

Physiological Responses to Oxidative and Heavy Metal Stress in Seedlings of Rice Paddy, *Oryza sativa* L.

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Organisms living under pollution stress varied responses, from changes in gross morphology to changes in biochemistry. Plants in particular have evolved diverse ways of responding to adverse changes in their environment. Plants overcome severe environmental stress by developing tolerance or adaptation through physiological and biochemistry mechanisms (Verkleij and Schat, 1990; Yu, 1992). Plants are also known to develop genetic resistance to specific pollutants have been identified (Scandalios, 1990; Duan, 1996). Oxidative stress is known to be generated by a number of environmental factors including light, temperature, water, mineral deficiency, toxic metals and air pollutants such as O_3 , SO_2 and NO_x (Hendry, 1994). Furthermore, plants after reacting with oxygen can exhibit a broad ranged of physiological responses including changes in gene expression (Elstner and Osswald, 1994). Heavy metals as well as oxidative agents are known to induce adaptive responses to chemical mutagens and heavy metals in plant cells (Yang et al., 1995; Panda et al., 1997). However, the physio-ecological mechanisms underlying the metallo- or oxidative adaptive responses are not very clear. In order to improve our understanding of the physiological basis of the adaptive response, this article studied the induction or inhibition of antioxidant enzyme, thiols, proteins and lipid peroxidation (LP) by oxidative stress compared with heavy metal stress, investigated in germinating seeds of rice paddy, *Oryza sativa* L.. Of the two oxidative agents used, hydrogen peroxide (H_2O_2) generated extracellular oxidative stress, whereas paraquat (PQ), a redox agent, served as the source of intracellular oxidative stress that mediated the transfer of electron from NADPH or NADH to O_2 , generating a flux of superoxide radical through redox cycling.

MATERIALS AND METHODS

Seeds of rice paddy (*Oryza sativa* L.) Tiefeng 241, supplied from Institute of Rice Paddy, the Chinese Academy of Agricultural Sciences, were treated with PQ (Sigma, USA), H_2O_2 (BCF, China) and cadmium chloride (BCF, China). Stock solutions of the chemicals were made in 0.1M phosphate (pH 7.0) to prepare solutions at the experimental concentrations.

Seeds were presoaked for 5 h, and then soaked for 5 h in a treatment solution, PQ, 5×10^{-5} , 10^{-4} , 10^{-3} M, H_2O_2 , 10^{-3} , 5×10^{-3} , 10^{-2} M and $CdCl_2$ 10^{-5} , 10^{-4} , 5×10^{-3} M. After treatment the seeds

were washed in running tap water for 2 h and placed on moist filter paper in petri-dishes for germination at room temperature($25\pm 1^{\circ}\text{C}$). For each treatment 100 seeds were used. Batches of plant tissue taken from the germinating seeds after 2, 5 and 8 days(embryonic shoot/first leaf) were used for physiological analyses. All the treatments had three replications. Seeds treated with phosphate buffer were used controls and handled in a similar manner to treated ones.

Weighed plant tissues were homogenized in 5 ml 0.1M phosphate buffer (pH 7.0) and centrifuged at $10000\times g$ at 4°C for 15 min., and the supernatant was used for enzyme measurement. The activity of enzymes peroxidase(POD), catalase(CAT), superoxide dismutase(SOD), glutathione reductase (Xue,1985) and protein were all estimated following standard procedure using a UV-vis spectrophotometer(SF 722, China).

Determination of total thiols(TSH), non-protein thiol (NPSH) and glutathione(GSH) in the supernatant were determined using 5,5'-dithiobis-2-nitrobenzic acid (Sigma, USA). Part of the supernatant was analysed for TSH and the remainder deproteinated with 5% trichloroacetic acid(TCA), and analysed for NPSH and GSH. Protein thiol(PSH) was calculated by subtracting the values of NPSH from TSH, and phytochelatin(PC) was calculated by subtracting the values of GSH from NPSH.

To estimate LP, the plant tissue was extracted with 5% TCA containing 0.5% thiobarbituric acid (TBA), heated at 95°C , cooled and centrifuged at $4000\times g$ for 25min. The absorbance of the supernatant was measured at 530nm and for non-specific absorbance at 600nm. The malondialdehyde(MDA) equivalents (TBA reactive materials) were calculated on the resulting difference using the extinction coefficient $155\text{ mM}^{-1}\text{cm}^{-1}$.

All the data represent means calculated from three replicate petri-dishes. And the least significant difference test was employed for comparison of the changes at $p\leq 0.05$ or $p\leq 0.01$.

RESULTS AND DISCUSSION

The data for antioxidant enzymes, POD, CAT, SOD and GR are presented in Fig.1. A dose-dependent induction of POD and CAT by PQ, H_2O_2 and CdCl_2 were found. Induction of POD and CAT by PQ and H_2O_2 was significant($p\leq 0.05$) at all the concentrations from day 2 onwards, but for CdCl_2 it was found to be significant($p\leq 0.05$) only at the higher concentrations 10^{-4} and/or 10^{-2} M , and this was more pronounced on days 2 and 8. Significant induction($p\leq 0.01$) of SOD was found for PQ and H_2O_2 at all concentrations and all days but CdCl_2 failed to induce SOD activity. The differences with respect to the activity of GR induced by the oxidative agents, and cadmium was remarkable. Both PQ and H_2O_2 significantly($p\leq 0.05$) induced GR activity from day 2 through to day 8 and this apparently followed a dose-response. Cadmium, on the other hand, not only failed to induce but actually inhibited GR activity significantly($p\leq 0.01$), again following a dose response.

The data for thiol levels are presented in table 1. The TSH levels increased on all days showing

Table 1. Estimations of total thiol (TSH), protein thiol (PSH) and non-protein thiol (NPSH; $\mu\text{mol g}^{-1}\text{FW}$) in growing rice paddy seedlings on different days of development following seed-treatment with paraquat(PQ), H_2O_2 and CdCl_2

Concentration(M)		Days of seedling development									
		Day 2				Day 5				Day 8	
		TSH	PSH	NPSH	TSH	PSH	NPSH	TSH	PSH	NPSH	
Control	0	12.1±0.27	8.76±0.88	4.5±0.8	14.9±0.01	8.7±0.06	6.08±0.26	13.5±0.05	8.22±0.2	5.26±0.08	
PQ	5×10 ⁻⁵	15.36±0.6*	9.25±0.18	6.05±0.7	15.03±0.06	6.9±0.44	7.6±0.01	15.33±0.12**	8.35±0.41	6.82±0.46	
	10 ⁻⁴	16.16±1.15**	9.68±0.81	6.75±0.45**	15.4±0.44	6.45±0.08	8.01±0.36	17.22±0.12**	9.32±0.56	7.66±0.12	
	10 ⁻³	15.8±0.46**	10.12±0.22	6.58±0.46**	-	-	-	-	-	-	
Control	0	13.1±0.2	8.8±0.11	5.1±0.3	11.6±0.13	5.43±0.38	6.1±0.03	12.4±0.2	7.11±0.18	5.4±0.03	
H ₂ O ₂	10 ⁻³	16.1±0.08**	11.86±0.2*	5.7±0.04	15.6±0.07**	8.96±0.21**	6.65±0.02	15.66±0.98**	9.4±0.73**	5.8±0.21	
	5×10 ⁻³	16.24±0.6**	12.32±0.49**	5.9±0.01	15.46±0.54**	8.2±0.68**	7.23±0.12	15.54±0.63**	9.19±0.17**	6.46±0.26	
	10 ⁻²	16.65±0.77**	9.64±0.43	6.8±0.23	17.1±0.46**	9.54±0.52**	7.32±0.03	16.26±0.2**	10.54±0.38**	6.68±0.33	
Control	0	11.1±0.01	5.3±0.33	5.64±0.26	10.34±0.87	3.66±0.33	5.89±0.16	12.4±0.8	5.86±0.36	6.58±0.54	
CdCl ₂	10 ⁻⁵	10.63±0.09	2.34±0.6	8.94±0.74*	15.89±0.34*	4.78±0.56	11.13±0.68**	16.5±0.16**	3.02±0.38	11.4±0.55**	
	10 ⁻⁴	12.66±0.18**	3.410.54	9.56±0.11**	16.67±0.41**	3.29±0.17	13.56±1.23**	18.74±1.88**	4.16±0.87	13.66±0.23**	
	5×10 ⁻³	14.86±0.25**	4.350.9	11.2±0.78**	17.3±0.44**	2.8±0.22	15.56±1.55**	21.13±1.22	5.16±0.88	15.68±0.29**	

a dose-response for all the chemicals tested. A significant ($p \leq 0.05$) increase for PSH was noted for H_2O_2 but no such increase was found for PQ, and $CdCl_2$. The increase of NPSH on the other hand was remarkably significant ($p \leq 0.01$) for PQ, and $CdCl_2$, but not for H_2O_2 .

A significant ($p \leq 0.05$) increase of GSH was noted for both PQ and H_2O_2 (Fig.2). In contrast, a dose-dependent decrease in GSH levels was noted for $CdCl_2$ at all days. Correspondingly the PC values determined for $CdCl_2$ gave a significant and dose-dependent increase ($p \leq 0.01$) from day 2 through to day 8. The PC levels calculated for either PQ or H_2O_2 , however, did not increase. The significant ($p \leq 0.01$) dose-dependent increase of protein contents (Fig.2) induced by all of the chemicals tested was noteworthy. LP measured as MAD equivalents in the present study was strongly induced by H_2O_2 and to a lesser degree by PQ and cadmium (Fig.2).

POD, CAT, SOD are the three major antioxidant enzymes responsible for scavenging the reactive oxygen species generated via different mechanisms in plant cells (Winston, 1990). GR also play an important antioxidant role by maintaining a high GSH/GSSG, oxidized glutathione ratio (Alscher, 1989). GSH is a major antioxidant that is known to protect plant cells from oxidative stress (Smith et al, 1990). Changes in processes that regulate GSH concentration and/or redox status are considered to be important in the adaptive mechanisms of plants exposed to stressful environmental conditions. Furthermore, GSH has been implicated as the substrate, for phytochelatin biosynthesis under metal stress (Steffens, 1990) and LP has been considered as a reliable measure of oxidative damage (DeVos and Schat, 1991).

In this study, PQ, H_2O_2 and $CdCl_2$ induced POD, CAT, LP and proteins. Since the protein content invariably was increased by the oxidative agents, as well as the heavy metal, enzyme activities were calculated on a fresh weight basis rather than on a protein basis (Brunschoen-Hart et al, 1995). The specific enzyme activities would appear to decrease if expressed on a protein basis. The activity of SOD was significantly induced by PQ and H_2O_2 only. The failure of induction of SOD by cadmium was noteworthy. Induction of antioxidant enzymes and LP in general is not restricted to oxidative agents and heavy metals but may be induced by a variety of environmental factors including temperature, water deficit, wounding, air pollution etc.

Under conditions of oxidative stress, such as exposure to O_3 , SO_2 , heat shock or drought, both GR activity and GSH level have been shown to increase in plant tissue (Smith et al, 1990). The differences with respect to GR activity, GSH and PC levels following exposure to PQ and H_2O_2 on the one hand, and $CdCl_2$ on the other, were therefore remarkable. The inhibition of GR activity accompanied by lowering GSH levels and concomitant increases of PC levels by $CdCl_2$ suggested the involvement of PC in the underlying cadmium-induced adaptive response in plant cells. In fact, GSH, which is the precursor of PC, is known to be depleted along with increase of PC levels, in plant cells *in vivo* or *in vitro* within a few minutes of exposure to low levels of cadmium (Yu, 1993; Rauser, 1995). The present results therefore provided biochemical evidence upholding our earlier suggestions that PC are involved in the cadmium-induced adaptive response in plant cells to chemical mutagens and/or heavy metals (Yang, 1998; Panda et al., 1997). Biochemical responses to environmental stress have in common the induction of

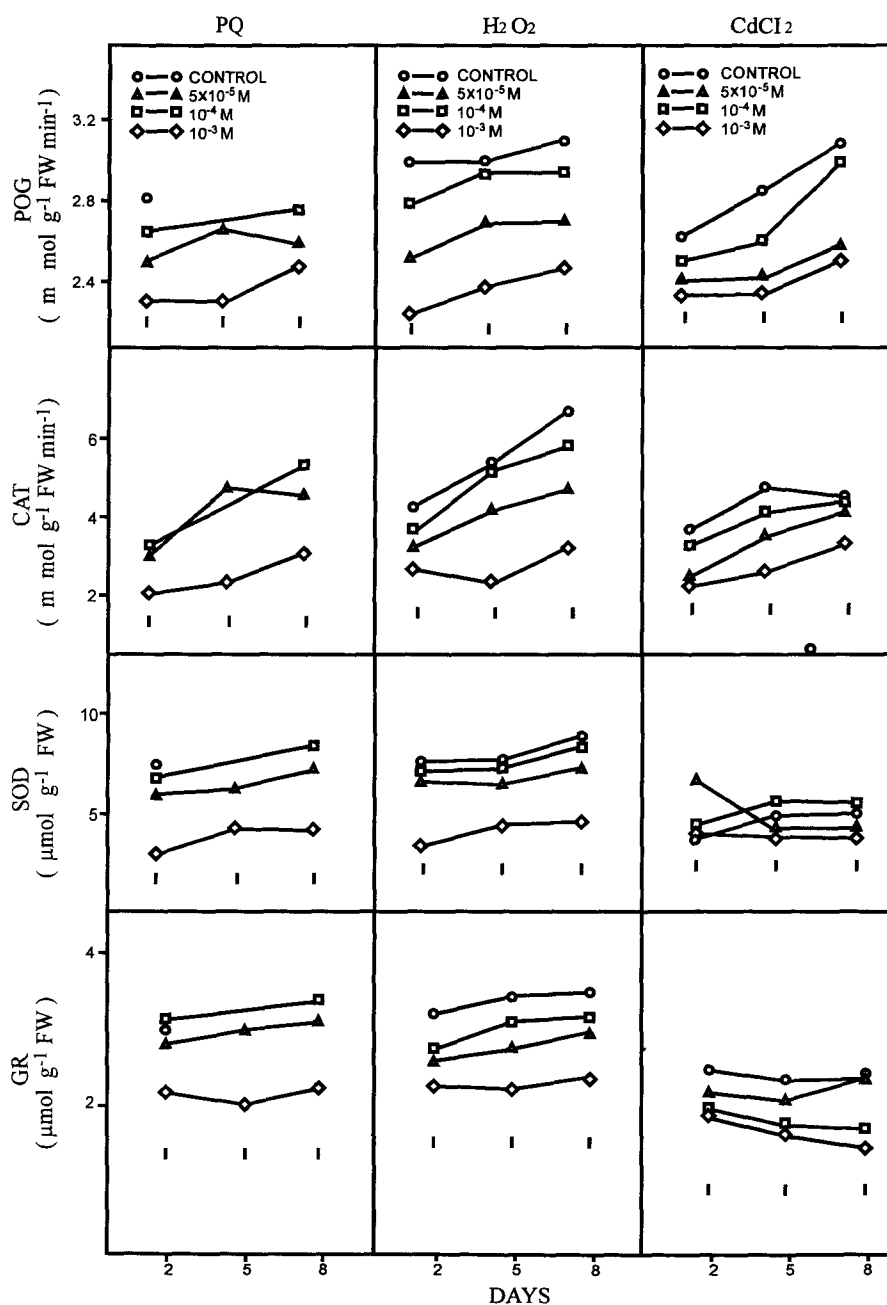


Figure 1. Activity of peroxidase (POD), Catalase (CAT), superoxide dismutase (SOD) and glutathione (GR) estimated on different days following treatment with paraquat (PQ), H_2O_2 and $CdCl_2$ in growing paddy seedlings. The vertical bars indicate the least significant difference values at $p < 0.05$.

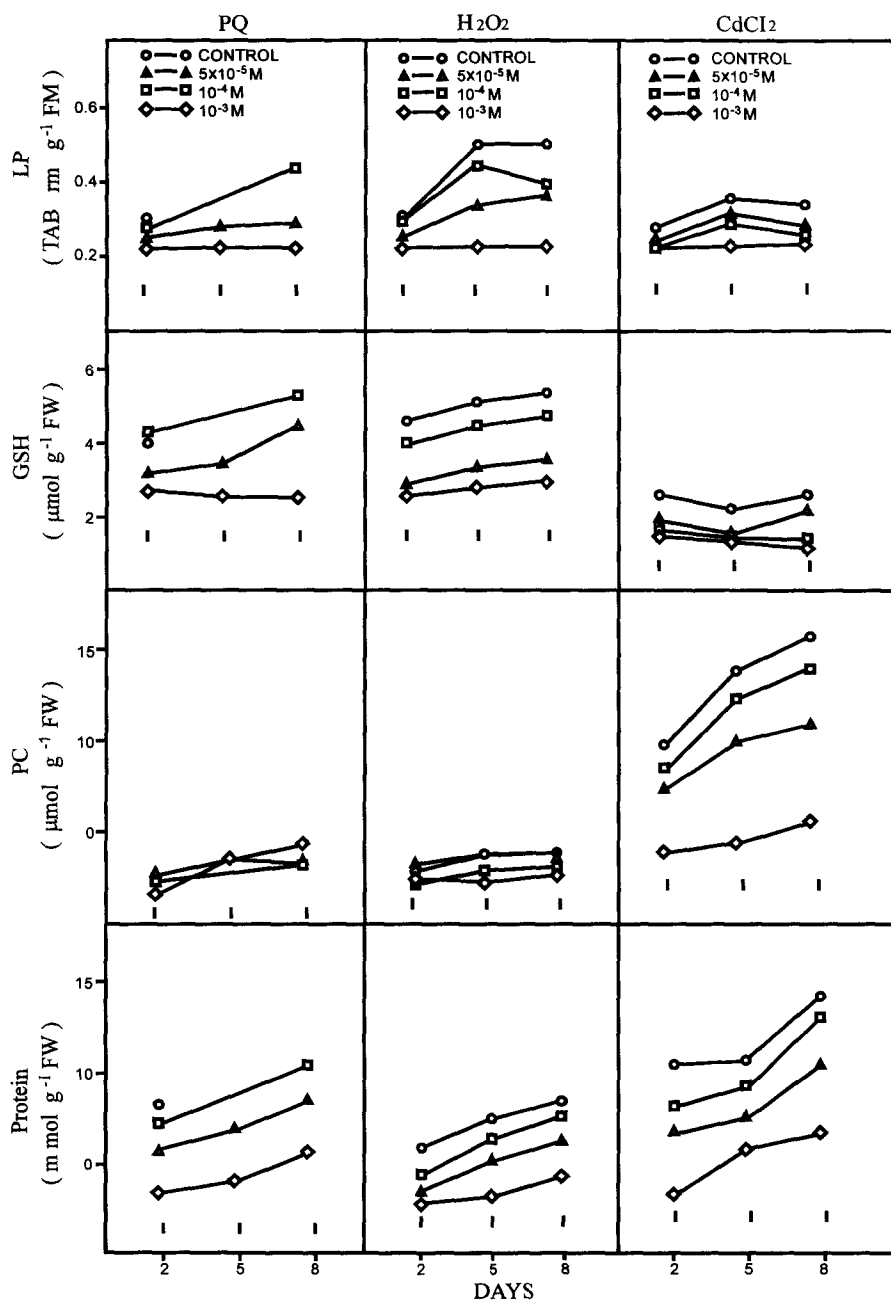


Figure 2. Level of lipid peroxidation(LP),glutathione(GSH),phytochelatin(PC) and protein estimated on different days following treatment with paraquat(PQ), H₂O₂ and CdCl₂ in growing paddy seedlings.The vertical bars indicate the least significant difference values at $p < 0.05$

some of the shock proteins which may have a role in the adaptive response (Wollgiehn and Newmann,1995). These results underlyed the crucial involvement of GSH and PC in the potential adaptive responses to oxidative stress and metal stress respectively.

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REFERENCES

- Alscher RG, (1989). Biosynthesis and antioxidant function of glutathione in plants. *Physiol Plant* 77:457-464
- Brunschon-Harti S, et al., (1995). Effects of ethylenediurea and ozone on the antioxidative systems in beans (*Phaseolus vulgaris L.*). *Environ Pollut* 90:95-103
- Devos CHR, Schat H, (1991). Free radicals and heavy metal tolerance. In: Rozema, J., Verkleij, J.A.C.(Eds.). *Ecological Responses to Environmental Stress*. Kluwer Academic Publishers, Amsterdam, The Netherlands, p: 22-30
- Duan C, Wang H, (1996). Effects of Cd, Pb, Cu, Zn, As on growth, cell division and membrane permeability of soybean. *Chinese J Environ Sci* 16: 256-261
- Elstner E F, Osswald W, (1994). Mechanism of oxygen activation during plant stress. *Proc. Royal Society Edinburgh* 102B, 131-154
- Hendry GAF, (1994) Oxygen and environmental stress in plants an evolutionary context. *Proc. Royal Society Edinburgh* 102B,155-165
- Panda KK, et al., (1997). Persistence of cadmium induced adaptive response to genotoxicity of maleic hydrazide and methyl mercuric in meristem cells of *Allium cepa L.*: differential inhibition by cycloheximide and buthionine sulfoximine. *Mut Res* 389:129-139
- Rausser WE, (1995). Phytochelatins and related peptides. *Plant Physiol* 109:1141-1149
- Scandalios, J.G., (1990) Response of plant antioxidants defense genes to environmental stress. *Adv Genet* 28:1-41
- Smith IK, et al., (1990). Glutathione. In: Alscher, R.G., Cumming, JR(Eds.). *Stress Responses in Plants: Adaptation and Acclimation Mechanisms*. Wiley-Liss New York. p 205-215
- Steffens JC, (1990). Heavy metal stress and the phytochelatin response. In: Alscher RG, Cumming, JG(Eds.). *Stress Responses in Plants: Adaptation and Acclimation Mechanisms*. Wiley-Liss, New York, p 377-394
- Verkleij JAC, Schat H, (1990). Mechanisms of metal tolerance in higher plants. In: Shaw AJ(Ed). *Heavy Metal Tolerance in Plants: Evolutionary Aspect*. CRC Press, Florida, p57
- Winston GW, (1990). Physiochemical basis for free radical formation in cell:Responses and defenses. In:Alscher,R.G., Cumming JG(Eds.). *Stress Responses in Plants: Adaptation and Acclimation Mechanisms*. Wiley-Liss, New York, p 57-86
- Wollgiehn R, Newmann D, (1995). Stress response of tomato cell cultures to toxic metals and heat shock: differences and similarities. *J. Plant Physiol.* 146: 736-742.
- Yang J, (1995) Movement and accumulation of heavy metals in soil—plant system. *Chinese J*

- Ecol 9: 89-94
- Yang J, (1998) Impact of Cd on growth and nutrient accumulation of different plants species. Chinese J Appl Ecol 9: 89-94
- Yu G, Wu Y, Wang H,(1992) Effects of differential cadmium compound and its interaction on wheat (*Triticum aestivum* L.). Chinese J Ecol, 12: 92-96
- Yu G, et al, (1993). Toxicity of cadmium, zinc and their interaction on the growth of wheat radical roots. J. Yunnan Univ. 37:52—56(In Chinese)