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Responses of Antioxidant Defenses in *Coprinus comatus* Exposed to Cadmium and Mercury Toxicity

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Pot culture experiments using *Coprinus comatus* were conducted to study the effects of soil cadmium or mercury concentrations (at doses of 0.5, 1.5, 5.0 and 15.0 mg kg^{-1}) on the growth, the metal accumulation, soluble proteins, lipid peroxidation, thiol compounds and the activities of antioxidant enzymes. The results showed that the biomass and soluble protein content of *C. comatus* were inhibited by Cd and Hg, but the presence of metals enhanced lipid peroxidation in stipes and caps. Both Cd and Hg were readily accumulated by fruit bodies, and their contents were greater in the caps than those in the stipes. Thiol compounds and antioxidant enzyme activities [catalase (CAT) and peroxidase (POD)] were induced by soil Cd, while the levels increased with Hg concentrations up to a maximum value and then decreased. However, superoxide dismutase (SOD) activity was enhanced exposed to both Cd and Hg.

Key Words: Antioxidant enzymes, Cadmium, *Coprinus comatus*, Mercury, Toxicity.

INTRODUCTION

Heavy metals are continuously being mobilized and dispersed into the ecosystems by an expanding variety of anthropogenic sources such as industrial processes, traffic, mining, solid waste management and agricultural activities, suggesting an increasingly important role for metal pollution[1,2]. They have long residence times in soils and may continue to exert harmful effects on the environment[3]. Among metals, cadmium (Cd) and mercury (Hg) are of special concern since they are considerably toxic to living organisms at ecologically relevant concentrations. They are ubiquitous in the environment and are not considered as essential elements to living organisms. Cadmium has long biological half-life and its tendency to form strong complexes with S-containing peptides/proteins renders this metal a notable hazard to a wide range of organisms[4]. Mercury, like cadmium, is considered one of the most poisonous microelement of our ecosystem[5], mainly because mercury compounds often exert clastogenic effects in eukaryotes, especially by binding -SH groups and acting as spindle inhibitors, thereby causing aneuploidy and/or polyploidy[6,7].

Therefore, controlling the uptake and accumulation of toxic metals is becoming a fundamental task for living organisms. The ability of some fungi to survive in environments with different levels of heavy metals may depend on a set of tolerance/resistance mechanisms. Possible strategies of detoxification include extracellular chelation or precipitation by secreted metabolites[8]; binding of metal to cell wall or complexation in vacuoles[8,9]; production of compounds containing thiol (SH) functional groups[8], which are capable to bind heavy metals[10]; synthesis of various antioxidants to combat increased production of reactive oxygen species (ROS) caused by metal[11].

Metal-induced toxicity observed in different living organisms, has been associated with oxidative damage[8,12]. Oxidative damages resulting from ROS may cause wide-ranging damage to biomolecules such as lipids, proteins and nucleic acids, eventually leading to cell death[11]. Therefore, tolerance of the fungi to different metals has been associated with its ability to clear away ROS[13]. Fungi have well-developed defense systems in order to scavenge ROS and to limit oxidative damages by using both enzymatic and non-enzymatic systems, including superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH)[11].

The accumulation of potentially toxic metals in different mushroom (macro-fungi) is well documented[14-18] and the concentrations are, commonly, considerably higher than those in green plants. This would suggest that macro-fungi possess a very special mechanism that helps them to tolerate oxidative stress. Responses of filamentous fungi in liquid medium to metals have been extensively studied but much less work was done on effect of cadmium and mercury toxicity on fruiting body of *C. comatus*, which is a common macro-fungus. Thus, it looks necessary to study antioxidative responses of the mushroom when exposed to heavy metals. This may help in getting

an insight into the mechanisms of metal tolerance for mushroom.

In the present study, we examined the responses upon different level concentrations of Cd or Hg exposure in cap and stalk of *C. comatus* by pot experiments. The major objectives of the investigation were to evaluate the metals toxicity by assaying metal stress indexes [*e.g.*, biomass, metal accumulation, soluble proteins and malondialdehyde (MDA)] and explore the potential metal tolerance mechanisms in the mushroom by determining thiol compound contents and antioxidant enzymes (SOD, POD and CAT) activities.

EXPERIMENTAL

Pot experiment and biomass: A strain of *C. comatus* (Cc173) was purchased from Huike, a mushroom production site in Chengdu of China and then cultured on PDA medium (potato extract 20 %, glucose 2 %, agar 2 %). The compost (30 % cottonseed hulls, 12 % cattle manure, 6 % wheat bran, 2 % $CaCO_3$ and 50 % water) was packed into plastic bags after routine fermentation and then autoclaved. Every bag weighing 0.75 kg was inoculated in one tube of mycelia of *C. comatus* (Cc173) together with the PDA medium.

The study was conducted in a greenhouse which was used to keep temperature constant and filtrate sunlight. Natural brown soil used in the study was collected from agricultural fields in Longquanyi, Chengdu and some physicochemical properties were listed in Table-1. Soil samples were air dried in room temperature and was screened through a 10-mm sieve after moving the visible weeds and small stones. After these steps, solutions of either Cd^{2+} ($CdCl_2$) or Hg^{2+} ($HgCl_2$) were added to the soil (0.5, 1.5, 5.0 and 15 mg kg^{-1}), control group added with the same volume of distilled water. Then the soil sample was incubated for 3 months.

TABLE-1
PHYSICOCHEMICAL PROPERTIES OF THE UNTREATED SOIL

Parameter	Soil value
pH	6.93 ± 0.05
Water capacity (%)	13.62 ± 0.19
cation exchange capacity (cmol kg^{-1})	11.45 ± 0.24
organic mater content (g kg^{-1})	18.18 ± 0.26
Total Cd (mg kg^{-1})	0.12 ± 0.03
Extracted Cd (mg kg^{-1})	0.05 ± 0.02
Total Hg (mg kg^{-1})	Not determinted
Extracted Hg (mg kg^{-1})	Not determinted

Data represent the mean ± SE of three replicates.

After incubation, 6 kg soil samples were placed in each plastic pot. Meanwhile, 0.75 kg uniform mycelia together with the compost (taken out from the bags) were transplanted into each pot. Tap water was added daily to reach the field water-holding capacity.

After 7 days fructification (formation of primordia), the ripened fruiting bodies were harvested and washed with deionized water for three times, then separated into caps and stalks. A half of samples were used to determine MDA content, thiolic compounds, soluble protein levels, antioxidant enzymes activity, the other half were dried at 50 ºC in an oven for 72 h to a constant weight for dry weight determination and then powdered in a mill for analyses of Cd and Hg.

Chemical analysis and sample preparation: The pH, water capacity and organic mater content and cation exchange capacity of the control soil were determined according to standard methods described by Lu[19]. The mushroom samples were digested with concentrated HNO_3 and 30 % H_2O_2 (3:1, v/v) in microwave (*ca.* 40 min). Soil samples were digested with mixture (HNO_3:$HClO_4$:HCl = 5:2:2 (v/v/v)) as above method. The bioavailable (EDTA-extractable) levels of Cd and Hg in soil were determined by the method of Tarvainen and Kallio[20]. The Cd concentrations were measured directly by graphite-furnace atomic absorption spectroscopy (VARIAN, SpectrAA-220Z). And mercury contents in fruit bodies and soils have been determined by flame atomic absorption spectrophotometer (VARIAN, SpectrAA-220Fs) equipped with VGA-77. Metal content was expressed in mg kg^{-1} dry weight (DW).

Fresh caps and stalks were quick-frozen in liquid nitrogen and then grinded using a pre-cooled mortar and pestle. The homogenate was suspended in 20 mM tris, 1 mM EDTA buffer (pH 7.5) and centrifuged at 15000 g for 20 min at 4 ºC. The resulting supernatant was named as the cell-free extract.

Lipid peroxidation: Malondialdehyde level (as an index of lipid peroxidation) was measured spectrophotometrically by reaction with thiobarbituric acid (TBA), according to the method of Heath and Packer[21]. The cell-free extract (0.6 mL) was mixed with 1 mL of 0.5 % TBA in 20 % trichloroacetic acid (TCA) (w/v) solution and incubated at 95 ºC for 0.5 h. After quickly cooling, the mixture was centrifuged at 10000 g for 10 min and then the absorbance of the supernatant was recorded at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from the 532 nm absorbance. The absorbance coefficient of MDA-55 mM^{-1} cm^{-1} was used in the calculation and results are expressed as nM of MDA g^{-1} of fresh weight (FW).

Thiol compounds and soluble protein contents: The SH-groups concentration was assayed according to Sedlak and Lindsay[22]. To measure total thiols (T-SH), the reaction mixture consisted of 50 µL of cell-free extracts, 150 µL of 0.2 M tris, pH 8.2 and 10 µL of 0.01M 5,5'-dithio-*bis*(2-nitrobenzoic acid) (DTNB) and was completed to a final volume of 1.0 mL with the absolute methanol. After 0.5 h reaction, the mixtures were centrifuged at 3000 g for 15 min and the absorbance of supernatants was read at 412 nm against a reagent blank (without sample) with reduced glutathione as a standard.

To measure nonprotein thiol (NP-SH), the reaction mixture consisted of 500 µL of the cell-free extracts, 400 µL of deionized water and 100 µL of 50 % TCA and was gently shaken for 15 min. Then reaction mixture was centrifuged at 3000 g for 15 min and 20 µL of the supernatant was mixed with 400 µL of 0.4 M tris, pH 8.9 and 10 µL of 0.01 M DTNB. The absorbance was recorded in 5 min at 412 nm against a reagent blank (without sample).

The soluble protein content was assayed by the Bradford[23] method, using bovine serum albumin as standard.

Activities of antioxidant enzymes: Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Beauchamp and Fridovich[24] by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. One unit of enzyme activity was defined as the quantity of enzyme causing 50 % inhibition of the initial

reduction of NBT under light. The reaction medium consisted of 1.5 mL of 50 mM phosphate buffer (pH 7.8), 300 µL of methionine (130 mM), 300 µL of NBT (750 µM), 300 µL riboflavin (20 µM), 300 µL EDTA-Na_2 (100 µM) and 50 µL of cell-free extract.

The activity of catalase (CAT, EC 1.11.1.6) was assayed according to Beers and Sizer[25]. The reaction mixture contained 800 µL H_2O_2 (100 mM), 100 µL cell free extract and completed to 3 mL volume with 50 mM phosphate buffer (pH 7.0). One enzyme activity unit was defined as absorbance at 240 nm changes 0.01 per minute. The activity was evaluated by measuring the decrease in absorbance at 240 nm due to H_2O_2 consumption.

Peroxidase (POD, EC 1.11.1.7) activities were determined spectrophotometrically following the increase in absorbance at 470 nm due to the oxidation of guaiacol according to Omran[26]. The reaction mixture in a total volume of 50 mL 100 mM phosphate buffer (pH 6.0) containing 19 µL H_2O_2 (30 %), 28 µL guaiacol was prepared immediately before use. Then 0.1 mL cell free extract with 0.9 mL phosphate buffer was added to 3 mL reaction mixture. One enzyme activity unit was defined as absorbance at 470 nm changes 0.01 per minute. Enzymatic activities were expressed as U mg^{-1} protein.

Data analysis: All experiments were arranged in a completely randomized design with three replicates. Data analysis was performed with SPSS (Statistical Package for the Social Science version 18.0) for Windows, by using one-way ANOVA with Duncan tests. Pearson correlations were used to study the relationship between heavy metal concentrations and biochemical responses. Results presented are mean ± standard deviation and were considered significantly different with $p < 0.05$.

RESULTS AND DISCUSSION

Effects of Cd and Hg on the biomass production: Heavy metal have been shown to affect growth and metabolism of fungi partly depending on the concentration of metals[8,27] and growth inhibition parameters are thought to be the important indicators of metal toxicity in fungi[28,29]. Thus, knowing the cascade of events produced by metals exposure and relating it with growth effects would be an important consideration for risk assessment in these organisms. In this study biomass of *C. comatus* (Fig. 1A) was slightly stimulated with the addition of 0.5 mg kg^{-1} cadmium, the lowest concentration used in the present study and at higher levels, Cd become strongly toxic to cells, which agrees with reports for *Aspergillus niger* under cadmium and arsenate treatment[27,30]. It is possible that low concentrations of Cd stimulated growth by forming complexes with constituents of the culture compost, which could allow essential trace elements to become available to the fungus. Exposure to Hg in the soil resulted in growth inhibition in *C. comatus* (Fig. 1B), as reflected by the general trend of decreased the dry biomass with increasing metal concentrations. Metal effects on *C. comatus* biomass indicated higher toxicity of Hg than Cd, which agrees with reports in alfalfa[31,32].

Effects of Cd and Hg on soluble protein levels: The present results indicate that Cd and Hg at low levels may not significantly affect the levels of soluble protein of *C. comatus*

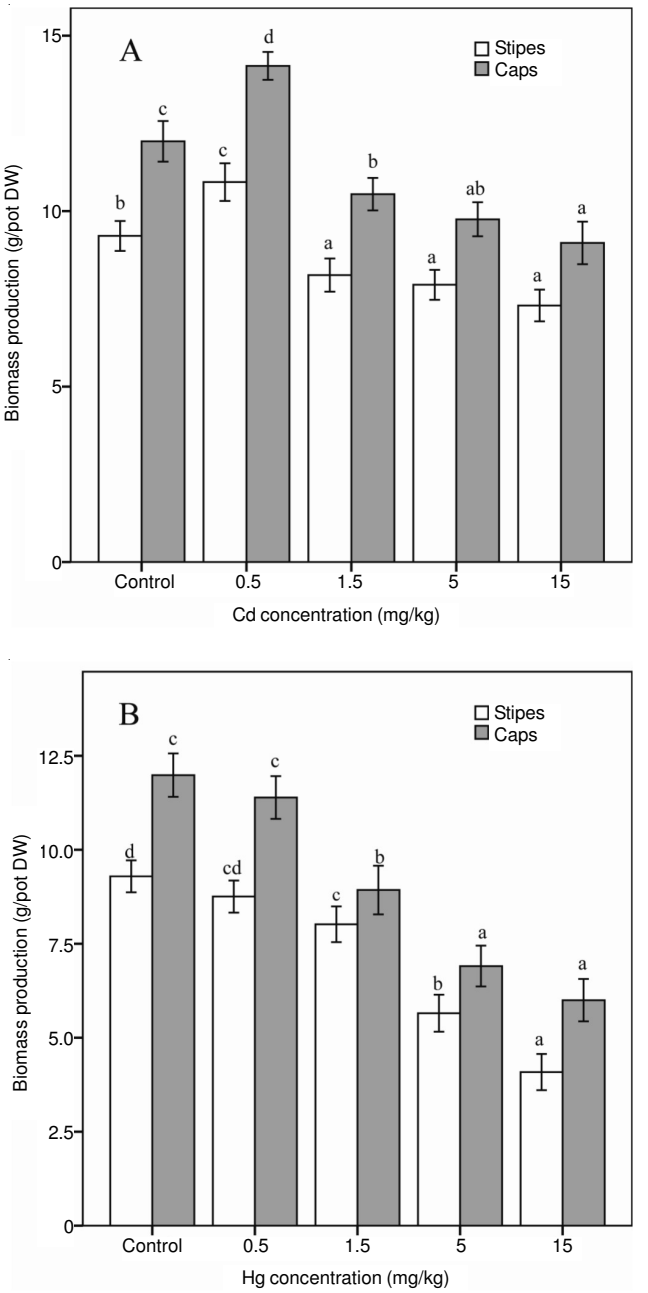

Fig. 1. Biomass (g DW) of stipes (white) and caps (gray) in *C. comatus* treated with 0 (control), 0.5, 1.5, 5.0 and 15.0 mg kg-1 Cd(A) or Hg(B). Data represent the mean ± SE of three replicates. Bars with different letters denote significant differences between treatments at $p < 0.05$, according to Duncan's test

(soil Cd or Hg concentration ≤ 1.5 mg kg^{-1}) and the protein content reduces significantly only at higher concentrations (Table-2). A similar trend of the protein inhibition was observed in *A. niger* and *Agaricus bisporus* in the presence of Cd or other metals[27,33]. The loss of soluble protein at the higher concentrations of Cd and Hg might be caused by chelating detoxification induced by excess metal in the cells. This also may be due to the autolysis of the *C. comatus* mycelium, with subsequent proteolytic breakdown at the higher concentrations of toxic metals[27].

Effects of Cd and Hg on malondialdehyde contents: The formation of MDA has been used as a general indicator of metal toxicity in cellular environment in some fungi[30,34]. In

TABLE-2
METAL ACCUMULATION, PROTEIN CONTENT IN *Coprinus comatus* TREATED WITH 0 (CONTROL), 0.5, 1.5, 5.0 AND 15.0 mg kg^{-1} Cd OR Hg

Soil (mg kg^{-1})	Metal content (mg kg^{-1} DW)		Protein content (mg g^{-1} DW)	
	Stipes	Caps	Stipes	Caps
Control	0.09±0.03a	0.11±0.03a	5.36±0.27b	5.40±0.28b
Cd 0.5	0.27±0.09ab	0.35±0.07b	5.22±0.37b	5.42±0.34b
Cd 1.5	0.54±0.1b	0.84±0.31c	5.04±0.35b	5.04±0.27b
Cd 5	0.89±0.25c	1.43±0.25d	4.87±0.28ab	4.34±0.29a
Cd 15	2.51±0.28d	3.36±0.42e	4.31±0.35a	4.31±0.28a
Control	ND	ND	5.36±0.27b	5.40±0.28b
Hg 0.5	0.19±0.02a	0.23±0.04a	5.49±0.22b	5.53±0.29b
Hg 1.5	0.27±0.03a	0.31±0.03a	5.34±0.28b	5.18±0.29b
Hg 5	0.37±0.04b	0.61±0.06b	4.71±0.22a	4.55±0.19a
Hg 15	0.72±0.07c	0.96±0.09c	4.55±0.27a	4.46±0.26a

Data represent the mean ± SE of three replicates. Values with different letters denote significant differences between treatments at $p < 0.05$, according to Duncan's test. ND = not determined.

the current study, there was a gradual increase in malondialdehyde content (Fig. 2), which was positively correlated ($R < 0.01$) with metal concentrations (both Cd and Hg), suggesting damage done to the membrane. The findings also might indicate that Hg has a higher capacity than Cd to induce oxidative damage in fruiting body of *C. comatus* (there were higher levels of lipid peroxides in Hg treated fungus compared with Cd treated mushroom (Fig. 2)).

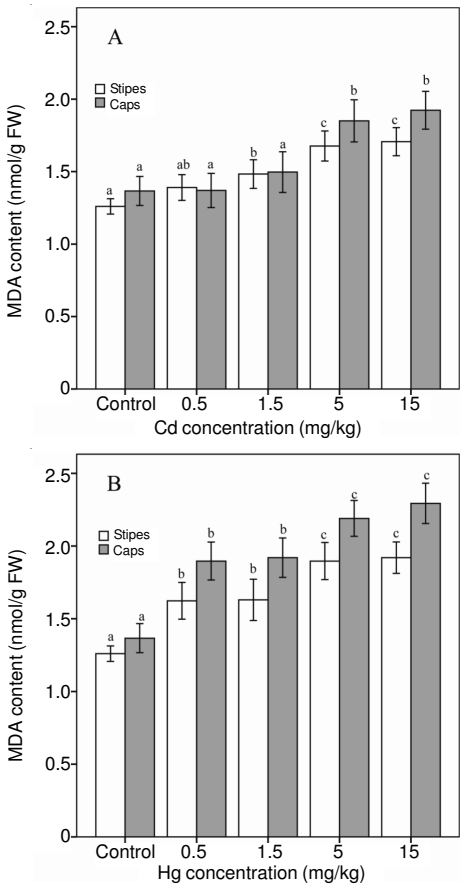

Fig. 2. MDA content (nmol g^{-1} FW) in stipes (white) and caps (gray) of *C. comatus* treated with 0 (control), 0.5, 1.5, 5.0 and 15.0 mg kg^{-1} Cd(A) or Hg(B). Data represent the mean ± SE of three replicates. Bars with different letters denote significant differences between treatments at $p < 0.05$, according to Duncan's test

Cd and Hg uptake: In the present study both cadmium and mercury accumulation in caps and stipes increased with the increasing metals spiked in the soils (Table-2), which was similar with other mushrooms[14]. Our results also indicated that the metals (both Cd and Hg) contents in the caps prevailed over that in the stipes (Table-2). Similar results have been found in wild mushrooms collected from natural contaminated soils[15,16]. In general, the highest level is observed in the sporophore (spores-forming part) and then the cap and the lowest in the stipe[35]. Partitioning of metals in stipes and caps might be a strategy to avoid toxicity in mushrooms and the high mobility of Cd and Hg may led to their translocation within the fruiting body and subsequent accumulation in caps.

Effects of Cd and Hg on the levels of thiol compounds: The metal toxicity to organisms is, in partly, related to the excess production of ROS and peroxyl radicals in cells and subsequent oxidative stress, which represent a threat to the cells[11]. SH-containing compounds are known to have high affinity for metal ions[10] and also have ability to scavenge ROS[11]. In this study, the production of T-SH and NP-SH in caps seemed to be remarkably dose-dependent ($r = 0.950$, $p = 0.000$ of T-SH and $r = 0.927$, $p = 0.000$ of NP-SH, respectively) under Cd treatments (Fig. 3A,C). The contents of T-SH were not significantly affected by Cd concentration in stipes and caps below 1.5 and 5 mg kg^{-1}, respectively. NP-SH content increased significantly in the caps at 1.5, 5.0 and 15.0 mg kg^{-1} Cd concentrations. Concentrations of T-SH in both stipes and caps (Fig. 3B) increased significantly at lower Hg concentrations (0.5, 1.5 and 5 mg kg^{-1}) compared to control. But the value showed a declined trend at higher Hg concentration (15 mg kg^{-1}). NP-SH in the mushroom treated with Hg (Fig. 3D) showed similar trend as T-SH. NP-SH content in stipes and caps increased significantly up to soil Hg concentration of 1.5 or 5.0 mg kg^{-1}, with maximum increases of 47 and 51 %, respectively.

The results indicated that *C. comatus* could produce SH-compounds to protect cells against cadmium and mercury toxicity. Similar trend was found in lots of studies. A linear increase in glutathione levels with increasing Cd concentration was found in a strain of *Articulospora tetracladia* isolated from a nonpolluted stream[36]. The induction of NP-SH in response to cadmium, depending on the dose, was also observed in *Fontanospora fusiramosa*, *Flagellospora curta*[37], the *Mucor racemosus*[36] and the *Paxillus involutus*[38]. Furthermore, the increase of Pb-SH was stimulated in Cd-adapted mycelia of *F. fusiramosa*[37]. However, our previous findings showed that exposure to Cd and Pb concentrations resulted in a decrease in SH-compounds of the *A. bisporus* mycelia[33]. This may suggested that there are no clear patterns relating biochemical responses to heavy metals of macro-fungi and other antioxidant defense system might be involved in metal-stress response.

Effects of Cd and Hg on antioxidant enzymes: Effects of Hg or Cd on the antioxidant enzyme activity in the caps and stipes of *C. comatus* were shown in Figs. 4 and 5. In general, exposure to metals led to increased SOD, CAT and POD activities compared to the control.

Activities of SOD, CAT and POD increased in a concentration-dependent manner ($r \geq 0.888$, $p = 0.000$) when treated with Cd (Fig. 4). Changes of SOD activity in both caps and

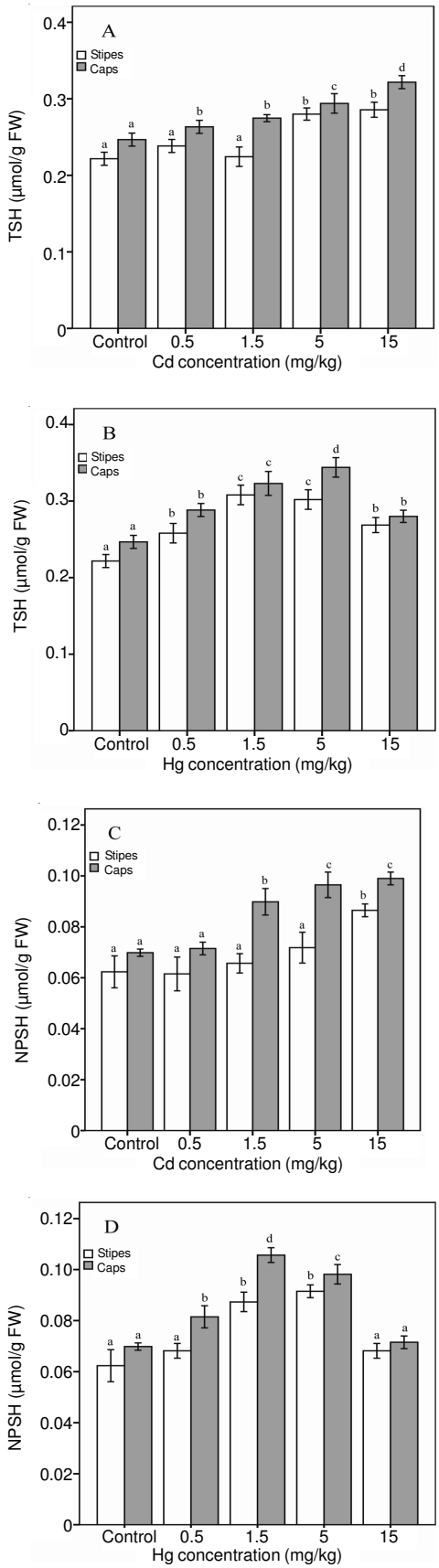

Fig. 3. Concentration of the total thiols (A and B) and the nonprotein thiols (C and D) (µmol/g FW) in stipes(white) and caps (gray) of *C. comatus* treated with 0 (control), 0.5, 1.5, 5.0 and 15.0 mg kg^{-1} Cd or Hg. Data represent the mean ± SE of three replicates. Bars with different letters denote significant differences between treatments at $p < 0.05$, according to Duncan's test

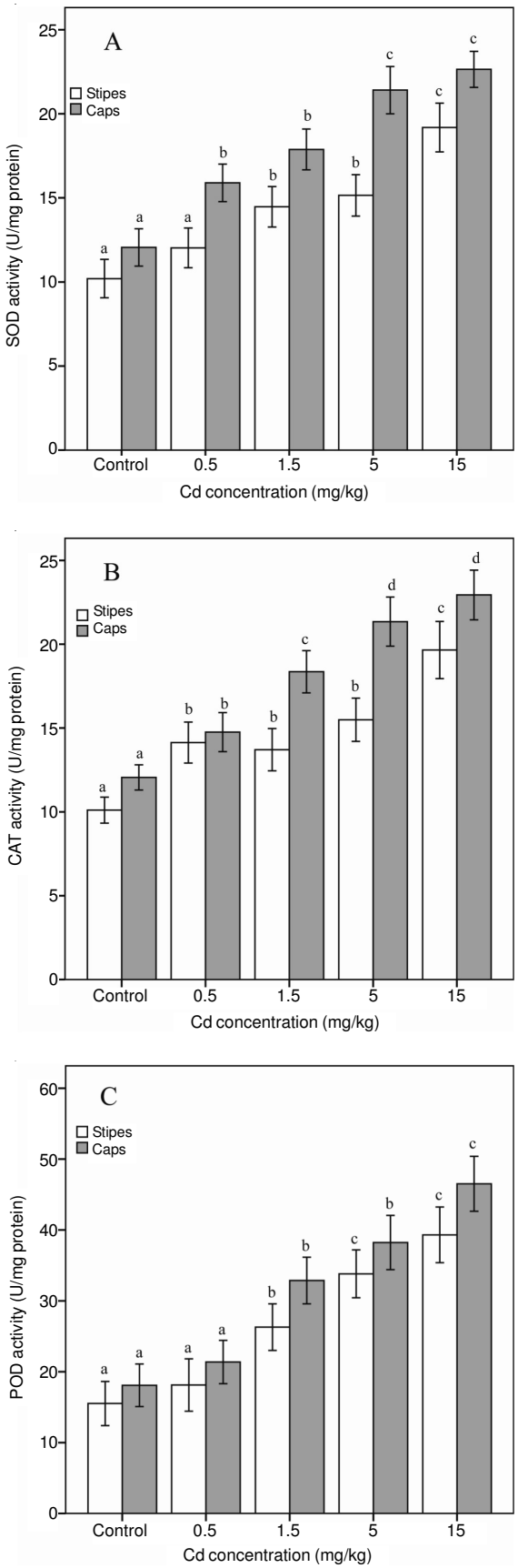

Fig. 4. Activities of the enzymes SOD (A), CAT (B) and POD (C) (U/mg protein) in stipes (white) and caps (gray) of *C. comatus* treated with 0 (control), 0.5, 1.5, 5.0 and 15.0 mg kg^{-1} Cd. Data represent the mean ± SE of three replicates. Bars with different letters denote significant differences between treatments at $p < 0.05$, according to Duncan's test

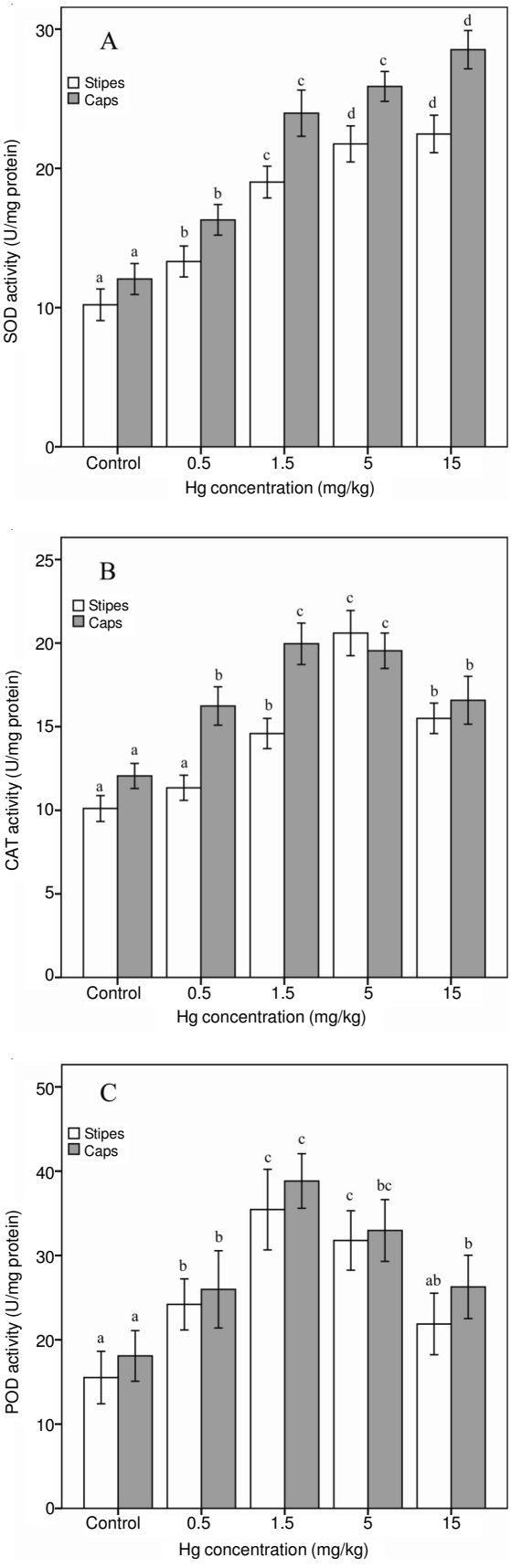

Fig. 5. Activities of the enzymes SOD (A), CAT (B) and POD (C) (U/mg protein) in stipes (white) and caps (gray) of *C. comatus* treated with 0 (control), 0.5, 1.5, 5.0 and 15.0 mg kg^{-1} Hg. Data represent the mean ± SE of three replicates. Bars with different superscript letters denote significant differences between treatments at $p < 0.05$, according to Duncan's test

stipes (Fig. 4A) were significant with increasing Cd concentrations in soils expect at 0.5 mg kg^{-1} in stipes. As cadmium increased from 0.5, 1.5, 5.0 and 15 mg kg^{-1} soils, the CAT in caps and stipes (Fig. 4B) increased significantly compared with the control. Exposed to Cd, POD activity also showed a significant increase compared to control since 1.5 mg kg^{-1} (Fig. 4C).

Under different conditions of Hg (0.5, 1.5, 5.0 and 15.0 mg kg^{-1}), we could observe bell-shaped concentration-response trends: CAT and POD activity (Fig. 5B,C) levels increased with Hg concentrations up to a maximum value and then decreased. However, SOD activity in stipes and caps (Fig. 5A) was increased with increasing metal concentration, with maximum increases of 1.20-fold and 1.36-fold, respectively. CAT activity in the stipes increased significantly up to 5 mg kg^{-1}, with maximum increases of 1.04-fold compared to the control. CAT activity in the caps increased gradually up to 1.5 mg kg^{-1}, the greatest value increasing by 66 % compared to the control. POD activity reached the peak at 1.5 mg kg^{-1}, increasing by 1.28-fold and 1.15-fold compared to the control in stipes and caps, respectively. In addition, SOD activity under Hg treatment well-correlated to Hg concentrations ($r = 0.949$, $p = 0.000$; $r = 0.960$, $p = 0.000$ of stipes and caps, respectively).

SOD is an important enzyme involved in scavenging the superoxide radicals[11]. Induction of SOD activity has been reported in *A. bisporus* and *A. niger* in response to zinc, copper, arsenic and cadmium toxicity[27,30,33]. In the present experiments, the enhancement of SOD activity in the test fungus indicated detoxification of superoxide radicals generated by Cd and Hg. CAT is ubiquitous enzyme, which protects aerobic organisms from the toxic effects of H_2O_2 by catalyzing the conversion of H_2O_2 to molecular O_2 and H_2O[11]. Exposure to Cd has been reported to stimulate CAT in *A. bisporus* and *A. niger*[27,33]. The increase in CAT activity at cadmium and lower concentrations Hg treatment would indicate that there is an effort on part of mushroom to neutralize or minimize the hazardous effect of ROS generated by Cd or Hg.

POD, which converts peroxide into oxide and oxygen, have often served as an indicator of metabolism in response to changes in growth conditions and/or environmental stress conditions[39]. The activity of POD in *C. comatus* was stimulated, suggesting that POD provide an additional protection against the oxidative damage induced by Cd and Hg toxicity. In the current work, the decrease in CAT and POD activity at 5 and 15 mg kg^{-1} Hg treatment was also observed. The decreases in antioxidant enzymes observed at high metal concentrations may reflect a cytotoxicity due to an overproduction of ROS created by the interactions between excess metals and cellular components[12]. The bell-shaped concentration-response trends of antioxidant enzymes in our study may be suggest the disturbances in anti-oxidative defense systems and intolerance of the test fungus at such a high metal concentration. These alterations also might represent a metal-specific response.

Conclusion

Summing up, both Cd and Hg showed a significant toxicity in *C. comatus* at elevated metals exposure. Induction of thiol compounds (T-SH, NP-SH) and antioxidant enzyme (SOD,

CAT, POD) was a defense against heavy metal stress. The results of thiol compounds, antioxidant enzymatic activity and stress indexes also provide support for the idea that the toxic mechanisms of Cd and Hg differ. The data confirm the inhibitory effect of heavy metals on the growth of the mushroom and Hg was found to be comparatively more toxic than Cd. These results may help to understand defense mechanisms of macro-fungi to metal stress.

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