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Effects of mercury and cadmium on the activities of antioxidative enzymes in the seedlings of *Phaseolus aureus*

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Abstract

Phaseolus aureus Roxb. was exposed to $HgCl_2$ and $Cd(NO_3)_2$ either at the germination stage in concentration 0.5, 5 and 25 μM for 48 and 96 h, or at the seedling stage (5th day of germination) in concentration 0.5, 5 and 20 μM for 6, 24 and 48 h. The germination and the growth of roots (germination stage treatment) were less in Hg than in Cd treatment. The seedlings (seedling stage treatment) were, however, more susceptible to Cd than Hg. Both root and leaf tissues of the plant treated at the germination stage showed enhanced lipid peroxidation and activities of the antioxidative enzymes (catalase, guaiacol peroxidase and ascorbate peroxidase), except the catalase in leaf in 25 μM Cd treatment. At seedling stage the content of malondialdehyde increased significantly only in the leaf tissue, during 6 h exposure. The activities of all the enzymes exhibited an increasing trend in both the tissue of the seedlings, particularly the leaf, at least after 24 and 48 h, except the catalase whose activity declined in response to Cd. Active involvement of the guaiacol and ascorbate peroxidases, rather than catalase, in scavenging cellular H_2O_2 was indicated. It was concluded that the two metals had little primary damaging effect on membranes.

Key words: bean, catalase, germination, lipid peroxidation, peroxidase.

Introduction

Environmental stresses, whether physical or chemical, which disturb the normal cellular metabolism can upset the balance of production and quenching of reactive oxygen species like superoxide radical, singlet oxygen and hydrogen peroxide (H_2O_2) (Okuda *et al.* 1991, Spychalla and Desborough 1990, Luna *et al.* 1994). Increased production/accumulation of $O_2^{\cdot-}$ and H_2O_2 in organisms under stress may lead to the generation of $HO^{\cdot}$ radical by Haber-Weiss reaction (Bowler *et al.* 1992)

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or more efficiently by Fenton type reaction (Gutteridge and Halliwell 1990) leading to extensive damage of membranes by peroxidation of their constituent lipids. Concomitant changes in the activities of the enzymes catalase, peroxidase and superoxide dismutase, responsible for removal and destruction of the reactive oxygen species, and hence referred as antioxidative enzymes, however, have got important bearings on the oxidative damage of membranes in organisms under stress (Cakmak and Horst 1991, Spychalla and Desborough 1990, Hendry *et al.* 1992, De Vos *et al.* 1993).

Although heavy metals are known to affect various metabolic processes drastically, their role in oxidative cytotoxicity has been taken up for critical investigation only recently (Cakmak and Horst 1991, Bishnoi *et al.* 1993, De Vos *et al.* 1993, Luna *et al.* 1994). The objective of the present study was to establish the relationship between the toxicity, the extent of lipid peroxidation and the changes in the activities of the antioxidative enzymes for Hg and Cd on which the information is very scant. The enzymes studied were catalase, guaiacol peroxidase and ascorbate peroxidase, all scavengers of H_2O_2. The superoxide dismutase was excluded from the study because the product of the reaction catalysed by it, the H_2O_2, is ultimately removed by the above enzymes. The paper also reports how the toxicity of the two metals is dependent on the stage of development at which the test species, *Phaseolus aureus*, is exposed to them.

Materials and methods

Seeds of *Phaseolus aureus* Roxb., presoaked overnight in distilled water, were germinated over nylon net remaining in touch constantly with 100 cm^3 Hoagland's solution in a growth chamber (temperature 25 ± 2 °C; photoperiod 12 h; irradiance 300 $\mu mol\ m^{-2}\ s^{-1}$ by cool white fluorescent lamps).

Analytical grade mercuric chloride ($HgCl_2$) and cadmium nitrate ($Cd(NO_3)_2$) were used for the treatment. The day on which the seeds were set for germination was considered as the '0' day and the subsequent days as the 1st, 2nd...days of germination. Two treatments were followed: *1*) germination stage treatment (started on the '0' day), concentrations 0.5, 5 and 25 μM, various parameters were studied after 48 and 96 h; *2*) seedling stage treatment (started on the 5th day of germination), concentrations 0.5, 5 and 20 μM, exposure 6, 24 and 48 h.

Both root and leaf tissues were harvested, and accurately weighed samples were frozen immediately in liquid N_2 and stored at -70 °C for the study of the enzymes activities. Lipid peroxidation was estimated in the fresh samples. Thiobarbituric acid (TBA) was used to determine malondialdehyde (MDA), an end product of lipid peroxidation (Heath and Packer 1968). Each set of the plant tissue (200 mg) was homogenized in 2 cm^3 0.1% trichloroacetic acid (TCA) solution. The resultant homogenate was centrifuged at 15 000 *g* for 10 min. The reaction mixture containing 0.75 cm^3 supernatant and 2.25 cm^3 TBA reagent (0.5 g TBA, 99 cm^3 20% TCA, 1 cm^3 2 % butylated hydroxytoluene in ethanol) was heated at 90 °C in a hot water bath for 20 min and then quickly cooled in an ice-bath. After centrifugation at

15 000 *g* for 10 min the absorbance of MDA-TBA complex was determined at 532 nm and the non-specific absorbance, which was mostly negligible, at 600 nm. The concentration of MDA-TBA complex was calculated from the absorbance coefficient ($A = 155$ mmol^{-1} cm^{-1}).

For studying the activities of either of the enzymes, the frozen plant material was homogenized in 50 mM potassium phosphate buffer (pH 7.8) having 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM EDTA, 1 % polyvinylpyrrolidone (PVPP), and 0.1 mM ascorbate for ascorbate peroxidase. The homogenate was centrifuged at 15 000 *g* for 15 min. All the operations were performed at 4 °C. The activities of the enzymes were measured in the supernatant at 25 °C.

The activities of catalase (EC 1.11.1.6) and guaiacol peroxidase (EC 1.11.1.7) were measured by the method of Chance and Maehly (1955) with slight modification. The reaction mixture for catalase contained 25 mM potassium phosphate buffer (pH 6.8), 20 mM H_2O_2 and the enzyme aliquot. The decomposition of H_2O_2 was measured by following the decrease in absorbance at 240 nm (ΔA = 39.4 mmol^{-1} cm^{-1}). For guaiacol peroxidase the reaction mixture contained 25 mM phosphate buffer (pH 6.8), 10 mM H_2O_2, 0.1% guaiacol and the enzyme aliquot. The oxidation of guaiacol to tetraguaiacol was measured at 470 nm (ΔA = 26.6 mmol^{-1} cm^{-1}).

The ascorbate peroxidase (EC 1.11.1.7) in the leaf sample was measured according to Nakano and Asada (1981). The reaction mixture consisted of 25 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 1 mM H_2O_2 and 0.5 mM sodium ascorbate. The oxidation of ascorbate was followed at 290 nm (ΔA = 2.8 mmol^{-1}cm^{-1}).

Each estimation was done 4 times in four independent sets of experiments. The results were expressed as mean ± standard deviation. *F*-test and *t*-tests were

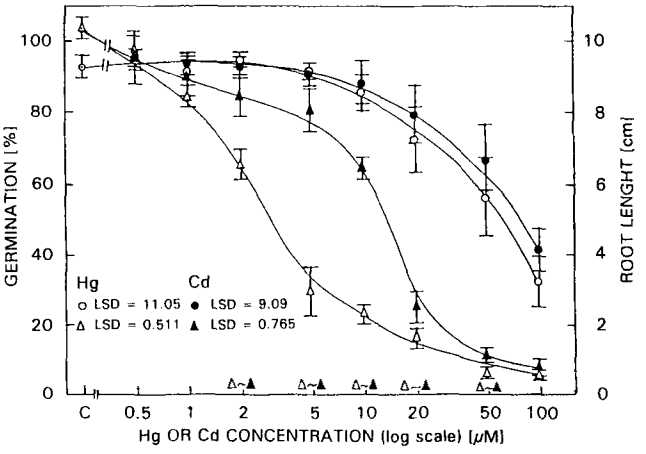

Fig. 1. The percentage of *P. aureus* germination (mean ± SD, n = 3, 7th day, *circles*) and the growth of roots (mean ± SD, n = 4, 4th day, *triangles*) on Hoagland's solution containing Hg (*open symbols*) and Cd (*closed symbols*) in various concentrations. The symbols in open and closed pairs at the bottom of the graph indicate significant difference between Hg and Cd treatment in that concentration.

performed following Gomez and Gomez (1983). Wherever the variation (F-test) was significant ($P \leq 0.05$), the least significant difference (LSD) has been given, otherwise not, and wherever the difference according to t-test was significant ($P \leq 0.05$), it has been suitably indicated.

Results

Both Hg and Cd significantly inhibited germination at 20 μM concentration and beyond that (Fig. 1). Their effect did not differ significantly. However, length of roots of the established seedlings was more reduced by Hg than by Cd in 2, 5 and 10 μM concentrations.

Table 1. The effect of Hg and Cd on the MDA content [nmol g^{-1} (f.m.)] of roots and leaves of *P. aureus* Roxb. at germination stage treatment (GST) and seedling stage treatment (SST). Values are means ± SD, n = 4. LSD at $P \leq 0.05$. NS = not significant. The mean values in Cd treatment marked with asterisk are significantly different ($P \leq 0.05$) from that in Hg treatment at corresponding concentration.

Hg/Cd [μM]	GST			SST					
	48 h	96 h		6 h		24 h		48 h	
	root	root	leaf	root	leaf	root	leaf	root	leaf
Control	56.33	49.71	44.17	51.65	42.76	46.03	39.98	44.19	44.07
	±5.17	±5.96	±5.03	±4.42	±4.58	±4.55	±5.57	±4.99	±3.63
Hg 0.5	54.14	50.35	41.28	50.05	55.94	43.80	51.44	46.29	47.38
	±5.25	±6.46	±5.61	±1.97	±5.79	±2.77	±5.14	±3.63	±4.88
Hg 5.0	59.31	62.02	52.20	52.24	58.54	45.17	49.50	41.30	51.36
	±4.78	±6.57	±5.46	±3.54	±5.31	±5.13	±6.32	±4.83	±4.54
Hg 20/25	69.35	65.95	59.04	47.82	58.67	35.22	42.89	38.34	48.24
	±6.75	±5.42	±4.75	±5.46	±4.74	±4.20	±4.17	±3.22	±3.22
LSD	9.24	10.58	9.19	NS	8.11	6.54	8.38	NS	NS
Cd 0.5	58.20	53.48	47.13	53.24	45.96*	45.43	40.87*	48.16	43.75
	±6.02	±3.37	±5.90	±5.09	±4.06	±3.23	±2.52	±5.29	±4.15
Cd 5.0	63.39	59.97	56.27	48.33	53.33	46.37	41.67	44.18	45.04
	±7.43	±4.42	±6.21	±3.26	±3.78	±5.59	±3.94	±4.08	±3.53
Cd 20/25	65.95	67.30	63.10	47.71	56.18	36.86	44.15	35.02	46.82
	±5.08	±6.02	±4.78	±3.92	±5.11	±5.61	±4.23	±3.11	±3.41
LSD	NS	8.85	8.01	NS	7.62	7.47	NS	6.71	NS

The seedlings were more susceptible to Cd than Hg. They died within 24 h at a Cd concentration as low as 30 μM, however not a single death among the seedlings was recorded in the case of Hg treatment even at a concentration as high as 100 μM (beyond which the result was not tested). Therefore, the highest concentration of both the metals for treatment of the seedlings was reduced to 20 μM.

At germination stage the MDA content of the tissues increased with the increase in Cd or Hg concentration in all the cases except the roots after 48 h exposure to Cd (Table 1). At seedling stage, however, MDA content of roots was not increased (Table 1). In leaves, on the other hand, the MDA content significantly increased in response to the metal treatment during the early periods of exposure (6 h) in all concentrations (Table 1). The effect of Hg in 0.5 μM concentration was significantly greater than that of Cd. However, after 24 and 48 h the MDA content of leaf declined more or less to control level except in the case of 24 h exposure to Hg.

Table 2. The effect of Hg and Cd on the activities of catalase [U mg^{-1}(protein)] in roots and leaves of *P. aureus* Roxb. at germination stage treatment (GST) and seedling stage treatment (SST). The other details as in Table 1.

Hg/Cd [μM]	GST 48 h root	96 h root	leaf	SST 6 h root	leaf	24 h root	leaf	48 h root	leaf
Control	9.98	16.88	101.0	20.52	113.0	24.15	129.1	27.23	132.0
	±0.86	±1.56	±5.51	±1.70	±5.63	±1.25	±2.73	±1.05	±3.28
Hg 0.5	10.50	17.87	102.1	20.84	111.9	26.55	127.8	28.36	132.2
	±1.92	±1.45	±4.02	±2.23	±7.19	±2.00	±4.57	±2.18	±5.47
Hg 5.0	10.04	15.96	105.7	20.22	120.5	26.63	132.8	29.42	134.9
	±1.31	±1.13	±5.32	±0.96	±5.46	±1.72	±4.81	±1.29	±2.45
Hg 20/25	12.77	19.84	108.4	17.91	127.8	25.65	146.1	22.06	145.9
	±2.14	±2.71	±3.53	±1.46	±2.90	±0.54	±4.77	±1.36	±3.75
LSD	NS	NS	NS	NS	8.50	NS	7.14	2.55	6.01
Cd 0.5	9.28	16.84	111.2	20.43	112.6	26.55	121.8	29.38	128.6
	±1.22	±1.34	±7.97	±1.72	±5.20	±1.44	±5.61	±1.83	±3.61
Cd 5.0	11.03	16.44	119.7*	19.46	115.9	23.85	124.3*	26.71*	134.2
	±0.89	±1.98	±5.44	±0.82	±5.17	±1.68	±3.82	±1.35	±1.90
Cd 20/25	15.46	23.91	101.2	17.25	110.5*	22.07*	117.4*	20.38	123.0*
	±2.58	±2.20	±8.50	±1.98	±4.40	±1.29	±3.52	±1.94	±2.22
LSD	2.41	2.81	10.22	NS	NS	2.24	6.36	3.14	4.38

The activities of catalase increased in both roots and leaves at germination stage (Table 2). The increase was mostly significant for Cd (with the exception of 25 μM concentration). At seedling stage, the activity in roots was significantly inhibited by both Hg and Cd after 48 h, together with a slight increase at 0.5 μM concentration. In leaves, on the other hand, while Hg significantly increased the activity of the enzyme, Cd inhibited its activity (Table 2).

Both the metals significantly increased the activity of guaiacol peroxidase (POX) in roots and leaves at germination stage, parlicularly in 25 μM concentration (Table 3). The activity also significantly increased at seedling stage, after 24 and 48 h; the increase was maximum in 0.5 μM concentration in roots and in 5 and 20 μM concentrations in leaves (Table 3). On short duration (6 h) exposure, however, the activity in roots significantly decreased.

Table 3. The effect of Hg and Cd on the activities of guaiacol peroxidase [nmol(tetraguaiacol) mg^{-1} (protein)] in roots and leaves of *P. aureus* Roxb. at germination stage treatment (GST) and seedling stage treatment (SST). The other details as in Table 1.

Hg/Cd [μM]	GST			SST					
	48 h	96 h		6 h		24 h		48 h	
	root	root	leaf	root	leaf	root	leaf	root	leaf
Control	193.3	461.1	10.68	483.6	11.51	446.8	9.90	550.1	11.55
	±12.36	±32.98	±0.97	±36.91	±1.24	±22.27	±0.41	±39.15	±0.33
Hg 0.5	187.1	525.4	10.15	486.8	11.22	577.1	10.74	756.3	9.51
	±8.04	±40.39	±0.84	±33.80	±0.92	±42.38	±0.75	±54.2	±0.81
Hg 5.0	219.5	439.8	12.81	396.5	12.12	496.5	20.90	591.6	16.61
	±18.45	±32.16	±1.02	±43.46	±0.79	±27.33	±0.97	±48.49	±1.02
Hg 20/25	420.2	771.9	17.40	326.3	10.56	483.3	20.51	600.9	17.88
	±52.5	±76.5	±1.27	±50.41	±1.01	±30.34	±1.73	±68.04	±0.41
LSD	44.37	75.54	1.97	64.93	NS	45.68	2.08	84.14	1.08
Cd 0.5	209.8	470.0	11.30	497.0	11.73	530.2	9.46*	683.4	9.04
	±17.45	±24.59	±0.42	±27.81	±0.94	±36.15	±0.20	±50.30	±0.34
Cd 5.0	207.21	421.7	11.23	424.5	12.81	550.3	21.16	634.1	18.34
	±16.98	±31.1	±1.01	±33.27	±0.48	±33.80	±1.29	±37.69	±1.15
Cd 20/25	358.3	549.4*	15.18	376.7	12.75*	581.0*	19.51	646.8	21.02*
	±14.58	±35.53	±1.50	±42.77	±1.13	±30.17	±0.42	±53.45	±0.67
LSD	23.86	45.32	1.83	55.46	NS	43.67	1.10	78.75	1.18

Table 4. The effect of Hg and Cd on the activities of ascorbate peroxidase [U mg^{-1}(protein)] in leaves of *P. aureus* Roxb. at germination stage treatment (GST) and seedling stage treatment (SST). The other details as in Table 1.

Hg/Cd [μM]	GST	SST		
	96 h	6 h	24 h	48 h
Control	0.127	0.121	0.154	0.140
	±0.009	±0.007	±0.010	±0.010
Hg 0.5	0.142	0.126	0.154	0.146
	±0.020	±0.013	±0.005	±0.012
Hg 5.0	0.175	0.120	0.160	0.158
	±0.014	±0.009	±0.010	±0.020
Hg 20/25	0.212	0.122	0.153	0.168
	±0.026	±0.004	±0.012	±0.015
LSD	0.036	NS	NS	NS
Cd 0.5	0.122	0.121	0.151	0.144
	±0.008	±0.010	±0.014	±0.004
Cd 5.0	0.168	0.121	0.161	0.168
	±0.011	±0.011	±0.018	±0.011
Cd 20/25	0.175	0.116	0.164	0.180
	±0.012	±0.009	±0.005	±0.024
LSD	0.022	NS	NS	0.028

Similar to POX, the activity of ascorbate peroxidase (APX) also significantly increased at germination stage (Table 4). At seedling stage, however, significant increase in its activity was only in response to Cd, after 48 h.

Discussion

Mercury has always been reported to be more toxic than Cd (Rai *et al.* 1981, Nordberg 1976, Fergusson 1990, Gadallah 1994), and the same is also evident from the present studies concerning the germination of seeds and the growth of roots. The seedling stage experiment, however, demonstrated that the toxicity of a metal, in comparison to another may not be the same at all the stages of plant development. The reason for this is not known and no report is available in support of this observation.

Significant increase in the MDA content of roots at germination stage appears to be the result of decrease in volume and increase in compactness of the cells in the tissue (Ernst *et al.* 1992) in response to the metals rather than increase in the peroxidation of membrane lipids as reported for other heavy metals (Cakmak and Horst 1991, De Vos *et al.* 1993) since no increase was observed at seedling stage. Further, Hendry *et al.* (1992) observed only insignificant changes in the MDA contents of roots of the seedlings of either Cd tolerant or Cd sensitive clone of *Holcus lanatus* on exposure to Cd. The decrease at seedling stage was probably because of inhibition of metabolic activities (De Lima and Copeland 1994) by the metals, and consequently less production of H_2O_2 and superoxide radicals.

The insignificant accumulation of MDA in leaf at seedling stage on long duration (48 h) exposure to the metals, even to Cd to which the seedlings are sensitive, is in contrast to the report of Hendry *et al.* (1992) who observed greatly enhanced levels of MDA in the shoots of the sensitive clone of *Holcus lanatus* even after 7-d exposure to Cd. The observed low level might be because of proper sequestration of the metals in *P. aureus* on long period exposure since during 6 h exposure the MDA content increased greatly (Table 1). The increase might be because of the inhibition of the enzymes of photosynthetic carbon reduction (PCR) cycle, which metals do (Clijsters and Van Assche 1985, Sheoran *et al.* 1990, Van Assche and Clijsters 1990), leading to the formation of superoxide radicals and singlet oxygen (Halliwell 1981). Comparatively greater increase in the MDA content of leaf in Hg treatment than that in Cd at 0.5 and 5 μM concentrations was probably because of the direct effect of Hg on the photosynthetic electron transport (PET) (Rai *et al.* 1991, Prasad *et al.* 1991) causing generation of singlet oxygen (Halliwell 1981); Cd does not affect PET directly (Krupa *et al.* 1993). The increase in the MDA content in leaves at germination stage (Table 1) was probably due to similar reasons.

Conflicting reports exist on the activities of catalase in plant tissues exposed to heavy metals; while Subhadra *et al.* (1991) reported an increase in its activity in roots, Cakmak and Horst (1991) and Luna *et al.* (1994) reported a decrease in roots and leaves, respectively. The increase in the activity in roots at germination stage (Table 2) may be an adaptive process; the roots emerge out in an environment already under stress. This is supported by the observation that the activity was

inhibited at exposure in post emergence condition (Table 2), *i.e.* at seedling stage, particularly in 20 μM concentration. The result was as expected since both the metals are effective inhibitors of enzymes in *in vitro* (Kagi and Hapke 1984). In addition, unlike Hg, Cd also seems to affect the pathway of synthesis of the enzyme as its activity in leaves at seedling stage was drastically inhibited by the metal; catalase being photosensitive needs constant fresh synthesis (Feierabend *et al.* 1992). Proper sequestration at germination stage probably prevented Cd from inhibiting the activity of the enzyme by a significant value even in 25 μM concentration (Table 2). Although the activities of catalase have been reported to bear a negative correlation with the levels of MDA in plant tissues under Al (Cakmak and Horst 1991) and Cu (Luna *et al.* 1994) stresses, the relationship was not observed in this study either for Hg or Cd.

The significant increase in the activity of guaiacol peroxidase (POX) in response to both the metals (Table 3) is in agreement with the reports available (Hendry *et al.* 1992, Subhadra *et al.* 1991, Cakmak and Horst 1991). This suggests active participation of the enzyme in scavenging H_2O_2 although it is present mostly in cell wall (Bowler *et al.* 1992). The decrease in the activity at germination stage after 6 h (Table 3) was probably due to direct interaction of the metals with the enzyme (Kagi and Hapke 1984).

Unlike POX and catalase, ascorbate peroxidase (APX) is present in stroma (Miyake *et al.* 1993), and therefore its role as an antioxidative enzyme is increasingly being investigated (Mishra *et al.* 1993, Luna *et al.* 1994). The significant increase of the activity of the enzyme in leaves at seedling stage at 48 h exposure to Cd (Table 4) might be one of the reasons of the low level accumulation of MDA in the tissue during that period in response to the metal when compared to Hg. The significant increase only for Cd suggests metal specific response of the enzyme. This is further supported by the report of Luna *et al.* (1994) who observed significant decrease in its activity in response to Cu.

In conclusion, the present study revealed that the two metals although similar in chemical nature, are very different in their effect *in vivo*. The lethal effect of Cd on the seedlings (at seedling stage) in 30 μM or higher concentrations appears to be because of its drastic inhibitory effect on catalase which plays important role in cellular metabolism. The increase in the activities of the H_2O_2 scavenging enzymes although gives a circumstantial evidence of enhanced generation of superoxide radicals and H_2O_2 in the tissues (Fridowich 1986), insignificant increase in the levels of MDA in leaf on long duration exposure, and rather a decrease in its level in root (Table 1), hardly suggest much generation of $HO^{\cdot}$ radical by Haber-Weiss or Fenton type reaction in tissues under Hg or Cd stress. The transient increase in the levels of MDA in leaf at seedling stage during 6 h exposure (Table 1) might be because of the peroxidative damage of membranes as a result of the generation of singlet oxygen. Thus, lipid peroxidation induced by these metals is a consequence rather than the primary cause of toxicity unlike the metal like Cu which has been reported to increase the levels of lipid peroxidation in leaf by as high as 100 % even in dark treatment (Luna *et al.* 1994), and in root by as high as 200 % (De Vos *et al.* 1993) by generating $HO^{\cdot}$ radical by Fenton type reaction (Luna *et al.* 1994).

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