in rice seedlings favours depletion of these antioxidants in the cells. Both AsA and GSH
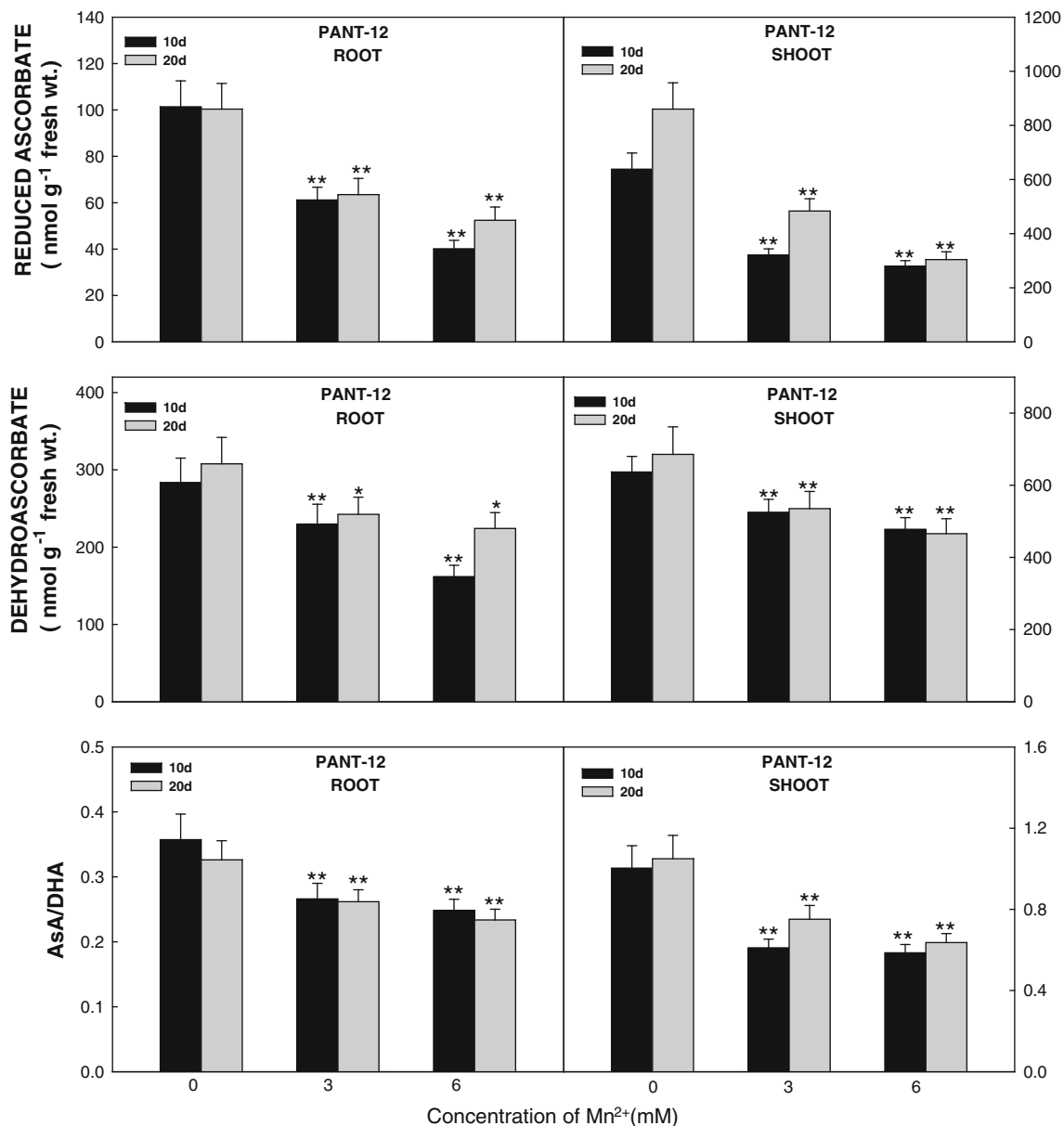
Fig. 4 Effect of increasing Mn concentration in the growth medium on the root and shoot levels of reduced ascorbic acid (AsA), dehydroascorbic acid (DHA) and their redox ratio (AsA/DHA) in rice seedlings at 10 and 20 days of growth. Values are mean ± SD based on three independent determinations and *bars* indicate standard deviations. * and ** indicate values that differ significantly from controls at $P \leq 0.05$ and $P \leq 0.01$, respectively according to Tukey's multiple range test

are involved in redox regulation of the cell cycle and their optimum levels in the cells and a high ratio of their reduced to oxidized forms is required for efficient removal of ROS and to maintain proper defense under abiotic stresses (Kocsy et al. 2004; Maheshwari and Dubey 2009). The depletion of AsA and GSH levels as observed in our studies under Mn toxicity could be due to enhanced rate of their utilization or decreased rate of their synthesis. A concomitant decline in the ratio of reduced to oxidized states of AsA and GSH in rice seedlings and a shift in redox state

of cells towards oxidized forms suggests that Mn toxicity induced oxidative damage in rice seedlings is associated with alteration in redox states of these antioxidants. Toxic levels of the metals Cu, Cd, Ni and Al have been shown to cause oxidation of glutathione in many plant species (Rao and Sresty 2000; Sharma and Dubey 2007). Rice plants exposed to Cd and Ni showed a decline in GSH level (Klapheck et al. 1994; Maheshwari and Dubey 2009). It is suggested that high AsA/DHA ratio prevents oxidative injury in plants (Fecht-Christoffers et al. 2003). In Ni
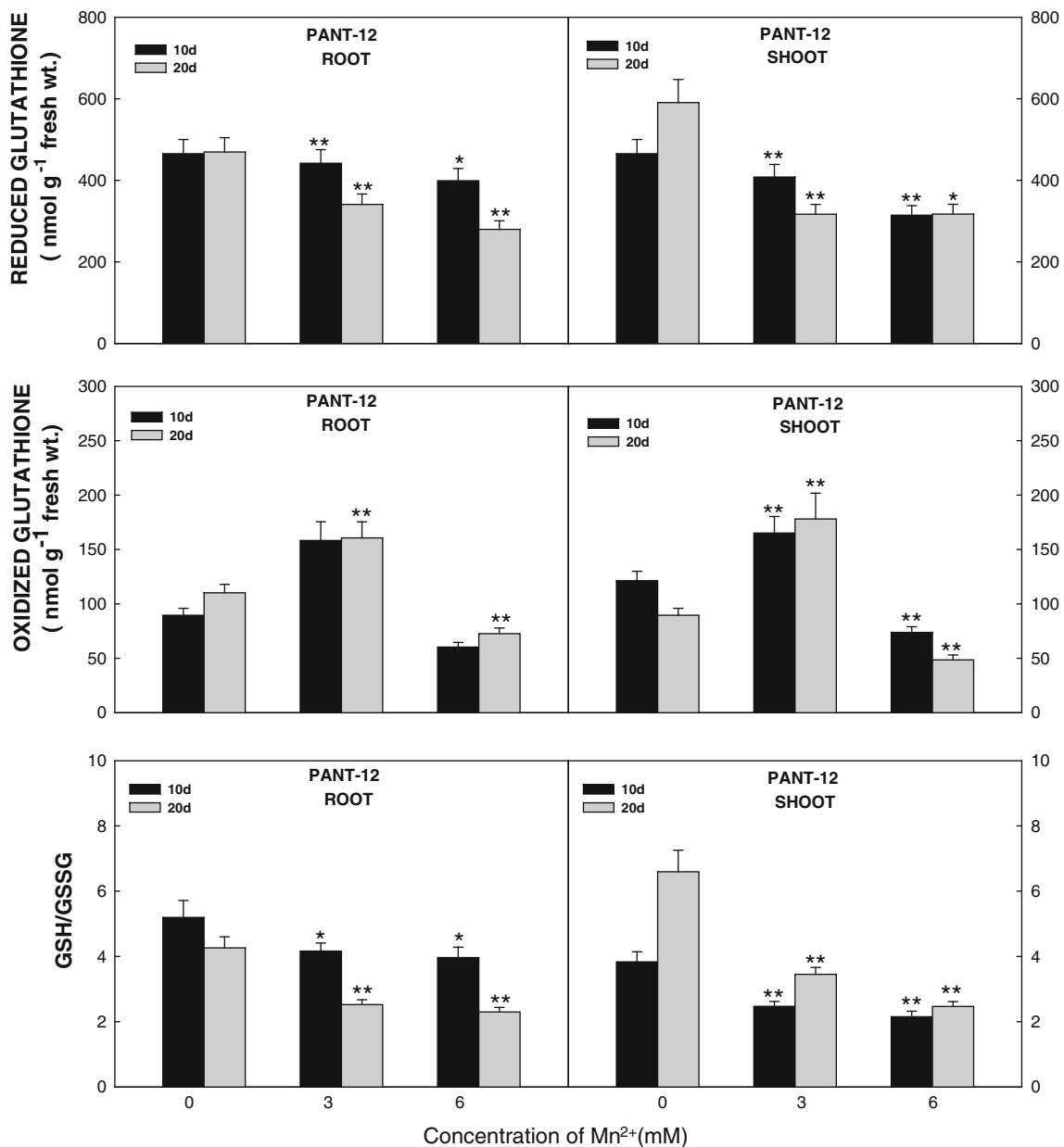
Fig. 5 Effect of increasing Mn concentration in the growth medium on the root and shoot levels of reduced glutathione (GSH), oxidized glutathione (GSSG) and their redox ratio (GSH/GSSG) in rice seedlings at 10 and 20 days of g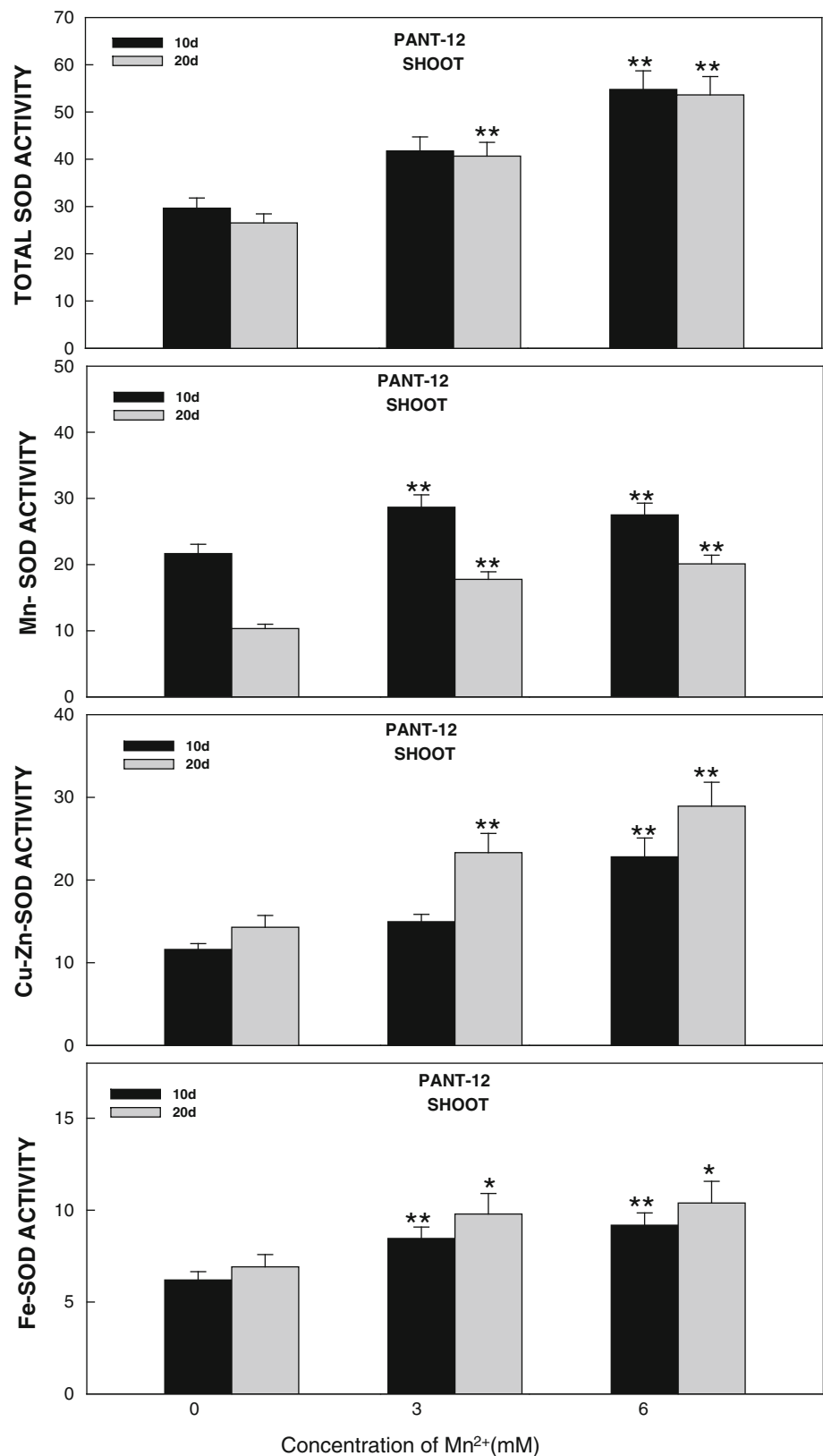rowth. Values are mean ± SD based on three independent determinations and *bars* indicate standard deviations. * and ** indicate values that differ significantly from controls at $P \leq 0.05$ and $P \leq 0.01$, respectively according to Tukey's multiple range test

treated rice plants decline in AsA/DHA ratio was attributed to decreased antioxidant capacity (Maheshwari and Dubey 2009).

The concentration of protein thiol declined in the seedlings with Mn exposure whereas specially in the shoots an increase in non-protein thiol level was observed under Mn toxicity. A decline in protein thiol level indicates oxidation of—SH groups of proteins under Mn toxicity. In rice the pool of non-protein thiol comprises of cysteine, γ-glutamyl cysteine, GSH, hydroxymethyl GSH, phytochelatins [PCs γ-(Glu-Cys)$_n$-Gly] and hydroxymethyl phytochelatins (hm-PCs) (De Vos et al. 1992; Klapheck et al. 1994). In our studies a decline in the level of GSH and an increase in the level of non-protein thiol specially in shoots indicate that Mn toxicity might favour induction of the synthesis of PCs and hm-PCs and other low mol wt thiol rich compounds which are known to play a role in the detoxification mechanism against metals in plants (Zenk 1996; Rauser 1999). GSH and its homologue hm-GSH are well known antioxidants in rice and are precursors for the

Fig. 6 Effect of increasing Mn concentration in the growth medium on the root and shoot activities of total superoxide dismutases (SOD), Mn SOD, Cu/Zn SOD and Fe SOD in rice seedlings at 10 and 20 days of growth. Activities are expressed as units mg^{-1} protein. Values are mean ± SD based on three independent determinations and *bars* indicate standard deviations. * and ** indicate values that differ significantly from controls at $P \leq 0.05$ and $P \leq 0.01$, respectively according to Tukey's multiple range test

synthesis of PCs and hm-PCs, respectively (Klapheck et al. 1994). Cd treated rice plants showed decline in GSH content and increased levels of γ-Glu-Cys peptides and PCs

(Klapheck et al. 1994). Metal induced decline in GSH level due to increased synthesis of PCs increases susceptibility of plants to oxidative stress (Galli et al. 1996). An increase in
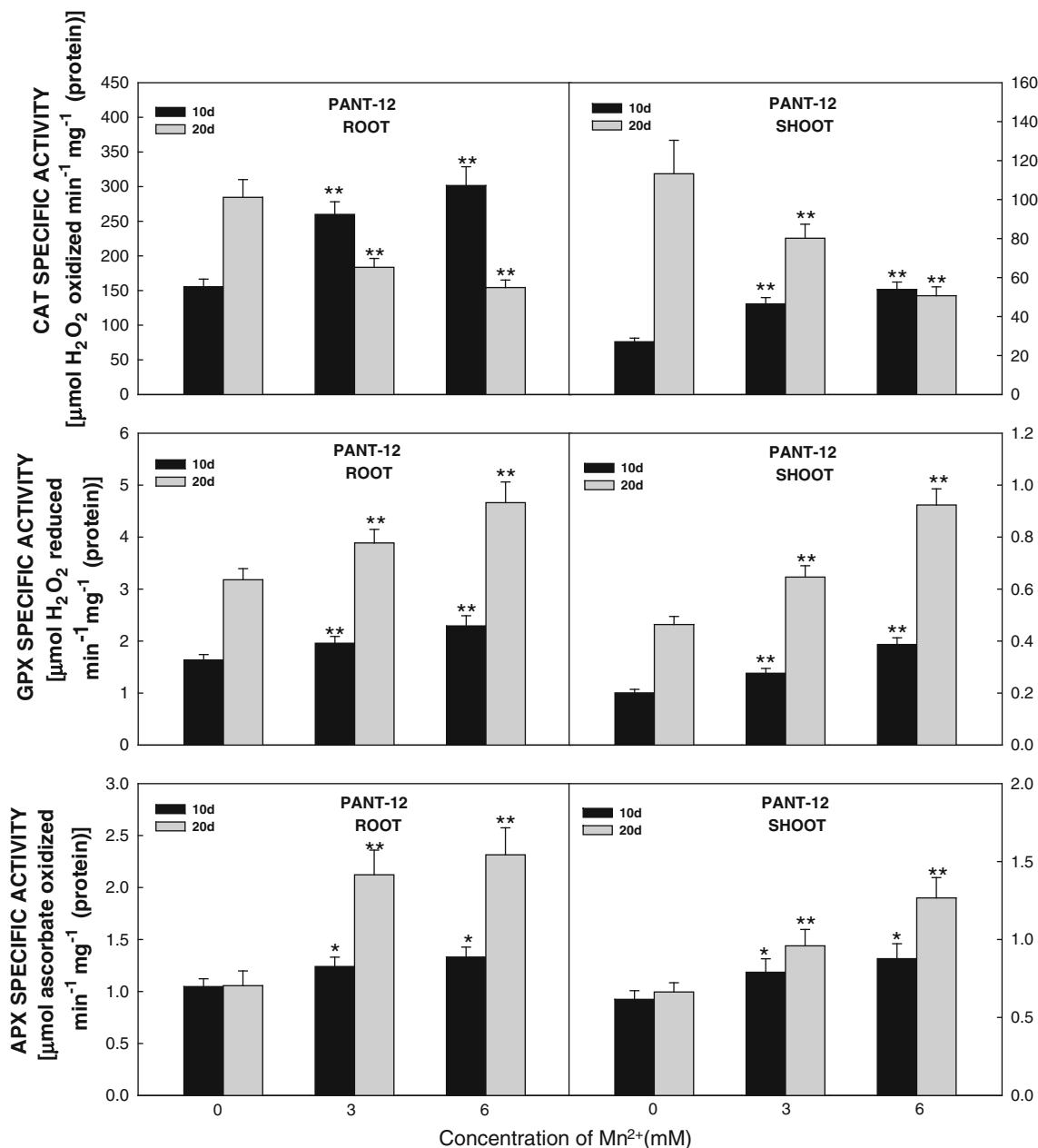
Fig. 7 Effect of increasing Mn concentration in the growth medium on the root and shoot activities of catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) in rice seedlings at 10 and 20 days of growth. Values are mean ± SD based on three independent determinations and *bars* indicate standard deviations. * and ** indicate values that differ significantly from controls at $P \leq 0.05$ and $P \leq 0.01$, respectively according to Tukey's multiple range test

non-protein thiol level has been documented in Cu-treated barley (Demirevska-Kepova et al. 2004) and Ni-treated rice seedlings (Maheshwari and Dubey 2009).

Reactive oxygen species are scavenged enzymatically through a complex and elaborate coordination of antioxidative enzymes (Apel and Hirt 2004). Among these enzymes SODs play important role in scavenging O_2^- by catalyzing the dismutation of two molecules of O_2^- into O_2 and H_2O_2 and serve as first line of defense against toxic

O_2^- (Wang et al. 2005). Changes in the activity of SOD are used as a indicator of changes in O_2^- production (Wang et al. 2005). In the present study increased activity of SOD and its three isoforms Mn SOD, Cu/Zn SOD and Fe SOD were observed due to Mn treatment in rice seedlings. Increased SOD activity under Mn toxicity suggests induction of a protection mechanism in Mn-stressed plants to protect the cells from oxidative damage caused by O_2^-. Similar increase in SOD activity was observed in common
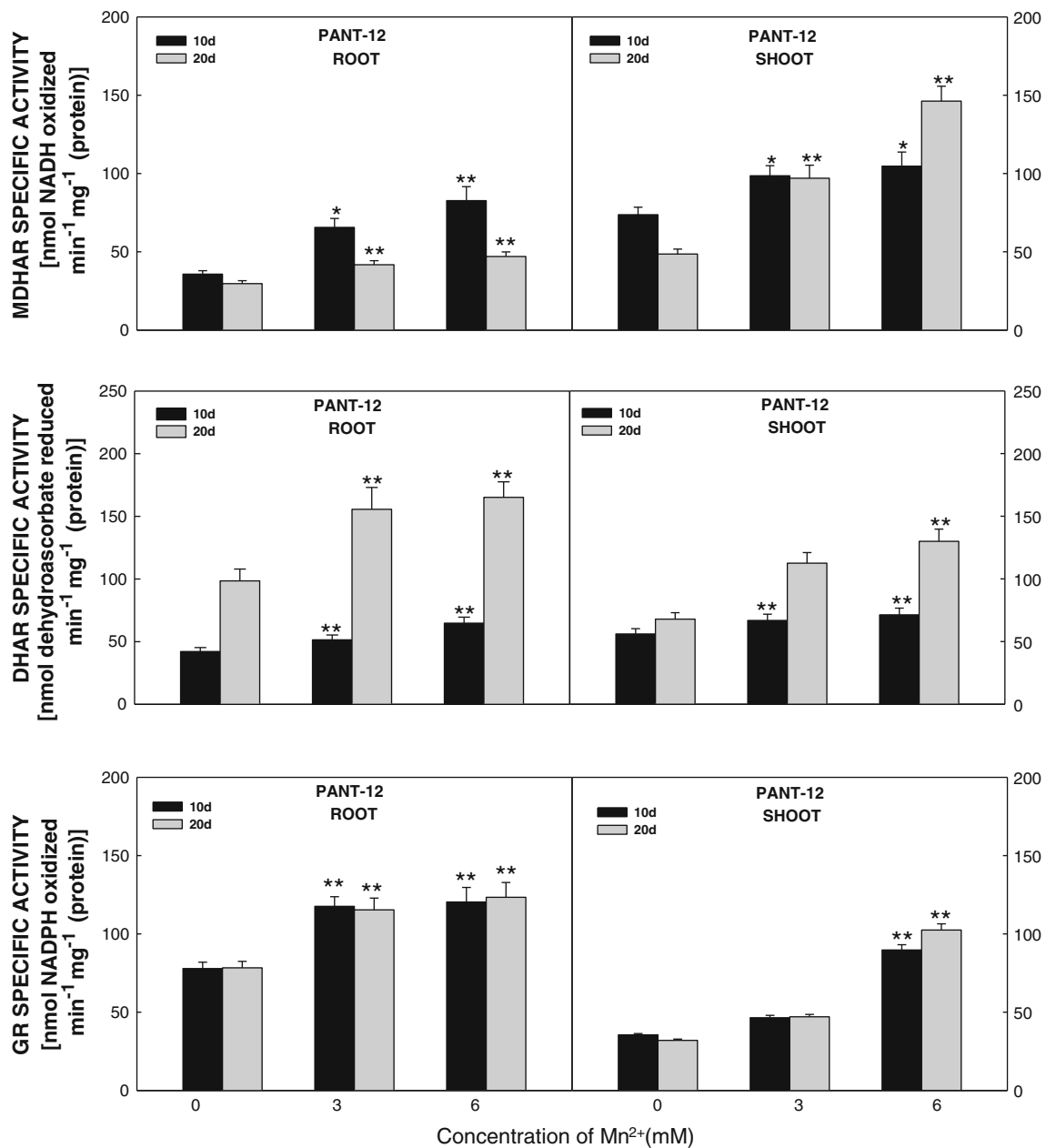
Fig. 8 Effect of increasing Mn concentration in the growth medium on the root and shoot activities of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) in rice seedlings at 10 and 20 days of growth. Values are mean $\pm$ SD based on three independent determinations and *bars* indicate standard deviations. * and ** indicate values that differ significantly from controls at $P \leq 0.05$ and $P \leq 0.01$, respectively according to Tukey's multiple range test

bean, cucumber and tomato plants on Mn exposure (Shi and Zhu 2008; Shenker et al. 2004), whereas in barley plants no alteration in SOD activity could be observed at Mn excess (Demirevska-Kepova et al. 2004). Our results thus suggest that different isoforms of SOD may confer protection to the rice plants from oxidative damage caused by O_2^- under Mn excess. Among different isoforms of SOD, the constitutive and induced levels of Cu/ZnSOD and MnSOD were greater than FeSOD in control and Mn

treated seedlings. Other studies have also reported low Fe-SOD activity compared to other SOD isoforms in lentil and rice plants (Bandeoglu et al. 2004; Maheshwari and Dubey 2009).

Catalase and peroxidase are the enzymes involved in the decomposition of H_2O_2 produced in cells due to higher SOD activity (Apel and Hirt 2004). In our studies, though an increase in CAT activity was observed during early growth stage (10 days) of rice seedlings due to Mn

treatment, with prolonged exposure of seedlings to Mn for 20 days a decline in CAT activity was observed. An increase in CAT activity due to Mn treatment during early growth stage (10 days) suggests that during this period H_2O_2 scavenging mechanism by CAT is very effective in the seedlings, whereas under prolonged Mn exposure CAT activity does not remain enough for adequate removal of H_2O_2. Varying observations have been reported for alterations in CAT activity under abiotic stresses. The activity of CAT increased in plants subjected to salinity and toxicity of certain heavy metals, allowing active scavenging of H_2O_2 (Hsu and Kao 2004; Kim et al. 2005), whereas plants exposed to the metals Pb, Cd, Cu,Ni and Al showed decline in CAT activity (Gonnelli et al. 2001; Verma and Dubey 2003; Sharma and Dubey 2007). No definite pattern of alteration in CAT activity could be noticed when rice seedlings were subjected to Ni treatment (Maheshwari and Dubey 2009). Decline in CAT activity in metal exposed plants could be attributed to either inactivation of enzyme due to its direct interaction with ions or ROS (Dat et al. 2000) or due to its decreased synthesis or impaired protein assembly (Ushimaru et al. 1999).

Peroxidases play important role in scavenging H_2O_2 in plants however, under excess Mn, its function becomes more complex. The oxidation of Mn^{2+} by a H_2O_2 consuming peroxidase has been proposed to be the key reaction leading to Mn toxicity symptoms (Shi et al. 2005). Activities of GPXs have been used as potential biomarkers for accessing sublethal, metal induced injury in plants (Radotic et al. 2000). Our results indicated a concomitant increase in the activity of GPX in the seedlings in response to increasing concentration and length of Mn exposure. Other studies have also reported increase in GPX activity in *Vigna*, barley and cucumber leaves under Mn excess (Fecht-Christoffers et al. 2003; Demirevska-Kepova et al. 2004; Shi et al. 2005). Our observations suggest a potential defensive role of GPX against Mn induced oxidative stress in rice plants in scavenging overproduced H_2O_2. Enzymes of Halliwell-Foyer-Asada cycle, which play important role in detoxification of ROS through successive oxidation and reduction reactions involving AsA and GSH (Kuźniak and Sklodowska 1999), were studied in 10 and 20 days grown rice seedlings with increasing concentration of Mn exposure. The activity of APX increased in the seedlings with increasing Mn concentration and exposure time. Several studies have reported increased activity of APX in plants exposed to toxic levels of the metals Cd (Shah et al. 2001), Pb (Verma and Dubey 2003), Ni (Gajewska and Sklodowska 2008; Maheshwari and Dubey 2009) and Mn (Shi et al. 2005). It is suggested that H_2O_2 acts as a systemic intracellular signal for the induction of APX under abiotic stresses (Hernandez et al. 2004).

In our experiments, under prolonged Mn-exposure of rice seedlings, among the enzymes involved in removal of H_2O_2 the activity of CAT declined whereas GPX and APX activities increased. This suggests that in Mn-stressed rice seedlings GPX and APX have important role in H_2O_2 scavenging, however, such increased activity is not enough to sufficiently eliminate H_2O_2 overproduced due to Mn excess.

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 with increasing Mn concentrations. Increased acitivies of all the enzymes MDHAR, DHAR and GR in rice seedlings due to Mn exposure suggests that Mn induces GSH and AsA regenerating system in order to maintain their requisite levels in the tissues under adverse conditions of Mn toxicity. Other studies have also reported enhanced activity of the enzymes of AsA-GSH cycle in different plant species exposed to the metals Zn, Mn, Cd and Al (Fecht-Christoffers et al. 2003; Sharma and Dubey 2007; Maheshwari and Dubey 2009). Despite increased activities of the AsA-GSH regenerating enzymes APX, MDHAR, DHAR and GR under Mn excess, a decline in the levels of these two key antioxidants AsA and GSH was observed in Mn-treated rice seedlings. This suggests that the enhanced activities of these enzymes are not sufficient for substantial reduction of oxidized forms of these antioxidants to their reduced forms and to maintain a proper cellular redox balance in Mn-stressed plants.

In conclusion, results of the present study suggest that Mn toxicity under acidic conditions is associated with induction of oxidative stress in rice seedlings leading to increased generation of O_2^- and H_2O_2, increased lipid peroxidation and protein oxidation and a decline in redox ratios of AsA and GSH. Among the antioxidative enzymes SOD, GPX APX and GR appear to play a key role in scavenging O_2^- and H_2O_2 under Mn excess.

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