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Expression of key antioxidant enzymes under combined effect of heat and cadmium toxicity in growing rice seedlings

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Abstract Effect of Cd^{2+} toxicity and heat stress in sensitive rice *cv.* DR-92 and tolerant rice *cv.* Bh-1 grown in North East region of India were studied in sand cultures. Increasing levels of 0–500 μM Cd^{2+} alone and/or heat stress showed increased activities of superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase enzymes which were associated with induced oxidative stress and altered enzyme activities. The values for SOD and POD activities were always more in *cv.* DR-92 whereas CAT and GR activities were higher in *cv.* Bh-1 in roots and shoots under Cd^{2+} or heat stress alone in sensitive *cv.* DR-92. Upon imposition of a combination of Cd^{2+} + heat the activities of SOD and POD decreased significantly in root/shoot of both the sensitive and tolerant rice varieties. A nine fold increase in GR activity under combination of heat + 100 μM Cd^{2+} stress in shoots of *cv.* Bh-1 at day 15 was noted when compared to controls. The dual stress combination of Cd^{2+} + heat did not alter catalase activity in vivo in both the rice varieties. Results suggest a time-specific and varietal distribution of the antioxidant enzymes in rice plants subjected to Cd^{2+} and/or heat stress. Tolerant *cv.* Bh-1 has better survival to combined stressors like Cd^{2+} and heat than sensitive rice *cv.* DR-92 and heat stress when given in combination with Cd^{2+} toxicity seem to mitigate the effect of Cd^{2+} stress alone in rice. The study indicates individual Cd^{2+} toxicity and heat stress and a combination of the two stresses to have separate implications on antioxidative defense mechanism in rice plants. Among enzymes of the defense apparatus ascorbate peroxidase and glutathione reductase appear to serve as an important component for better survival of rice plants under combination of Cd^{2+} + heat stress.

Keywords Abiotic stress · Antioxidant enzymes · Heat shock · Heavy metal · Reactive oxygen species

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
GR	Glutathione reductase
HSP	Heat shock protein
MDAR	Monodehydroascorbate reductase
POD	Peroxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Plants are continually under threat due to adverse environmental conditions. In nature, plants encounter a number of biotic and abiotic stress factors simultaneously that may include drought, heat shock and heavy metals (both from air and water) (Kochhar and Kochhar 2005). The altered geochemical and biochemical balance of heavy metals due to human activities presently makes metal pollution a major environmental concern (Lei et al. 2007). Heavy

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metals can bind to functionally important domains of biomolecules and thereby inactivate them or render oxidative stress by direct electron transfer or inhibition of normal metabolic reactions (Hall 2002). Heat stress affects plant growth throughout its ontogeny, though heat threshold level varies considerably at different developmental stages (Wahid et al. 2007). Heat stress has been shown to alter the plant metabolism either through synthesis of heat-shock proteins (HSPs) (Vierling 1991) or by altered antioxidant enzyme activities due to enhanced production of reactive oxygen species (ROS) (Kochhar and Kochhar 2005). Thus increasing contamination and consequential accumulation of heavy metals in the soil as well as increasing temperature have become serious problems to crop yield and productivity in agricultural terms (Shah et al. 2001; Sharma and Dubey 2005; Howarth 2005).

In general, heavy metals and heat stress result in oxidative damage to plants. ROS are always formed by the inevitable leakage of electrons onto molecular oxygen from the electron transport activities of chloroplast, mitochondria and plasma membrane or as a byproduct of various metabolic pathways localized in different cellular compartments (Xiaozhong and Huang 2000; Polle 2001). ROS may cause lipid peroxidation and subsequent membrane injury as well as protein and nucleic acid damage (Gao et al. 2008). The production of ROS under normal growth conditions in cells is low (240 μMs^{-1} $O_2^{\cdot-}$) and in a steady state (0.5 μM H_2O_2) in chloroplasts, however, an enhanced production of ROS under various stressful conditions of the environment is reported to result in altered cellular homeostasis (upto 720 μMs^{-1} $O_2^{\cdot-}$) and a steady state of 5–15 μM H_2O_2 in chloroplast (Polle 2001).

Plant cells contain a range of protective and repair systems, which under normal circumstances, minimize the occurrence of oxidative damage. There are systems which either react with reactive forms of oxygen and keep them at a low level or are antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidases (POD), ascorbate peroxidase (APX), glutathione reductase (GR) that quench ROS supported by antioxidants like glutathione, ascorbic acid, ά-tocopherol and carotenoids (Sairam et al. 2000; Shah et al. 2001) or systems that regenerate oxidized antioxidants (glutathione, mono- and dehydroascorbate) (Markovska et al. 2009).

The non-specific enzyme guaiacol peroxidases so named as they can metabolize guaiacol as substrate and can function as effective quencher of ROS and peroxy radical induced by stressful conditions in the cell (Van Assche and Clijsters 1990). Usually an altered peroxidase activity is reported to be associated with the response of higher plants to an uptake of toxic amounts of heavy metals (Van Assche and Clijsters 1990; Shah et al. 2001) or to heat injury (Kochhar and Kochhar 2005). Very often therefore, peroxidases are known as stress ameliorating enzymes which actively responds to damages in plant metabolism and may act as early and sensitive indicators of heavy metal toxicity (Shah et al. 2001). In coordination with enzyme superoxide dismutases, catalase and ascorbate peroxidase, the peroxidases play an essential protective role in the scavenging process. Once SOD converts superoxide radical to H_2O_2, it is reduced to water and oxygen either by APX in ascorbate–glutathione cycle or by POD and CAT in cytoplasm and in other cellular compartments (Howarth 2005). An enhanced or altered expression of these key antioxidant enzymes in response to biotic and abiotic stresses is known (Bowler et al. 1992; Rizhsky et al. 2004; Sharma and Dubey 2005).

Rice can be grown under various climatic conditions at latitudes ranging from 53°N to 40°S by long period induction and domestication (Lu and Chang 1980). Generally, several latitude-dependent indica rice cultivars are grown in the North-East Region of India. Of these two rice *cv*. DR-92 and *cv*. Bh-1 were studied for their sensitivity to cadmium toxicity and heat stress. The present study was undertaken with the objective to examine the effect of low and high cadmium toxicity in combination with heat stress on the antioxidant enzymes in sensitive rice *cv*. DR-92 and tolerant rice cultivar Bh-1 grown at low and high altitudes, respectively in North East India. Attempts are also made to test the hypothesis that whether there occurs a cross-talk between the various antioxidant enzymes and that cadmium and heat stress either individual or combination have different oxidative response in rice seedlings. If either of the two stresses when given in combination help in better survival.

Materials and methods

Plant material and stress conditions

Seeds of two rice (*Oryza sativa*) *cvs.* DR-92 (sensitive) and Bh-1 (tolerant) and grown at low and high altitudes in North-East region of India were surface sterilized with 0.1% sodium hypochlorite solution and imbibed in water for 24 h. Seeds were germinated in petriplates for 5 days and seedlings were raised for 20 days in sand cultures, saturated either with Hoagland nutrient solution (Hoagland and Arnon 1938) that served as control or nutrient solution supplemented with 10, 50, 100 and 500 μM $Cd(NO_3)_2$ as treatments (Shah and Dubey 1998). Cd^{2+} levels were ascertained as low toxic (10, 50 μM) and high toxic (100, 500 μM) concentrations with full viability of seeds. Pots were maintained at field saturation capacity at pH 7.0 and irrigation done when required. Seedlings were maintained in the growth chamber for 20 day at 28 ± 1°C, 80% relative

humidity and 12-h light (irradiance 40–50 μmol m^{-2} s^{-1}) followed by a dark period. Seedlings were uprooted at 5 day intervals, roots and shoots were separated which served as Cd^{2+} treated plant samples. For heat treatments the control as well as Cd-treated seedlings were uprooted and kept at 40°C for 2 h. The roots and shoots were separated and used for experimental studies. All the estimations were carried out in triplicate. Cadmium was estimated in plant parts according to the method of Shah and Dubey (1995) using Atomic Abbsorption Spectrophotometer (AAS) fitted with Perkin-Elmer-2380.

Assay of guaiacol peroxidase (POD) activity

Guaiacol peroxidase (POD) (EC1.11.1.7) activity assay was performed spectrophotometrically according to the method of Egley et al. (1983). POD was extracted by homogenizing about 200 mg of root and shoot samples in 5 ml of 60 mM phosphate buffer (pH 6.0) using a chilled mortar and pestle at 4°C. The homogenates were centrifuged at 22,000×*g* for 10 min and supernatant were used for enzyme assay. Assay mixture in a final volume of 2 ml contained 50 μl enzyme, 200 μl guaiacol and 50 μl H_2O_2 in 1.7 ml of buffer. The increase in absorbance was measured at 470 nm (extinction coefficient 26.6 mM^{-1} cm^{-1}) using a Beckman DU- 530 (Germany) spectrophotometer. Enzyme specific activity is expressed as μmol H_2O_2 reduced mg^{-1} protein min^{-1}.

Assay of superoxide dismutase (SOD) activity

The superoxide dismutase (SOD) (EC 1.15.1.1) was assayed according to the method of Mishra and Fridovich (1972). For extraction of the enzyme, about 200 mg fresh root and shoot samples were homogenized in 5 ml of 100 mM potassium-phosphate buffer (pH 7.5), containing 1.0 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) of soluble polyvinyl pyrrolidone (PVP) using prechilled mortar and pestle. After centrifugation at 22,000×*g* for 10 min at 4°C, the supernatant was dialyzed in cellophane membrane tubings against the cold extraction buffer for 4 h with 3–4 changes of the buffer. After dialysis the SOD activity was assayed in the supernatant. The assay mixture contained 50 mM sodium carbonate-bicarbonate buffer (pH 9.8), containing 0.1 mM EDTA, 0.6 mM epinephrine and 0.1 ml enzyme in a total volume of 3 ml. Epinephrine was the last component to be added. The adrenochrome formation during the next 4 min was recorded at 470 nm in a UV–Vis spectrophotometer (ELICO, SL-159, India). One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions.

Assay of ascorbate peroxidase (APX) activity

Ascorbate peroxidase (APX) (EC1.11.1.11) was assayed as given by Nakano and Asada (1981). About 200 mg of fresh roots and shoots were homogenized in 5 ml of 50 mM potassium-phosphate buffer (pH 7.8) containing 1% PVP, 1 mM EDTA and 1 mM ascorbic acid (added just before use) in a chilled mortar and pestle. The homogenate was centrifuged at 22,000×*g* at 4°C for 10–15 min and the supernatant was used for enzyme assay. Reaction mixture in a total volume of 3 ml contained 50 mM potassium-phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 0.1 ml dialyzed enzyme extract. H_2O_2 was the last component to be added and the absorbance was read at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}). Enzyme specific activity is expressed as μmol ascorbate oxidized mg^{-1} protein min^{-1}.

Assay of catalase (CAT) activity

Catalase (CAT) (EC 1.11.1.6) was assayed as given by Beers and Sizers (1952). The enzyme was extracted from 200 mg fresh root and shoot samples by homogenization in 5 ml of 50 mM Tris-NaOH buffer (pH 8.0) containing 0.5 mM EDTA, 2% (w/v) PVP and 0.5% (v/v) Triton X-100 using a chilled mortar and pestle. The homogenate was centrifuged at 22,000×*g* for 10 min at 4°C and after dialysis supernatant was used for enzyme assay. Assay mixture in a total volume of 1.5 ml contained 1 ml of 100 mM potassium-phosphate buffer (pH 7.0), 400 μl of 200 mM H_2O_2 and 100 μl dialyzed enzyme extract. The rate of H_2O_2 decomposition was monitored at 240 nm (extinction coefficient 0.036 mM^{-1} cm^{-1}). Enzyme specific activity is expressed as μmol of H_2O_2 oxidized mg^{-1} protein min^{-1}.

Assay of glutathione reductase (GR) activity

Glutathione reductase (GR) (EC 1.6.4.2) was assayed based on the method of Carlberg and Mannervik (1985) with minor modification (Dalton et al. 1986). About 200 mg of fresh root and shoot tissues were homogenized in 5 ml of 0.1 M potassium phosphate buffer (pH 7.0) using chilled mortar and pestle. The homogenate was centrifuged at 22,000×*g* for 10 min at 4°C and the supernatant so obtained was used for determination of enzyme activity. The reaction mixture contained 0.25 mM GSSG, 0.125 mM NADPH, 50 mM tricine (pH 7.8), 0.5 mM EDTA and 50 μl of extract in a final volume of 2 ml. The decrease in absorbance due to NADPH oxidation was recorded at 340 nm (extinction coefficient of 6.22 mM cm^{-1}) and expressed in terms of nmol NADPH oxidized mg^{-1} protein min^{-1}.

In all the enzymatic preparations protein was determined by the Lowry's method (1951) using bovine serum albumin (BSA, Sigma) as standard.

Statistical analyses

The seedlings were distributed over a completely randomized samples. All the experiments were performed in triplicate. Values in the figures indicate mean values ± SD. based on three independent experiments and were significantly different as assessed by the analysis of variance (ANOVA) test.

Results

Effect of Cd^{2+} toxicity and heat on guaiacol peroxidase (POD) activity

Increasing levels of Cd^{2+} led to a concomitant increase in guaiacol peroxidase activity in both roots and shoots of sensitive rice *cv*. DR-92 whereas as expected an opposite trend was observed for POD activity in tolerant *cv*. Bh-1 (Table 1, Fig. 1). The POD activity in *cv*. DR-92 were always higher in Cd^{2+} treatments than that in controls. Under low Cd^{2+} levels a decline in POD activity at day 15 followed by a significant increase at 20 day of growth period were observed in the roots of *cv*. DR-92. A high toxic (100 and 500 μM) level of Cd^{2+} however, resulted in a dip in POD activity much earlier i.e. at day 10 followed by a gradual increase till 20 day of growth. Almost two fold increase in the POD activity in shoots of *cv*. DR-92 were noted under 50 μM Cd^{2+} treatments at day 10 and a threefold increase under 500 μM Cd^{2+} treatments at day 5 of the growth period. Seedlings grown under 500 μM Cd^{2+} for 20 days showed about 50 to 80% increase in POD activity in roots whereas a 30 to 72% increase in POD activity in shoots were observed at day 15 when compared to control grown seedlings of rice *cv*. DR-92. Heat stress alone led to a significant elevation in POD activity in sensitive *cv*. DR-92. A combination of Cd^{2+} treatments + heat resulted in a significant decline in POD activity at 20 d of growth in *cv*. DR-92, the values being lower than those under heat stress alone.

The levels of POD activity were always higher in roots and shoots of controls and all treatments in *cv*. Bh-1 than that in *cv*. DR-92, and declined gradually in former throughout the 5–20 day growth period and under all treatments. Cd and heat stress alone resulted in a lowered POD activity in tolerant *cv*. Bh-1 except at day 10 where 50 μM Cd^{2+} concentration caused a slight elevation (5–15%) in enzyme activity. The combined effect of 500 μM Cd^{2+} and heat stress resulted in decrease in the POD activity in roots at 10 day in *cv*. Bh-1 which declined thereafter. In all Cd^{2+} + heat treated seedlings a significant decrease in the activity of POD is noted beyond 10 day in the rice *cv*. Bh-1 (Fig. 1, Table 1).

Effect of Cd^{2+} toxicity and heat on superoxide dismutase (SOD) activity

Table 1 and Fig. 2 show the activity levels of superoxide dismutase in control, Cd^{2+} stressed, heat stress as well as Cd^{2+} and heat stressed seedlings in roots and shoots of the rice sensitive *cv*. DR-92 and tolerant rice *cv*. Bh-1. SOD activity was higher in *cv*. DR-92 than in *cv*. Bh-1 in both roots and shoots. An increase in SOD activity during

Table 1 : Summary of the effect of individual and combination of Cd^{2+} + heat stress on sensitive rice *cv*. DR-92 and tolerant *cv*. Bh-1 during 5–20 days of growth period

Rice cultivar	Plant materials	Enzyme activity	Stress conditions			Days of growth
			Cd toxicity	Heat shock	Cd + Heat stress	
DR-92 (sensitive)	Root/shoot	Guaiacol peroxidase	↑	↑*	*↓ than heat shock alone	20
		SOD	↑*	↑* in shoots	in shoots	15
		APX	↑	↑* in roots	↑ in both roots/shoots	10
Bh-1 (tolerant)	Root/shoot	Guaiacol peroxidase	↓	*↓	*↓ under all stress conditions	10–20
		SOD	↑	↓ in roots than Cd treatments alone	↓ in both roots/shoots	10–20
		APX	No significant change in shoots, slight ↑ in roots	Slight ↑ followed by gradual ↓	Gradual ↓	10–20

↑ and ↓ denote increase and decrease in enzyme activities, respectively. * represent a significant change

Values for enzyme Guaiacol peroxidase and Glutathione reductase were always higher in both roots and shoots of tolerant *cv*. Bh-1 throughout the growth period

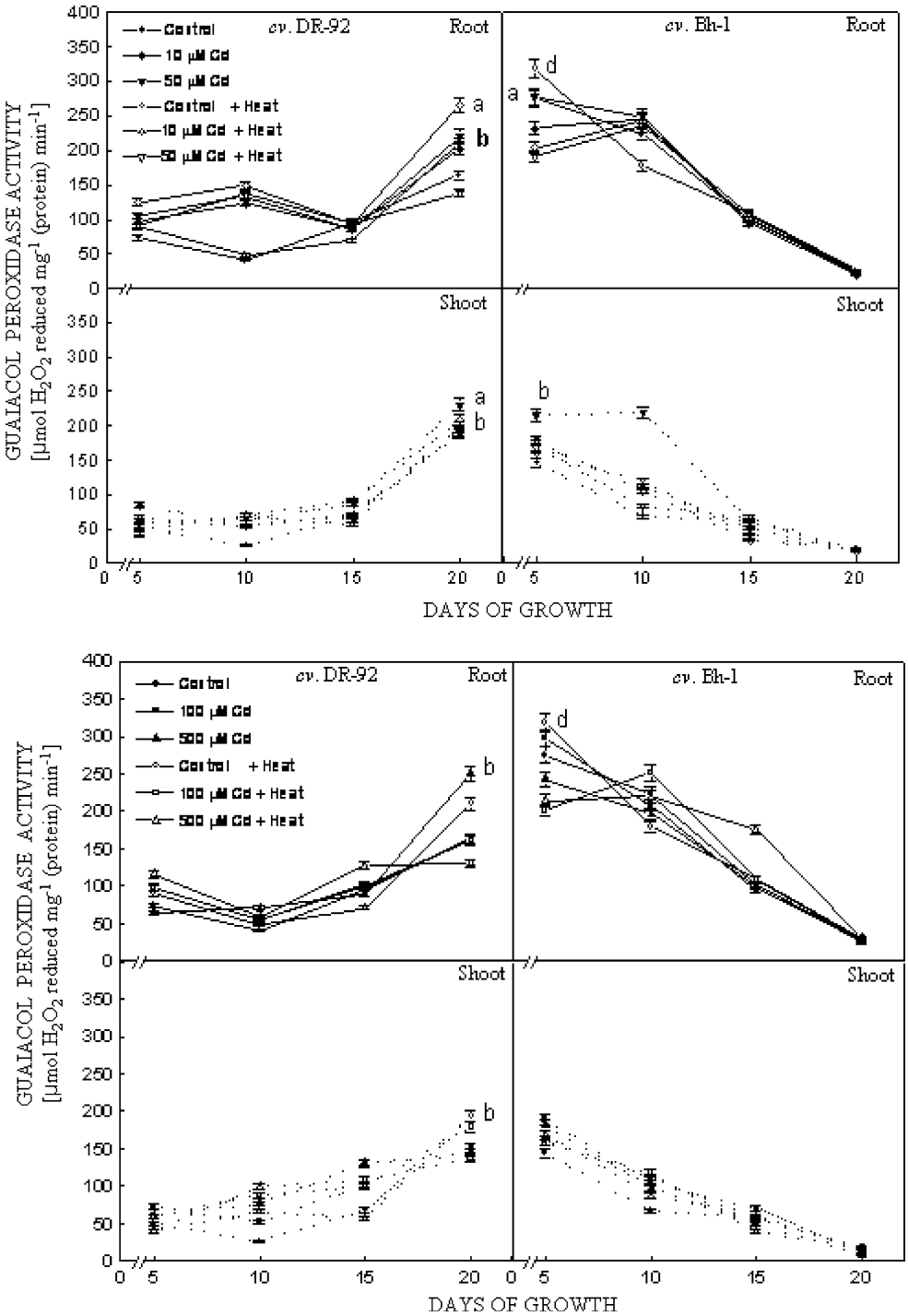

Fig. 1 Effect of 10 and 50 μM and 100 and 500 μM Cd^{2+} toxicity and heat stress on the activity of guaiacol peroxidase in the roots and shoots of rice *cvs.* DR-92 and Bh-1 grown in sand cultures. The data are mean of three replicates ± SD. ANOVA significant at $P \leq 0.01$. Values with *different letters* are significantly different at $P < 0.05$

5–15 day growth period with 30–50% higher activity in roots and shoots of 10 μM Cd^{2+} and 50 μM Cd^{2+} treatments than that in controls were noted in *cv.* DR-92 followed by a decline thereafter. Under 500 μM Cd^{2+} toxicity a significant increase of ~1.8 fold in SOD activity at 15 day in shoots and a similar increase under 100 μM Cd^{2+} in roots of *cv.* DR-92 were recorded. In tolerant *cv.* Bh-1 however, a 500 μM Cd^{2+} with heat stress revealed ~2 fold increase in SOD activity at day 15 in both roots and shoots as compared to controls. Heat treatments alone led to a decline in SOD activity in roots of *cv.* Bh-1 when compared to that in Cd treatments alone, whereas in sensitive *cv.* DR-92, heat stress led to a significant increase in enzyme activity in shoots. The combined effect of 100 and 500 μM Cd^{2+} + heat stress caused moderate amplification in SOD activity in roots and shoots *cv.* DR-92 during 10–15 day (Table 1), whereas the enzyme activity either remain unchanged or decreased in both roots and shoots of

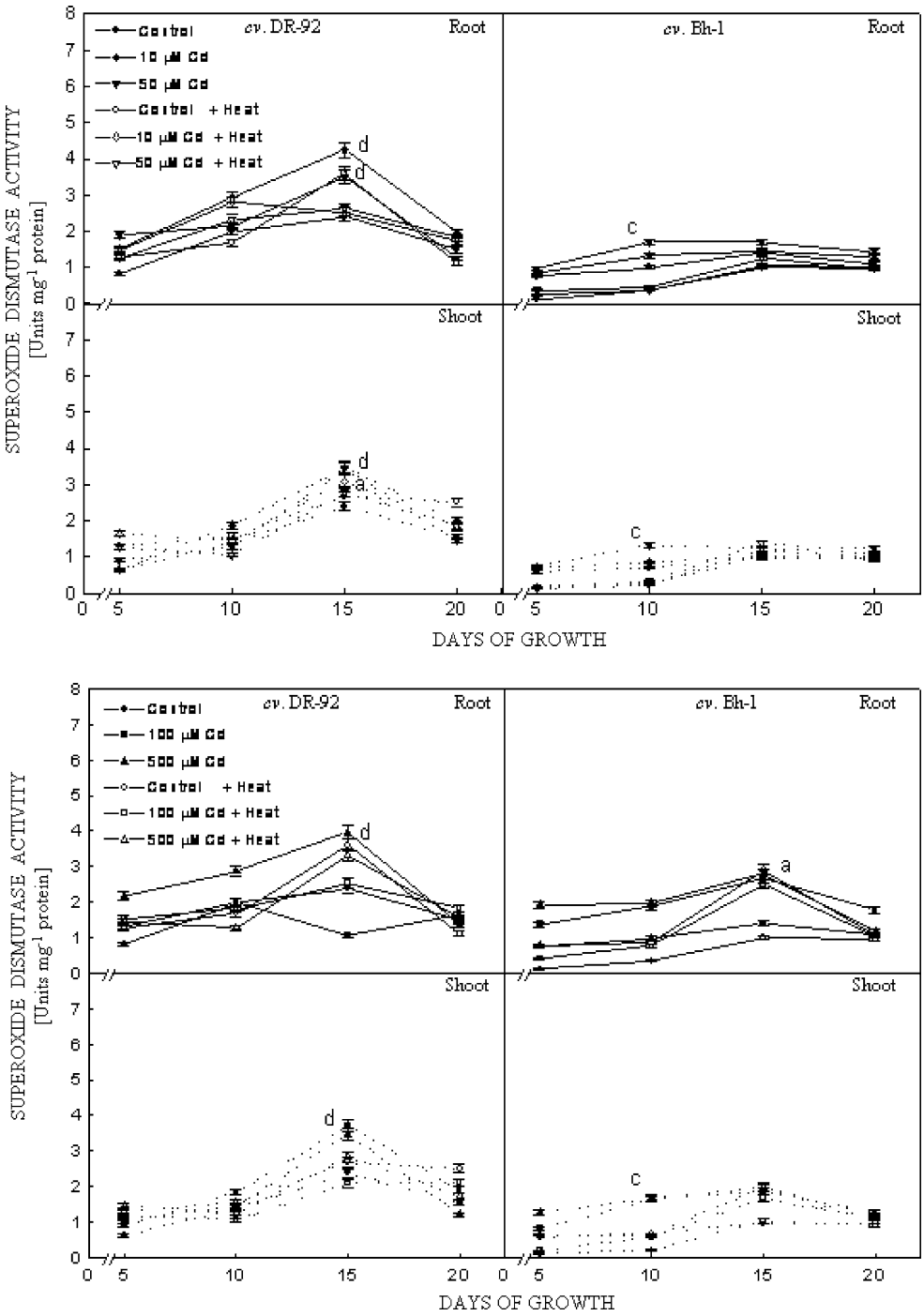

Fig. 2 Effect of 10 and 50 μM and 100 and 500 μM Cd^{2+} toxicity and heat stress on the activity of superoxide dismutase in the roots and shoots of rice *cvs.* DR-92 and Bh-1 grown in sand cultures. The data are mean of three replicates ± SD. ANOVA significant at $P \leq 0.01$. Values with *different letters* are significantly different at $P < 0.05$

tolerant *cv*. Bh-1, suggesting a protective effect of heat over Cd stress.

Effect of Cd^{2+} toxicity and heat on ascorbate peroxidase (APX) activity

The specific activity of APX under Cd^{2+} treatments and combination of Cd^{2+} and heat stress is shown as Table 1, Fig. 3. A 10 and 50 μM Cd^{2+} treatments alone caused a significant increase in APX activity during 10–15 days of growth period in sensitive *cv*. DR-92, when compared with controls that further enhanced under higher Cd levels. The values for activity of APX remained lower in tolerant *cv*. Bh-1 than that in sensitive *cv*. DR-92. Heat stress led to ~2.0 fold increase in APX activity in roots of *cv*. Bh-1 and ~2.5 fold elevation in roots of *cv*. DR-92 at day 10 which gradually declined during 5–20 days of growth period in *cv*. Bh-1 but continued to increase in *cv*. DR-92. Cd^{2+} treatments in combination with heat stress caused an elevation in APX activity during 10–15 days in both roots and

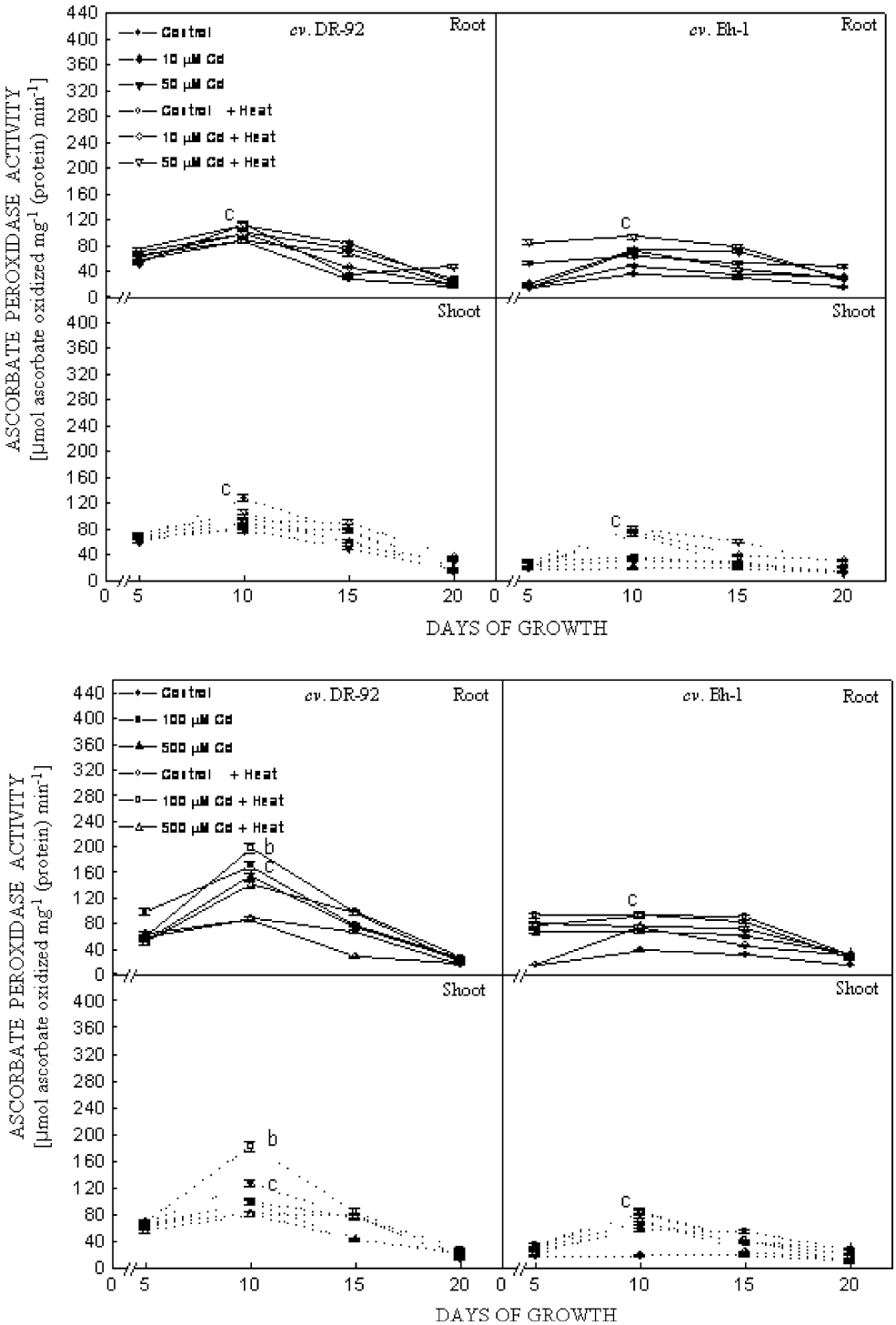

Fig. 3 Effect of 10 and 50 μM and 100 and 500 μM Cd^{2+} toxicity and heat stress on the activity of ascorbate peroxidase in the roots and shoots of rice *cvs.* DR-92 and Bh-1 grown in sand cultures. The data are mean of three replicates ± SD. ANOVA significant at $P \leq 0.01$. Values with *different letters* are significantly different at $P < 0.05$

shoots of *cv*. DR-92, however a gradual decline was noted for *cv*. Bh-1 under similar conditions.

Effect of Cd^{2+} toxicity and heat on catalase (CAT) activity

The activity of CAT under low/high Cd^{2+} levels, heat stress alone and Cd^{2+} + heat stress is shown as Fig. 4. Activity of CAT was always higher under 50 μM Cd^{2+} stress at day 10, in both the cultivars, with a further increase at day 15 under combined effect of 50 μM Cd^{2+} + heat stress in *cv*. DR-92 in roots. In *cv*. Bh-1 also a similar trend was noted but at rather a lower Cd^{2+} treatments of 10 μM Cd^{2+} alone. Seedlings grown under high Cd^{2+} level of 100 μM always exhibited a high catalase activity at 15 day in the two cultivars which declined

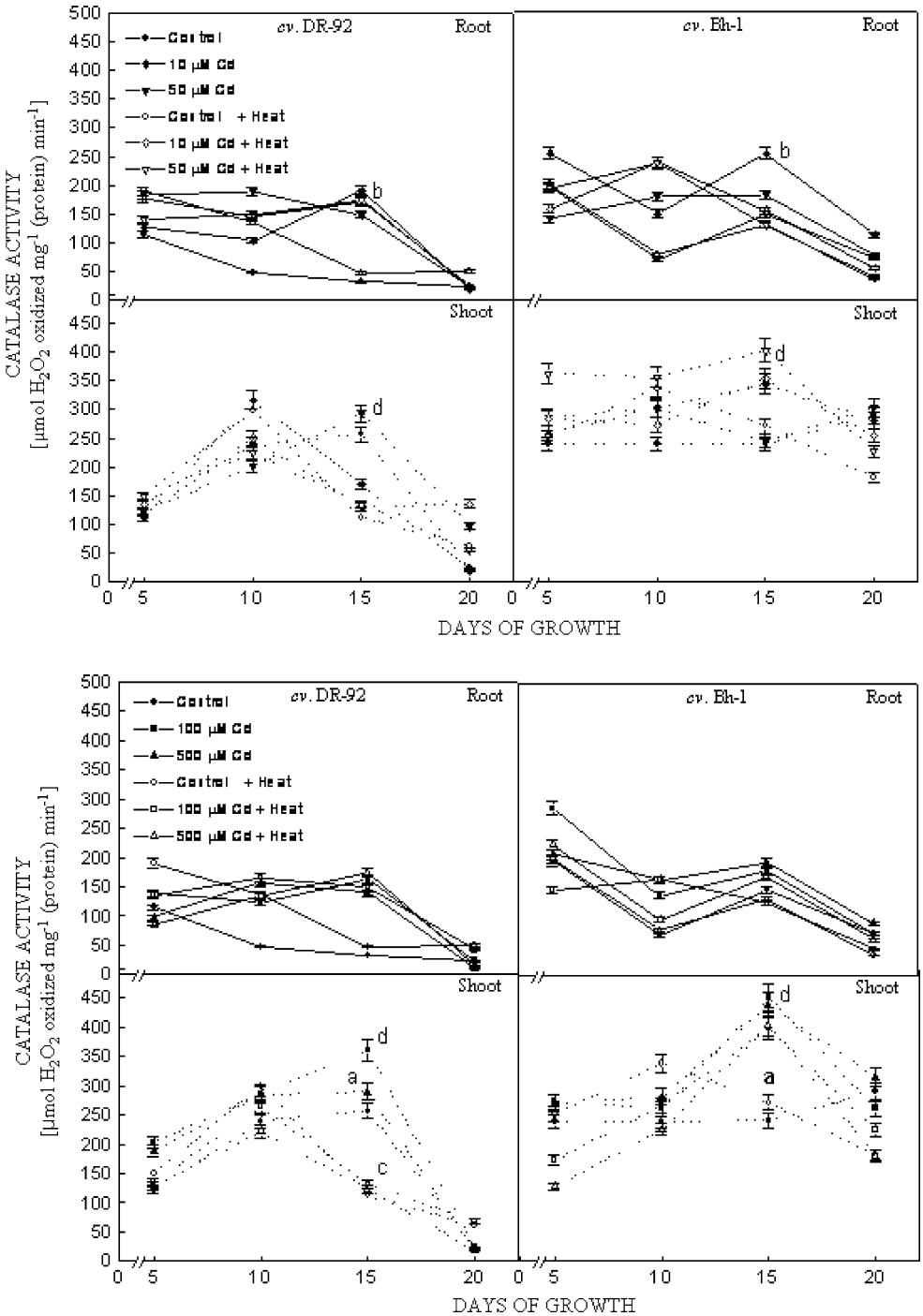

Fig. 4 Effect of 10 and 50 μM and 100 and 500 μM Cd^{2+} toxicity and heat stress on the activity of catalase in the roots and shoots of rice *cvs.* DR-92 and Bh-1 grown in sand cultures. The data are mean of three replicates ± SD. ANOVA significant at $P \leq 0.01$. Values with *different letters* are significantly different at $P < 0.05$

sharply thereafter. Heat stress caused a slight rise in CAT activity in shoots of tolerant *cv*. Bh-1 as well as sensitive *cv*. DR-92 at day 10 with a decline thereafter. The Cd^{2+} + heat treatments did not exhibit any significant variation in the CAT activities and remained almost unchanged except on day 10 where *cv*. Bh-1 had a 1.5 to 2.0 fold elevation in the CAT activity in roots than that in *cv.* DR-92. A 100 μM Cd^{2+} + heat stress together caused 1.6 fold elevation in CAT activity in shoots of *cv.* DR-92 at day 15. A similar increase was noted in *cv*. Bh-1 in corresponding plant samples. A higher Cd^{2+} levels of 100 and 500 μM in combination with heat caused a 30% inhibition in catalase activity in roots of *cv*. Bh-1 at 15 day of growth.

Effect of Cd^{2+} toxicity and heat on glutathione reductase (GR) activity

Figure 5 show the specific activity of glutathione reductase subjected to Cd^{2+} and/or heat stress during 5–20 days of

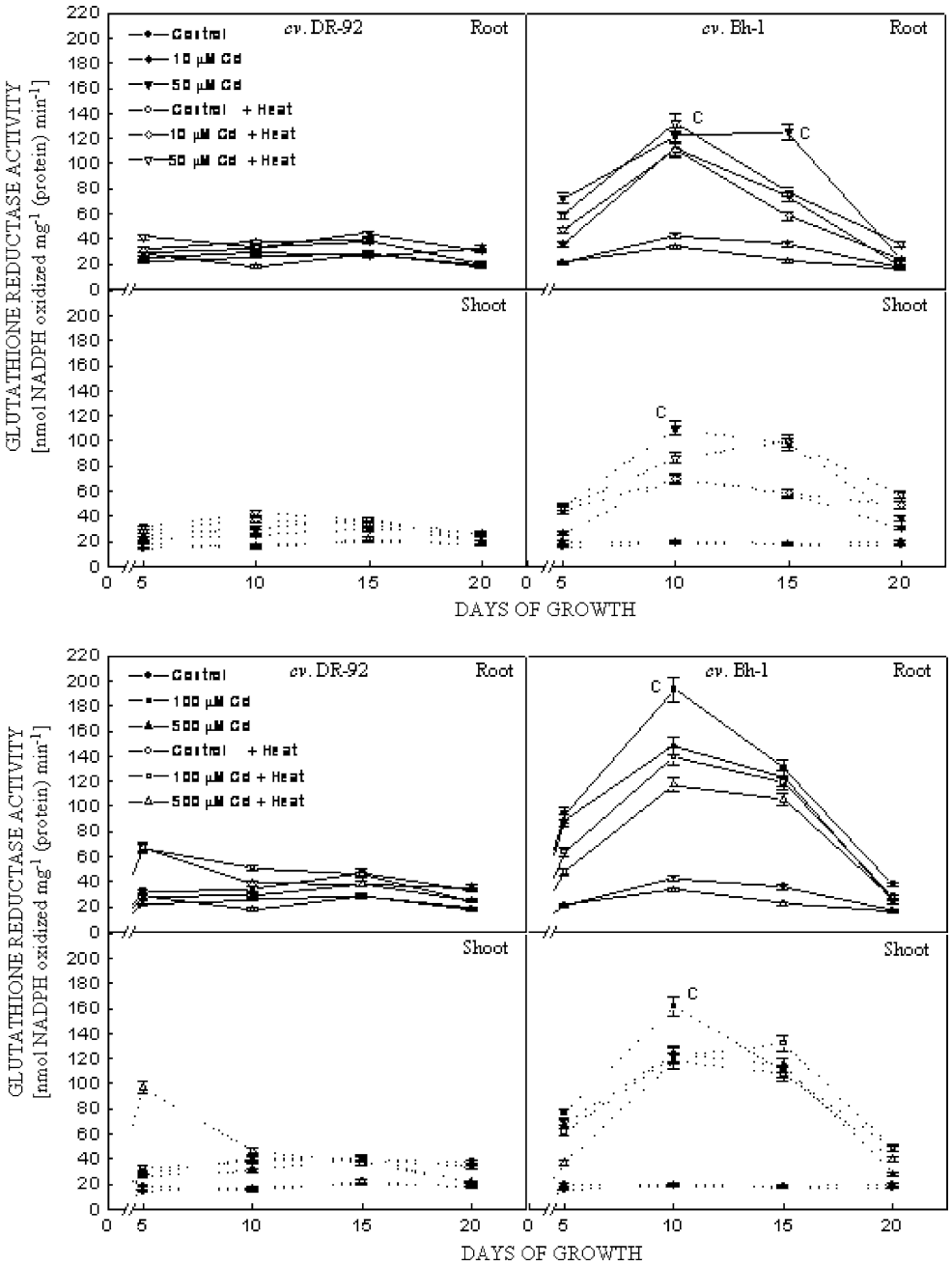

Fig. 5 Effect of 10 and 50 μM and 100 and 500 μM Cd^{2+} toxicity and heat stress on the activity of glutathione reductase in the roots and shoots of rice *cvs.* DR-92 and Bh-1 grown in sand cultures. The data are mean of three replicates ± SD. ANOVA significant at $P \leq 0.01$. Values with *different letters* are significantly different at $P < 0.05$

growth period in the roots and shoots of rice *cvs*. DR-92 and Bh-1. Under Cd^{2+} alone and stress combination of Cd^{2+} and heat a slight increase in GR activity were observed in *cv.* DR-92 at day 15 followed by a gradual decline thereafter. A marked change in GR activity could be observed in roots and shoots of tolerant *cv.* Bh-1, where a 10 and 50 μM Cd-treatments led to a ~2.5 to 3.0 fold increase in GR activity at day 10 in roots that further enhanced by ~3.3 to 4.0 folds under combined effects of Cd^{2+} + heat stress. Maximum GR activity were noted in 100 μM Cd-treated roots and shoots of *cv.* Bh-1 at day 10 of growth, where a 2.0 fold increase in GR activity in roots of *cv.* Bh-1 followed by a sharp decline thereafter were noticed. Under combined effects of 100 μM Cd^{2+} and heat, a nearly ninefold increase in GR activity were observed in shoots of *cv*. Bh-1 at 15 day of growth, when compared with controls. Shoots maintained higher GR activity than roots in both the rice cultivars and under all stress treatments. Heat alone increased GR activity in roots and shoots of tolerant *cv*. Bh-1 during 10 days and declined thereafter, however in sensitive *cv*. DR-92 the activity of GR did not exhibit any significant change throughout the growth period. Unlike the combined effect of Cd^{2+} and heat stress where activity was high at day 10, a 100 μM and 500 μM

Cd^{2+} treatments alone led to a significant increase in GR activity in shoots later at day 15 of the growth period. The activity of glutathione reductase declined beyond 15 day of growth in all test samples in both roots and shoots of the two cultivars.

Discussion

Cadmium toxicity and elevated temperature are the two major risks in crop plants. There is an increasing evidence suggesting that oxidative stress is a key damaging factor in plants exposed to a variety of stressful conditions including metal toxicity and that the plants resist damage due to oxidative stress by inducing the activities of antioxidative enzymes (Bowler et al. 1992). Therefore, the present study has been carried out to develop an understanding towards a combination of Cd^{2+} toxicity and heat stress, induced oxidative stress and the antioxidative defense system in growing rice plants. Results indicate induction of a high degree of oxidative stress paralleled with increasing Cd^{2+} levels and heat stress alone with altered activities of key antioxidative enzymes. Combinatorial effects of Cd + heat stress indicate a protective role of heat on Cd induced oxidative stress in rice seedlings.

An elevated activity of enzyme guaiacol peroxidase under increasing concentration of Cd^{2+} stress alone has been observed earlier in rice (Shah et al. 2001). In bean roots Cd^{2+} although caused oxidative stress it did not change the activity of POD. In contrast, in pea roots Cd^{2+} strongly stimulated POD activity mainly at higher Cd^{2+} concentration (Chaoui et al. 1997, 2004). When the root/shoot samples from rice *cv.* DR-92 were subjected to a combination of Cd^{2+} and heat stress a significant elevation in the activity of POD was noted suggesting a synergistic effect of the two stresses leading to an increased guaiacol peroxidase activity in this cultivar. Similar reports in different plants subjected to various abiotic stresses like drought, heat, cold, pathogen attack etc. are available in literature (Rizhsky et al. 2004). Most of these studies show that peroxidase activity and oxidative stress are induced by different physical, chemical or biological agents. An expressive enhancement of POD is also observed in soybean plants exposed to 150 μM Al (Shamsi et al. 2008). Enhancement in POD activity in Cd^{2+} stressed rice plants suggest the role of POD in removal of excess H_2O_2 produced under Cd^{2+} toxicity and/or heat stress. A decrease in POD activity as observed under stress conditions in roots and shoots of rice *cv.* Bh-1 could be due to varietal differences and better adaptability of *cv.* Bh-1 towards elevated ROS levels.

The result show a concomitant increase in SOD activity in the two *cvs.* DR-92 and Bh-1, the effect being more profound in roots than in shoots. This could be due to enhanced oxidative damage involved in roots of plants. With increase in Cd levels an enhanced uptake in roots were observed with less translocation to shoots (results submitted for publication elsewhere). An elevated SOD activity in leaves and roots under Cd^{2+} stress were also reported in *Phragmites australis* (Iannelli et al. 2002) and also in soybean plants exposed to low pH, Al alone and under combined effects of Al with Cd^{2+} (Shamsi et al. 2008). The decline in SOD activity beyond 15 days of growth is also reported in penncross grass at 28 days of growth (Xiaozhong and Huang 2000). Increase in SOD activity in response to stresses has also been attributed to the de-novo synthesis of enzymatic protein (Shah and Dubey 1998). Activities of Cu/Zn SOD, Fe-SOD and Mn-SOD have been shown to increase in plant cells under stressful conditions, which appear to be a part of defense mechanism under oxidative stress generated in cytosol, mitochondria and chloroplasts (Ushimaru et al. 1995). However, a Cu/Zn-SOD precursor was found to be down-regulated by heat stress in rice leaves (Lee et al. 2007). Sato et al. (2001) reported that SOD activity was not altered in rice seedlings exposed to heat (42°C), suggesting that SOD might respond to thermal stress in a complex manner or different isoforms could be differentially regulated.

Increased SOD activity during short-term heat stress may provide protection from oxidative stress, however prolonged periods of heat stress could be related to the reduction in SOD activity, which cause accumulation of $O_2^{\cdot-}$ especially in chloroplasts and mitochondria (Xiaozhong and Huang 2000). It is quite pertinent that the overall change in the ratio of $O_2^{\cdot-}$ scavenging enzyme (SOD) to H_2O_2 scavenging enzyme (CAT and POD) activity rather than individual changes in enzyme activities would result in the net oxidative stress in roots/shoots of rice seedlings (Kanazawa et al. 2000; Shah et al. 2001).

An increase in the activity of enzyme APX under increasing Cd^{2+} levels in sensitive *cv.* DR-92 as observed in our experiments suggest that cadmium inhibits plant growth and causes alterations in plant metabolism due to its interference with activities of enzymes and growth processes (Sharma and Dubey 2005). Cd stress alone did not lead to an increase in APX activity in tolerant *cv.* Bh-1 however under heat stress an increase in APX in tolerant *cv.* Bh-1 was noted suggesting thereby that oxidative stress is induced under heat stress and that *cv.* Bh-1 is tolerant to Cd alone. A combination of Cd + heat caused a decrease in APX activity suggesting that heat counteracts the effect of Cd in tolerant rice cultivar by lowering of APX in plants parts. High temperature stress can on the other hand account for significant decline in enzyme activities (Anderson 1997; Shah and Dubey 2005). However it is the

oxidative stress which is the major damaging factor in plants exposed to abiotic and biotic stresses, albeit the magnitude of stress varies (Alcazar et al. 1995). Furthermore an increase in APX activity under increasing levels of Cd^{2+} suggest an important role of APX in scavenging H_2O_2 under stressful conditions. Among H_2O_2 decomposing enzymes, APX has higher affinity for H_2O_2 than CAT and POD (Wang et al. 1999). Since ascorbate is the primary antioxidant and H_2O_2 is the major stable oxidant, the ratio of these redox components is indicative of the redox balance within the tissues (Markovska et al. 2009).

As observed in this study, an increase in the activity of enzyme catalase is also reported in some plant species exposed to toxic concentrations of heavy metals Cu, Pb, Zn (Prasad et al. 1999) but in other environmental stresses like drought (Zhang and Kirkham 1994; Sairam et al. 2000), senescence (Kanazawa et al. 2000), chilling (Anderson 1997), salinity (Singh et al. 2007) and highlight (Anderson 1997), an overall decline in CAT activity is reported. In the leaves of *Brassica juncea* (Markovska et al. 2009) decrease in activities of both APX and CAT was observed under Cd^{2+} stress suggesting an involvement in competition to remove H_2O_2. However, under Al^{3+} toxicity, CAT was reported to be less efficient scavenger of H_2O_2 as compared with APX enzyme (Sharma and Dubey 2005). It is also reported that Cd^{2+} metal may inhibit enzymes in H_2O_2 removal (CAT and APX) causing H_2O_2 accumulation in plants (Hatata and Abdel-Aal 2008).

In both roots and shoots of sensitive *cv.* DR-92 almost a constant activity of GR throughout the growth period was observed. A low activity of GR in *A. maritimum* is also reported under Cd stress (Schickler and Caspi 1999). Recently Zhou et al. (2008) showed that GR activities in leaves of alfalfa were not affected by Hg^{2+} exposure at low (1–10 μM) concentrations as compared to controls, but raising the Hg^{2+} concentrations (20–40 μM) remarkably increased the GR activities. It is known that GSH is oxidized during removal of the accumulating H_2O_2 under abiotic stress conditions (Szalai et al. 2009). Stress induced changes in level of H_2O_2 affects GSH/GSSG ratio, that affects the signaling causing thereby, changes in GR levels. With imposition of Cd^{2+} and heat stress, an increased GR activity in *cv.* Bh-1 observed herein could possibly be a result of changes in the expression of gene, regulating its synthesis. Miller and Mittler (2006) reported that the heat shock, transcription factors (Hsfs) that control the rapid induction of heat shock proteins (HsPs) in response to heat shock also function as molecular sensors and directly sense reactive oxygen species (ROS) and control the expression of oxidative stress response genes during oxidative stress. In the present study this oxidative stress was caused due to Cd toxicity. A recent report from Chao et al. (2009) suggest that accumulation of H_2O_2 during heat shock signals the increase in GSH levels thereby protecting the plant from oxidative damage caused by other abiotic stressors. Cd stress caused increase in HsfA4a expression, together with the metallothionein gene (MT) in roots of wheat and rice (Shim et al. 2009). These authors suggested that this HsfA4a confers Cd tolerance by upregulating MT gene expression in planta. The indirect regulation of protein by H_2O_2 and GSH may occur due to a cross-talk between the two species that affects thiol-disulfide transitions reported specially for GR (Dixon et al. 2005) playing thereby a role in signaling and responses to abiotic stress (Rausch et al. 2007). An interaction between the H_2O_2/GSH/GR redox system in combating a combination of stresses has also been suggested by Riechheld et al. (2007). Enhanced activities of enzymes of ascorbate–glutathione cycle (glutathione reductase) observed in Cd^{2+} stressed rice seedlings appear to be due to the need to maintain a favourable redox status, by maintaining sufficient level of reduced ascorbate and reduced glutathione and to overcome the possible problems of oxidation (Ranieri et al. 1996). Higher GR values in shoots than in roots suggest a tissue specific distribution of the enzyme in rice seedlings. The tolerant rice *cv.* Bh-1 possibly has enhanced formation of PCs resulting from more utilization of GSH and elevated GR activity, the effect being enhanced significantly in Cd treatments at day 10 in both roots/shoots than under Cd + heat treatments or heat stress alone. Results therefore also suggest better performance of GR under Cd stress alone than under heat or Cd + heat.

In conclusion results suggest that the Cd^{2+} toxicity + heat combinations should be regarded as a new state of abiotic stress in rice plants that requires a new defense or acclimation response perhaps in addition to the existing one. The potential effects of Cd^{2+} or/and heat stress combinations could vary depending on the relative level of each of the different stresses taken together and varietal differences. An involvement of cellular antioxidant defense systems in the adaptation of *Oryza sativa* to Cd^{2+} toxicity and/or heat stress, is indicated and rice *cv.* Bh-1 seems to be better adapted to combat Cd^{2+} metal concentrations and heat stress than *cv.* DR-92. The effect of Cd^{2+} concentrations and heat stress on growth and metabolism of rice seedlings when given individually is different than that observed in stress combination. In all there seems to be no linear relationship in antioxidant enzymes and stress combination in rice *cvs.* DR-92 and Bh-1, as both up and down regulations of antioxidant enzymes were observed that perhaps occur at both transcriptional and translational levels and also vary with the type and duration of stress and the plant variety. Heat stress alone causes enhanced oxidative stress but when given in combination with Cd largely seem to mitigate the effect of latter, in rice seedlings in *cv.* DR-92. APX activity play an important role in

conferring better survival of rice seedlings of *cv*. DR-92 under combination of Cd + heat stresses whereas GR confers better survival towards Cd stress in tolerant *cv*. Bh-1.

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