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Note

Responses of *Ganoderma lucidum* to heavy metals

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The levels of seven heavy metals and their toxicity toward *Ganoderma lucidum* under various cultivation conditions were assessed. The contents of Mn, Cu, Zn, Cd, Hg, Pb and U in the fruitbodies of cultivated *G. lucidum* and sawdust substrates were determined to be at trace levels for U, 0.01–0.1 μg/g for Cd and Hg, and 1–5 μg/g for Pb, 10–120 μg/g for Mn, Cu and Zn. The effects of heavy metals on the growth of mycelia of *G. lucidum* in pure cultures were examined over a wide range of concentrations (10–3,000 μg/ml), and their toxicities were found to decrease in the order: Hg>Cd>Cu>U>Pb>Mn=Zn. The translocation and accumulation of Zn from contaminated substrates (at 10 μg/g) in fruitbodies were investigated by using ^{65}Zn tracer, and *G. lucidum* was found to take up Zn with an efficiency of >60%, leading to accumulation of >100 μg/g in fruitbodies and >80 μg/g Zn in basidiospores.

Key Words——*Ganoderma lucidum*; heavy metal; toxicity; tracer; translocation.

The potential for environmental contamination by heavy metals, mainly due to use of fossil fuels in energy production, mining, metallurgical and galvanizing technologies and increasing quantities of sewage sludges is assuming crucial importance. For example, in the European Community, Cu, Zn, Cd, Hg, Pb, U are released in considerable amounts in atmospheric stack emissions (38.1, 341, 2.7, 60.1, 191.5 and 5.1 t/yr, respectively) (Sabbioni et al., 1984). Muramatsu and Yoshida (1997) have reported the contents of heavy metals in soils around the Tokyo region, which are dominated by Cu, Mn, Zn (100–1,000 μg/g), and Pb (up to 20 μg/g). However, the impacts of heavy metals on higher fungi are rarely investigated and their responses to such elements have not been assessed. Byrne and Ravnik (1976) found alarming accumulations of heavy metals in many of 27 species of wild mushrooms in Slovenia. Tyler (1980) analyzed many heavy metals (Mn, Cu, Zn, Cd, Pb, etc.) in 130 species of basidiomycetes in Skane, South Sweden. Such data are of interest in establishing the role of heavy metals in biogeochemical prospecting and in environmental contamination studies. The establishment of baseline or normal levels is, therefore, important in checking the incidence of local or global contamination, in view of the important role of fungi in biological recycling and the quality of cultivated fungi for foods and materia medica. However, only a limited number of commonly cultivated mushrooms (e.g., *Pleurotus* spp., *Agaricus bisporus* (Lange) Imbach (=*A. bisporus* (Berk.) Singer), *Volvariella volvacea* (Bull.: Fr.) Singer) have been examined for heavy metal contamination (Aichberger and Horak, 1975; Bano et al., 1981; Li and Chang, 1989).

Ganoderma lucidum (W. Curt.: Fr.) Karst. is a well-known medicinal mushroom in Oriental countries, now cultivated on a large scale and commercialized widely in China, Japan, Korea, Taiwan, Vietnam, Thailand, Malaysia, etc. (Chang, 1996). However, the accumulation of heavy metals in harvested products have not been examined in detail. In the present study, we report the levels of Mn, Cu, Zn, Cd, Hg, Pb and U in fruitbodies grown on mixed substrates such as those commonly used in cultivation in Vietnam and Japan, and compose them with levels in various sawdusts. We also report the effects of heavy metals on the growth of *G. lucidum* and the ability of this mushroom to translocate and accumulate Zn added to the substrate by using ^{65}Zn tracer.

Materials and Methods

Strains of *G. lucidum* Three strains of *G. lucidum* from Sichuan (Southwest China), Habac (North Vietnam) and Dalat (South Vietnam), which were cultivated in Dalat, Vietnam, and one strain from Takasaki (Central Japan), which was cultivated in Takasaki, were used in this study.

Substrates for *G. lucidum* cultivation Substrates for *G. lucidum* cultivation were prepared with mixtures of sawdust from unidentified trees as basic substratum (100%), and complemented with rice bran (15%), bean powder (5%), $CaCO_3$ (2%), $(NH_4)_2SO_4$ (0.5%), KH_2PO_4 (0.5%) and $MgSO_4$ (0.05%).

Analysis of heavy metals The fruitbodies harvested at 75 d of three strains cultivated in Dalat, Vietnam, and

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sawdusts of the rubber tree (*Hevea brasiliensis* L.), the lim tree (*Erythrophloeum fordii* Oliv.), and mixtures of unidentified sawdust (S1) were dried at 65°C for 3 d, then ground to fine powders for analysis. Pb, Zn, Cu, Mn and Cd were determined by polarography techniques with a Metrom Model 646 VA; and Hg, Zn, Mn and Cu were determined by neutron activation analysis (Campbell and Bewick, 1978) in Dalat Nuclear Reactor IVV-9 with a neutron flux of $\sim 2 \cdot 10^{12} n \cdot cm^2 \cdot s^{-1}$ with a multichannel gamma spectroanalyser (8,192 channels) with NIM standards and HP Ge detector. Fruitbodies of Takasaki strain and mixtures of unidentified sawdust collected in Japan (S2) were analyzed by ICP techniques.

Heavy metal supplements Seven to 9-d-old mycelial fragments of 7–9 mm in diam were inoculated onto PDA media supplemented with heavy metals and incubated at 26±0.3°C in the dark as follows.

Hg supplements were prepared with $HgCl_2$ and Hg acetate at 50–3,000 μg/ml Hg on PGA in Petri dishes with 5 replicates per treatment. Cd was used as $CdCl_2$ at 25–1,425 μg/ml Cd, Cu as $CuSO_4$ at 128–2,048 μg/ml Cu, U as uranyl acetate at 10–3,000 μg/ml U, Pb as $PbCl_2$ and Pb acetate at 10–3,000 μg/ml Pb, Zn as $ZnSO_4$ at 200–3,000 μg/ml Zn, and Mn as $MnCl_2$ at 50–3,000 μg/ml Mn. Diameters of colonies formed were determined 4–15 d after inoculation to assess the effects of heavy metals on the growth of mycelia.

The uptake and translocation of Zn were investigated by using 25 μCi ^{65}Zn with specific activity of about 5 μCi/mg Zn in each of five pots as previously described (Tham et al., 1999), based on the method of Brunnert and Zadražil (1983).

Results and Discussion

Heavy metals in *G. lucidum* cultivated on mixed substrates *Ganoderma lucidum* was cultivated on mixed sawdusts of various trees in Dalat City, Vietnam and Takasaki, Japan. Table 1 shows the levels of heavy metals in strains from Sichuan (Southwest China), Habac Province (North Vietnam), Dalat (South Vietnam) and Takasaki (Central Japan). Hg and Cd were extremely low in the former three strains and Cd was similarly low in the Takasaki strain (Hg was not determined). Pb was slightly higher, beeing highest in the China strain. Similar levels of Cu, Zn and Mn were found by both methods of analysis, and these accorded with earlier reports (Mizuno, 1992; Loi et al., 1993). U was found in Takasaki strain and Japanese sawdust at trace level (Table 2). Compared with many other mushrooms, this cultivated *G. lucidum* contains very low levels of heavy metals. High values were obtained for Cd in fungi, averaging 5 μg/g, as Byrne and Ravnik (1976) showed with some extraordinary cases of *Hygrocybe punicea* (Pers.: Fr.) Fr. (~40 μg/g) and others of about 10 μg/g. On the basis of the Joint FAO/WHO Codex Alimentarius Commissionſs maximum recommended weekly intake of 6.7–8.3 μg Cd/ kg body weight, or 500 μg for adult, only a modest consumption of such mushrooms would be acceptable. Woidich and Pfannhauser (1975) determined Hg in edible fresh, dried and canned mushrooms and found great variety, from 0.01–10 μg/g dry weight. Stegnar et al. (1973), and Byrne and Ravnik (1976) found an average more than 2 μg/g Hg in 27 higher fungi in Slovenia, with the highest values in *Boletus* spp., *Agaricus* spp., *Lycoperdon perlatum* Pers., and particularly *Lactarius deliciosus* (L.) Fr. (up to ~40 μg/g). Therefore, it can be concluded that *G. lucidum* and the sawdusts commonly used in its cultivation are safe from Hg and Cd contamination. Muramatsu and Yoshida (1997) reported Pb levels in mushrooms in the Tokyo region of around 2 μg/g, 10 times lower than that in the soils where they were collected. They also found extremely low contents of U in mushrooms, about 200 times lower than that in the soils (~2 μg/g U). The accumulation factors of Zn, Cu and Mn were about 1, i.e., levels of approx. 1,000 μg/g were found in both mushrooms and soils. Byrne and Ravnik (1976) found some special cases such as *Scleroderma vulgare* Horn. with 434–600 μg/g Zn, *L. perlatum* with

Table 1. Contents of heavy metals in *Ganoderma lucidum*.

Metal	Contents of heavy metals in 75 d old fruitbodies (μg/g dry weight, means of 4 samples)				
	Dalat strain	Lim strain	Sichuan strain	Takasaki strain[a]	error (%)
Cu	33	47	31	26	5
Mn	15	12	19	13	5
Zn	18	27	33	24	5
Cd[b]	<0.15	<0.15	<0.15	<1	—
Hg	0.07	0.01	0.01	nd	15
Pb	3.4	3.8	5.7	<5	10
U	nd[c]	nd	nd	trace	—

a) Determined only by ICP.
b) Determined only by NAA.
c) Not determined.

Table 2. Contents of heavy metals in sawdusts.

Metal	Contents of heavy metals in various sawdusts (μg/g dry weight, means of 4 samples)				
	Rubber tree[a]	Lim tree[b]	S 1[c]	S 2[d]	error (%)
Cu	24	17	13	<1	5
Mn	31	32	41	20	5
Zn	31	21	29	17	5
Cd[e]	<0.05	<0.05	<0.05	<1	—
Hg	0.01	0.01	0.01	nd[f]	—
Pb	2.0	1.2	1.7	<5	10
U	nd	nd	nd	trace	—

a) *Hevea brasiliensis* cultivated around Ho Chi Minh City, South Vietnam.
b) *Erythrophloeum fordii* cultivated in North Vietnam.
c) S 1: unidentified sawdust from Vietnam.
d) S 2: unidentified sawdust from Japan, determined only by ICP.
e) Determined only by NAA.
f) Not determined.

200–262 μg/g Zn, 396 μg/g Cu, *Macrolepiota procera* (Scop.: Fr.) Sing. with 381 μg/g Zn, 225 μg/g Cu and *Calvatia utriformis* (Bulliard: Pers.) Jaap with 166 μg/g Mn. Bano et al. (1981) conducted comparative analysis of fruitbodies and straw substrate on which they were grown and found that the Cu content of *Pleurotus* species was comparable to that of *Agaricus* and never exceeded that of straw substrate. Hg and Cd were found to be absent in all species of *Pleurotus*. Tyler (1980) reported the mineral analysis of 130 species of fruiting basidiomycetes, revealing a bioconcentration of many undesirable heavy metals. Grabbe and Domsch (1976), Collet (1977), Woggon and Bickerich (1978), Enke et al. (1979), Brunnert and Zadražil (1980), Anderson et al. (1982), Ciusa et al. (1982), Martinez et al. (1983), and Kawai et al. (1986) reported heavy metals in many edible mushrooms. The fungi found to be the best heavy metal collectors also showed the highest substrate degradation rates. Fruitbodies of fungi grown on lignino-cellulolytic materials contained less heavy metal than those collected from soft substrates, and the mobilization of heavy metals from the subatrate and their subsequent translocation into the fruitbodies was concluded to be related to the degree of substrate degradation. This suggests the need for a periodical monitoring of fruitbodies for metal contaminants under varied conditions of cultivation. Mizuno (1989a, b, 1991, 1992) analyzed many metals in mushrooms cultivated in Japan and found low contents of Zn, Mn, and Cu in *Hericium erinaceum* (Fr.) Pers., *Agaricus blazei* Murr., and *Grifola frondosa* (Dicks.: Fr.) S. F. Gray. The same authors (Mizuno et al., 1988; Mizuno, 1989b) also determined about 10 metals in *G. lucidum* cultivated on wood logs, showing an wide range of Zn, Cu and Mn from 1 to >100 μg/g, but provided no data on heavy metals. Loi et al. (1993) reported about 20 elements in *G. lucidum* cultivated on sawdust of rubber trees (*H. brasiliensis* L.) in Ho Chi Minh City, Vietnam, among which Zn ranged 20–30 μg/g, Cu 2–3 μg/g, and Mn 15–19 μg/g. Therefore, it can be concluded that *G. lucidum* can be safely cultivated. In future, comparative studies of heavy metal accumulations in wild and cultivated fungi are desirable.

Responses of *G. lucidum* to heavy metals Little is known about the degrees of toxicity of metals toward fungi. The Dalat strain was selected for these experiments because of its relatively high growth rate. The levels of growth of mycelia of *G. lucidum* in pure cultures with various heavy metal applications indicated the following order of toxicity:

$$Hg > Cd > Cu > U > Pb > Mn = Zn$$

The data in Table 3 show that the growth of mycelia was inhibited critically by Hg at 100 μg/ml. The LG 50 value, as defined previously (Tham et al., 1999), was about 75 μg/ml. The respective values of latter metals were 400, 1,000, 2,000, 2,600 and 3,000 μg/ml. Zn and Mn showed no inhibition over the wide range of concentrations tested. Therefore, it is concluded that the toxicity of Zn, Mn, Pb and even U are not so strong for *G. lucidum*. The data show that Hg has highest toxicity, while Cd is modelately toxic, more so than Cu, but less toxic than V and Se (Tham, 1996a, b; Tham et al., 1999). $HgCl_2$ and Hg-acetate showed similar toxicity, and did $PbCl_2$ and Pb-acetate.

Data on the growth responses of higher fungi to heavy metals are scant. Khor (1979) found that straw mushrooms (*V. volvacea*) are quite sensitive to metals: biomass production was decreased to one-sixth when the level of $MnCl_2$ was increased from 2 mM to 4 mM. On the other hand Kurtzman and Changˇ-Ho (1989) found very little inhibition of mycelial growth by Mn at concentrations up to 8 mM (~400 μg/ml). Data on other heavy

Table 3. Growth of mycelia of *Ganoderma lucidum* under various levels of heavy metals.

Conc of metals (μg/ml)	Colony diam (mm) 9 d after inoculation						
	Hg	Cd	Cu	U	Pb	Mn	Zn
0	88.6±4.3	86.7±5.0	90.7±5.1	88.4±4.3	87.1±4.2	87.5±4.1	88.2±5.6
50	57.7±4.3	82.9±5.1	91.1±4.2	93.1±4.2	87.7±5.2	87.6±4.6	89.9±4.9
75	44.7±6.1	—[a]	—	—	—	—	—
100	21.3±3.6	68.7±5.5	89.7±5.3	91.2±3.7	86.3±4.8	—	—
200	17.2±3.3	53.3±4.3	89.2±4.7	—	—	90.3±3.7	87.3±5.1
300	no growth	—	—	89.3±4.8	85.8±5.3	—	—
450	—	39.2±3.2	—	—	—	92.4±4.5	89.2±4.7
550	died	—	92.5±4.1	87.6±6.2	—	89.7±5.8	—
750		—	51.3±4.5	—	87.7±6.0	87.7±5.8	85.9±5.6
1,000		19.5±3.1	41.5±4.2	84.7±5.7	—	86.3±4.8	86.6±4.4
1,500		died	—	57.6±5.3	88.9±5.5	—	88.8±5.6
1,800			—	—	—	—	—
2,000			26.9±4.8	46.6±4.4	79.8±6.1	—	88.6±4.6
2,050			—	—	—	85.6±5.7	—
3,000			died	29.2±4.3	31.4±3.1	72.6±3.8	76.6±4.7

a) Not determined.

Table 4. Zn uptake and translocation in *Ganoderma lucidum* by using ^{65}Zn tracer.

Time after inoculation (d)	Accumulation of Zn in[a)]					
	stipe		pileus		basidiospores	
	a	b[b)]	a	b[b)]	a	b[b)]
30	487	5.65±1.4	638	7.55±1.7	—[c)]	—
35	496	5.90±1.3	665	7.90±1.3	—	—
40	597	7.10±1.7	723	8.55±2.8	—	—
45	633	7.50±1.7	792	9.40±3.1	—	—
55	617	7.30±2.0	864	10.25±2.7	451	5.35±1.3
65	611	7.25±2.5	1,233	14.60±2.5	569	6.75±1.6
75	625	7.40±1.8	1,194	14.15±2.8	707	8.30±1.7

a) a: cpm 100 mg^{-1} dry weight; error of measurements <5%.
b: amount of Zn derived from the total Zn supplement (5 mg) into substrates (μg 100 mg^{-1} dry weight).
b) Mean value±standard deviation.
c) Not determined.

metals such as Pb and U are limited to the report by Rothstein (1965) on the effects of U on yeasts. Although uranyl ions, UO_2^{2+}, can not penetrate into the yeast cells, the transport system for glucose is specially inhibited, and associated with binding to carboxyl groups, the enzyme invertase, located on the outer surface of the cells, is inhibited too. High toxicities of metals, particularly heavy metals, were determined in higher plants (Macnicol and Beckett, 1985). The high tolerance of *G. lucidum* to Cu, Mn and Zn might correlate with its wood-decaying ability, particularly with the functions of ligninocellulolytic enzymes (Adaskaveg et al., 1990), and this may be a general feature of wood-rotting fungi.

Zn uptake and translocation *in G. lucidum* Zn was selected as an example of heavy metals capable of incorporation into biomass of the Dalat strain of *G. lucidum*. When Zn labeled with ^{65}Zn was added to substrates at the level of 10 μg/g, it was effectively taken up and translocated into fruitbodies. Accumulation of Zn in pilei increased up to 75 d of cultivation and exceeded that in stipes throughout. Traces of ^{65}Zn were found in discharged basidiospores after 50 d of cultivation (Table 4). Harvested fruitbodies (~5% compared with dry weight of substrates) were analyzed for total Zn content (by polarography), and the amount of Zn derived from the Zn supplement was calculated from the specific activities of ^{65}Zn supplemented and in the fruitbody. The respective values obtained, approximately 136 and 120 μg/g, indicated that 90% of the Zn accumulated in biomass of fruitbodies was derived from that added artificially to substrates. In discharged basidiospores, the amount of Zn derived from fruitbodies was up to 80 μg/g, as calculated both from specific activities of ^{65}Zn and by NAA. Overall, supplemented Zn was used with efficiency of about 60%. Bano et al. (1981) indicated that *Pleurotus* mushrooms have a tendency to accumulate Zn in their fruitbodies. Brunnert and Zadražil (1983) found very high translocation rates of externally applied Cd and Hg in *Pleurotus djamor* (Fr.: Fr.) Boedijn. and *P. ostreatus* (Jacq.: Fr.) Kummer. Aichberger and Horak (1975) reported that the commercial champignon, *A. bisporus*, was capable of high Hg uptake up to 30 μg/g from artificially contaminated substrates (10 μg/g Hg supplemented). However, the application of radiotracers, e.g., ^{203}Hg and ^{109}Cd (Brunnert and Zadražil, 1983), should lead more exact data for the transportation and accumulation of polluted Hg or heavy metals by mushrooms.

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