Journal of Environmental Sciences Vol. 12, No. 4, pp. 458—462, 2000

ISSN 1001—0742
CN11—2629/X

Article ID: 1001-0742(2000)04-0458-05 **CLC number:** Q948 **Document code:** A

Comparison of physiological responses to oxidative and heavy metal stress in seedlings of rice paddy, *Oryza sativa L.*

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Abstract: Physiological responses on the bases of activities of antioxidant enzymes: peroxidase, catalase, superoxide dismutase and glutathione reductase as well as estimation of total protein, lipid peroxidation and thiols in the form of protein, non-protein, glutathione and phytochelatin measured in growing seedlings of paddy, *Oryza sativa L.*, from day 2 to 8 were compared following treatment of seeds for 5h with oxidative agents, paraquat 5×10^{-5}, 10^{-4}, 10^{-3} mol/L, H_2O_2 10^{-3}, 5×10^{-3}, 10^{-2} mol/L, and $CdCl_2$ 10^{-5}, 10^{-4}, 5×10^{-3} mol/L. A significant induction of all antioxidant enzymes along with an increase in the levels of protein, lipid peroxidation and glutathione was noted in response to oxidative stress, $CdCl_2$ induced significant peroxidase and catalase activities but not superoxide dismutase. In a marked contrast from oxidative stress, $CdCl_2$ decreased glutathione reductase activity as well as glutathione levels but increased phytochelatin level. The different physiological responses thus underlined the crucial involvement of glutathione and phytochelatin in the oxidative and heavy metal-linduced adaptive responses respectively.

Key words: adaptive response; oxidative stress; heavy metal; antioxidant enzymes; glutathione; phytochelatin

Introduction

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, 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 NOx (Hendry, 1994). Furthermore, plants after reacting with oxygen can exhibit a broad ranges of physiological responses including changes in gene expression (Elstner, 1994). Heavy metals as well as oxidative agents are known to induce adaptive responses to chemical mutagens and heavy metals in plant cells (Yang, 1995; Panda, 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-cycling 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.

1 Materials and methods

1.1 Plant material and chemical treatments

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 0.1 mol/L phosphate (pH 7.0) to prepare solutions at the experimental concentrations.

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

After treatment the seeds were washed in running tap water for 2h and placed on moist filter paper in petri-dishes for germination at room temperature (25 ± 1℃). For each treatment concentration 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 as controls and handled in a similar manner to treated ones.

1.2 Biochemical extractions and estimations

Weighed plant tissues were homogenized in 5 ml 0.1 mol/L phosphate buffer (pH 7.0) and centrifuged at 10000 × g at 4℃ 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-nitrobenzoic acid (Sigma, USA). Part of the supernatant was analyzed for TSH and the remainder deproteinated with 5% trichloroacetic acid (TCA), and analyzed for NPSH and GSH. Protein thiol (PSH) was calculated by subtracting the values of NPSH from TSH, and phytochelatin (PC) was calculated by substracting 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℃, cooled and centrifuged at 4000 × g for 25 min. The absorbance of the supernatant was measured at 530 nm and for non-specific absorbance at 600 nm. The malondialdehyde (MDA) equivalents (TBA reactive materials) were calculated on the resulting difference using the extinction coefficient 155 mmol/cm.

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 $p \leqslant 0.05$ or $p \leqslant 0.01$.

2 Results

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 ($p \leqslant 0.05$) at all the concentrations from day 2 onwards, but for $CdCl_2$ it was found to be significant ($p \leqslant 0.05$) only at the higher concentrations 10^{-4} and/or 10^{-2} mol/L, and this was more pronounced on days 2 and 8. Significant induction ($p \leqslant 0.01$) 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 ($p \leqslant 0.05$) induced gractivity from day 2 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 ($p \leqslant 0.01$), again following a dose response.

The data for thiol levels are presented in Table 1. The TSH levels increased on all days showing a dose-response for all the chemicals tested. A significant ($p \leqslant 0.05$) 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 ($p \leqslant 0.01$) for PQ and $CdCl_2$, but not for H_2O_2.

A significant ($p \leqslant 0.05$) 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 ($p \leqslant 0.01$) from day 2 to day 8. The PC levels calculated for either PQ or H_2O_2, however, did not increase. The significant ($p \leqslant 0.01$) 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).

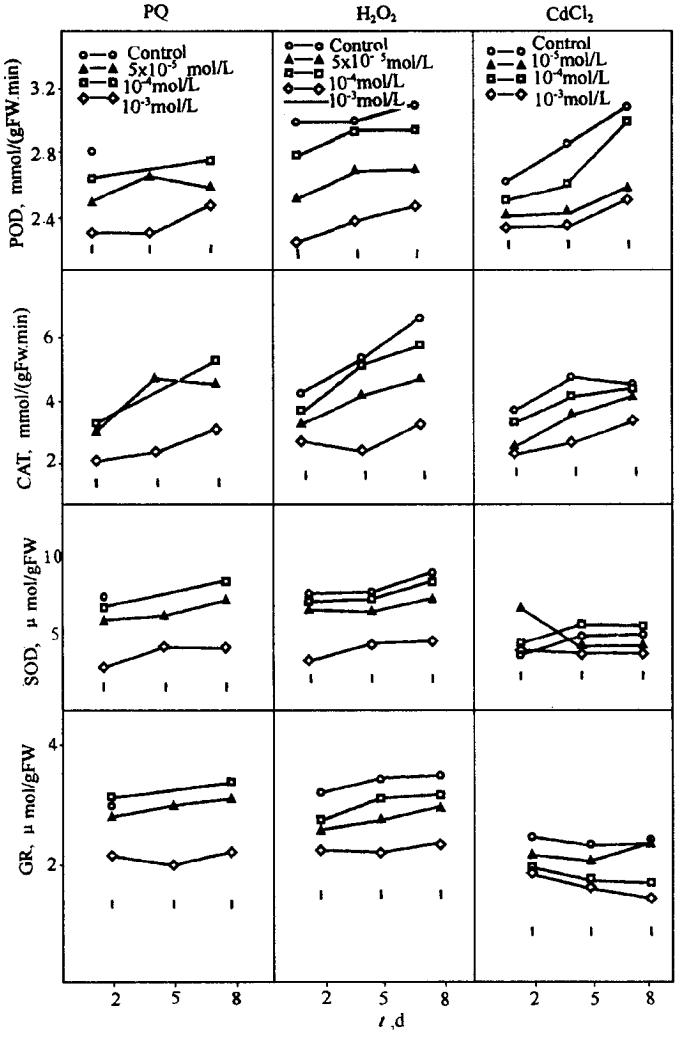

Fig. 1 Activity of POD, CAT, SOD and GR estimated on different days following treatment with PQ, H_2O_2 and $CdCl_2$ in growing paddy seedlings. The vertical bars indicate the least significant difference values at $p<0.05$

Table 1 Estimations of TSH, PSH and NPSH (μmol/g FW) in growing rice paddy seedlings on different days of development following seed-treatment with PQ, H_2O_2 and $CdCl_2$

Chemical	Conc., mol/L	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±0.05	6.9±0.44	7.6±0.01	15.33±0.12**	8.35±0.41	6.82±0.46
	10^{-4}	16.16±0.15**	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_2O_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±1.18**	3.41±0.54	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**	4.35±0.9	11.2±0.78**	17.3±0.44**	2.8±0.22	15.56±0.55**	21.13±1.22	5.16±0.88	15.68±0.29**

Increase significant with control at $p\leqslant0.05$ (*) or $p\leqslant0.01$ (**); values represent mean ± *SEM* calculate from three replications

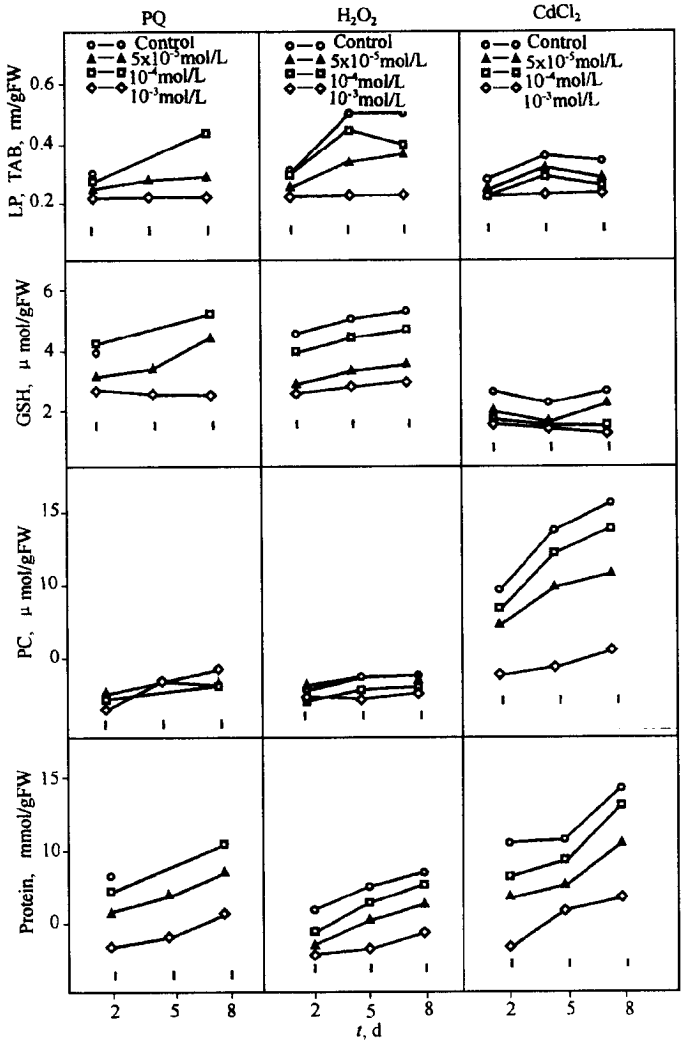

Fig. 2 Level of LP, GSH, PC and protein estimated on different days following treatment with PQ, H_2O_2 and $CdCl_2$ in growing paddy seedlings. The vertical bars indicate the least significant difference values at $p < 0.05$

3 Discussion

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 plays 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, 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, 1991).

In this study, PQ, H_2O_2 and $CdCl_2$ induced POD, CAT, LP and proteins. Since the protein content invariable 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 (Brunschon-Hart, 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 pollutants and so on.

Under condition 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, 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 metal (Yang, 1996; Panda, 1997). Biochemical responses to environmental stress have in common the induction of some of the shock proteins which may have a role in the adaptive response (Wollgiehbn, 1995). These results underly the crucial involvement of GSH and PC in the potential adaptive responses to oxidative stress and metal stress respectively.

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(Received for review May 23, 1999. Accepted January 8, 2000)