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Note

Growth and fruitbody formation of *Ganoderma lucidum* on media supplemented with vanadium, selenium and germanium

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Responses of mycelia of *Ganoderma lucidum* to vanadium, selenium and germanium were examined over a wide range of concentrations (10–1,120 μg/ml) in pure culture. Se and V were found to be highly toxic, but Ge was not toxic at the levels tested. *Ganododerma lucidum* cultivated on substrates of sawdust with V (30–80 μg/g) developed mature fruitbodies, but the bioaccumulation of V was quite low (2.5–7 μg/g in pileus, 12.5–21.5 μg/g in stipe and <1 μg/g in basidiospores). Se as Na_2SeO_4 labeled with ^{75}Se was effectively taken up from substrates and accumulated in fruitbodies (mainly in pileus), then depleted by discharge of basidiospores. Ge as $GeCl_4$ labeled with ^{77}Ge was easily uptaken and translocated into fruitbodies.

Key Words——accumulation; *Ganoderma lucidum*; germanium; selenium; vanadium.

The trace elements in fungi are a neglected area of study, and data on responses of higher fungi to such elements are scant. For example, selenium (Se) has been studied sporadically in mycology for its distribution in fungal biomass and its effects on fungal growth and development. On the other hand, it is regularly reported to be present in bioactive antioxidants and to be an important microelement in foods, whose deficiency in the diet results in serious heart diseases (Ferretti and Levander, 1976). Some *Amanita* species such as *Amanita muscaria* Hooker, *A. mappa* Batsch., *A. pantherina* (DC.: Fr.) Secretan and others such as *Paxillus involutus* Fr., *Lycoperdon gemmatum* Batsch., *Calvatia caelata* Merg., *Boletus edulis* Bull.: Fr. etc. can accumulate 1–20 μg/g of Se (Bao and Thuy, 1983). Although vanadium (V) is widely distributed in nature, particularly in sea ecosystems, data on V in fungi are very few, whereas its bioactivity in mammalian, namely, human cell lines and in medicine has been reported recently (Sabbioni et al., 1989). In some higher fungi, accumulation of V paralleled that of Se (Byrne and Ravnik, 1976). Mizuno et al. (1988), Tong and Khoong (1994), and Chen and Fu (1996) have reported germanium traces in *Ganoderma lucidum* (W. Curt.: Fr.) Karst. fruitbodies grown under normal conditions on various substrates in Japan, Taiwan, and Malaysia. They attempted Ge-bioenrichment in mycelial fermentations and fruiting cultivations of *G. lucidum* on large scales in order to improve yields of compounds with biopharmacological activities in the products from this well-known medicinal fungus.

In view of the biochemical importance of the toxic and beneficial actions of mineral elements in higher fungi, particularly medicinal mushrooms, we examined the responses of *G. lucidum* to about 30 elements that are commonly distributed in fruitbodies. Of these, V, Se and Ge were found only in trace levels in *G. lucidum* commonly cultivated in Dalat, Vietnam (Tham, 1996a, b). To elucidate further the physiological responses of *G. lucidum* to bioactive elements, particularly their bioaccumulation, in order to improve the pharmacological qualities of the mushroom, we investigated the growth of mycelia and fruitbody formation of *G. lucidum* varieties on media supplemented with V, Se and Ge.

Materials and Methods

The newly found strains of *G. lucidum* in Dalat, Central Highlands of Vietnam (about 1,500 m above sea level) and Takasaki, Japan, were isolated and cultured purely on PDA medium.

V supplements were prepared with VCl_3 and $(NH_4)_2VO_3$ at 50–1,020 μg/ml V on PDA in Petri dishes with 5 replicates per treatment. Fragments of 7–9 d-old mycelial of 7–9 mm in diam were inoculated and incubated at 26°C in the dark. Diameters of colonies formed were determined at 4–9 d after inoculation to assess the effects of V on mycelial growth. Se supplements were prepared with Na_2SeO_4 at 10–1,000 μg/ml Se, and Ge

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was used as $GeCl_4$ at 10–1,120 μg/ml Ge.

Substrates for *G. lucidum* cultivation were prepared with sawdust as basic substratum (100%), and added 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%) to the basic substratum. The moisture content of substrate was adjusted to about 65%. Polypropylene (PP) bags containing substrate (0.5 kg of dry substrate/bag) were irradiated for sterilization at 32 kGy (8 kGy/h) with γ rays of ^{60}Co (Malek et al., 1994). V as VCl_3 solution was supplemented at 30 and 80 μg/g into each of 5 bags. The bags were inoculated with seed and incubated at 26°C under 85% humidity in the dark for 23–26 d to allow full colonization of mycelia. All bags with young primordia were then transferred to the greenhouse for fruiting under daily irrigation and attenuation of illumination. The contents of V in harvested fruitbodies were analyzed by ICP techniques.

Fifty μCi ^{75}Se/5 mg Se as Na_2SeO_4 in 30 ml H_2O was injected into substrates in 5 bags 25 d after inoculation (primordia well formed). Translocation and accumulation of Se in fruitbodies were determined by γ measurements and calculations from specific activity of ^{75}Se in dry matter of stipe, pileus and basidiospores harvested from 5 random samples (L'Annunziata and Legg, 1984). For Ge analysis, 150 μCi ^{77}Ge/50 mg Ge as $GeCl_4$ was injected into 5 bags in the same way as ^{75}Se but 35 d after inoculation.

Results and Discussion

Effect of V, Se and Ge on mycelial growth of of *G. lucidum* Mycelial growth of *G. lucidum* was measured as colony diam in pure cultures supplemented with VCl_3 and NH_4VO_3. We found that *G. lucidum* is more sensitive to V both in cation and anion forms (V^{3+}, VO_3^-) compared with Se. Mycelia showed healthy growth at 50 μg/ml V and slight inhibition at 100 μg/ml V. At higher V concentrations, mycelial growth was inhibited critically (150–200 μg/ml V). Inhibition of mycelial growth by 50% (LG50: limitation of growth by 50%) occurred at about 180 μg/ml V. At 510 and 1,020 μg/ml V, growth was interrupted and the mycelia died completely 5–7 d later (Table 1).

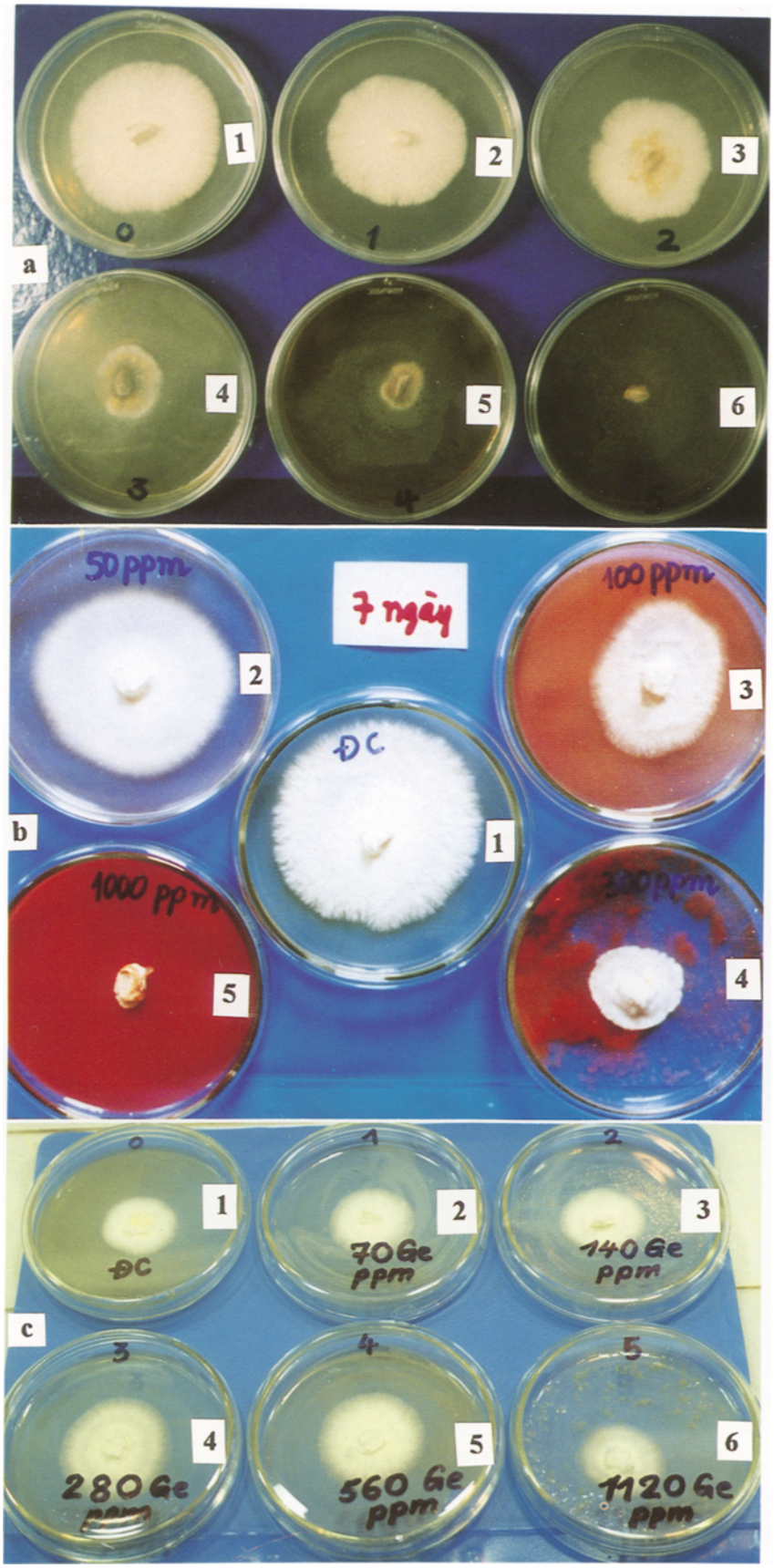
Table 1 also shows the clear effects of Se on the growth of mycelia. Se was considerably toxic to *G. lucidum* at 100 μg/ml or more. The LG50 value was about 200 μg/ml Se. The mycelia died after growth for 7–9 d in the presence of 500 or 1,000 μg/ml Se, whereas they survived with very weak growth at 300 μg/ml Se in the media.

Control and Ge-treated colonies both grew healthily. No toxic effect on *G. lucidum* mycelia at any concentration of Ge up to 1,120 μg/g. Our results agreed with the data of Chen et al. (1994a), who found similar effects of Ge both in cation and anion forms on *G. lucidum*. The responses of mycelia to V, Se and Ge are illustrated in Fig. 1.

Table 1. Mycelial growth of *G. lucidum* on PDA supplemented with V, Se and Ge.

Mineral supplement (μg/ml)	Colony diam 9 d after inoculation (mm)			
	V in VCl_3	V in $(NH_4)_2VO_3$	Se in Na_2SeO_4	Ge in $GeCl_4$
Control (0)	84.2±4.1	87.5±4.4	87.4±2.9	85.4±3.8
10	—	—	77.8±2.2	85.8±2.2
50	77.2±4.2	83.3±4.1	68.9±4.2	—
70	—	—	—	86.9±4.0
100	72.7±3.7	71.9±2.6	53.7±2.9	—
140	—	—	—	89.7±3.6
150	69.9±3.3	69.1±3.2	51.4±3.4	—
180	42.6±2.5	43.9±4.3	—	—
200	41.4±3.6	40.9±3.1	45.2±2.4	—
280	—	—	—	88.4±2.5
300	—	—	23.2±1.4	—
500	—	—	7.7±1.7	—
510	18.3±2.1	22.1±2.8	—	
560	—	—	—	89.5±2.6
1,000	—	—	7.1±1.2	—
1,020	8.5±1.3	10.2±1.7	—	—
1,120	—	—	—	85.2±2.9

Fig. 1. Growth of *G. lucidum* on media supplemented with V, Se and Ge at 7 d after inoculation.
a. V suplement (μg/ml): 1, 0; 2, 50; 3, 100; 4, 200; 5, 510; 6, 1,020.
b. Se suplement (μg/ml): 1, 0; 2, 50; 3, 100; 4, 300; 5, 1,000.
c. Ge suplement (μg/ml): 1, 0; 2, 70; 3, 140; 4, 280; 5, 560; 6, 1,120.


a
1
2
3
4
5
6
b
50 ppm
7 ngày
100 ppm
ĐC
1000 ppm
300 ppm
c
ĐC
70 Ge ppm
140 Ge ppm
280 Ge ppm
560 Ge ppm
1120 Ge ppm

Chen et al. (1994a) examined the responses of mycelia of *G. lucidum* to GeO_2 added to liquid media and showed increases in the contents of free amino acids and of Ge up to 3,000 μg/g dry biomass. On yeast (*Saccharomyces cerevisiae* Hansen), Se was shown to be quite toxic, and biomass in submerged fermentation being drastically decreased when 30–100 μg/ml Se was added (Thuong et al., 1995). However, Se bioenrichment was successful up to 150–500 μg/g dry matter, supporting the results obtained by Meng et al. (1990) suggesting the improvement of antioxidative activities. Recently, we obtained biomass of mycelia of a special Lingzhi fungus in Vietnam, *Humphreya* sp., with high concentrations of Se (150–1,000 μg/g) in pure cultures (Tham et al., 1997). Therefore, it seems possible to accumulate Se in *G. lucidum* by optimizing culture conditions.

Biological effects of V on microorganisms have been intensively studied: these include the adverse impacts on ATPase activity and ATP content in the fungus *Neurospora crassa* Shear et Dodge at 1.5–15 μM VO_4 (Bowman and Slayman, 1979), and the effect of stimulation on the oxidation of NADH in plasma membranes of *S. cerevisiae* (Minasi, 1990). Ulaszewski et al. (1987) showed similar effects of vanadate (0–20 μM) on wild and mutant strains of *Schizosaccharomyces pombe* Lindner. A low-molecular-weight compound $C_{12}H_{20}N_2VO_{11}$ was isolated from *A. muscaria* by Kneifeld and Bayer (1973) and Felcman and Da Silva (1984), which was called "amavadine." However, the physiological significance of V in higher fungi and other organisms is still not clear. Arnon and Wessel (1953) reported that V is an essential element for the growth of the green alga *Scenedesmus obliquus* (Turpin) Kutzing, which required 0.1 μg/ml V in the nutrient medium. Beneman et al. (1972) found that V can substitute for Mo in N_2-fixation in *Azotobacter*, and Robson et al. (1986) showed the alternative nitrogenase from *Azotobacter chroococcum* Beijerink is a V-enzyme. Meish and Becker (1981) found that V interferes with chlorophyll synthesis and photosynthetic electron transport in *Chlorella fusca* Shihira et Krauss and even in higher plants. However, Welch and Huffman (1973) concluded that if V were essential to higher plants, it is required at a level of 1/2,500 to less than 1/250 that required for the normal growth of *S. obliquus*, rat, or chicks. Further study is required to clarify the biological functions of V for the growth of fungi.

Cultivation of *G. lucidum* on substrates supplemented with V, Se and Ge On the substrates supplemented with 30 and 80 μg/g V as VCl_3, the mycelia grew normally and young primordia formed well at 20–25 d after inoculation. The fruitbodies of both strains isolated in Takasaki, Japan, and in Dalat, Vietnam, developed completely and discharged spores from 50–60 d after inoculation. There was no difference in morphology of fruitbodies grown on substrates with and without V supplements. However, the strain from Takasaki showed a delay in maturation of about 7–11 d (the glossy cortex on the upper surface of pilei formed and expanded later as shown in Fig. 2). The samples of the strain from Dalat were harvested for analysis of V-bioaccumulation in at 75 d (Table 2). Without V addition, the fruitbodies of *G. lucidum* contained only a trace of V (< 1 μg/g), due to the low contents of V in the substrate. The addition of 30 μg/g V to substrate produced the effective accumula-

Fig. 2. Fruitbody formation of *G. lucidum* on substrates supplemented with V.
a. Dalat strain, Vietnam; b. Takasaki strain, Japan. Two strains were inoculated at the same time. In Takasaki strain, the periphery surface of pileus is white, but turns brown later.

Table 2. Accumulation of V in fruitbodies of *G. lucidum*.

Fruitbodies	V content in fruitbodies (μg/g)		
	Levels of V applied to substrates		
	without V	30 (μg/g)	80 (μg/g)
Stipe	< 1	12.5	21.5
Pileus	< 1	2.5	7.0
Basidiospores	< 1	—	< 1

tions of V in biomass. In the pilei, we found quite low contents of V, 4–6 times lower than that in stipe tissues. Accumulation of V to double the control level occurred when *G. lucidum* was grown with 80 μg/g V supplied to substrate. V was clearly accumulated at higher level in stipes (older parts of fruitbodies) than in pilei, especially in young pilei. Basidiospores contained only traces of V. Thus, V was not translocated from fruitbodies into spores. High contents of V in fruitbodies of higher fungi was found only in *A. muscaria*, naturally grown in V-polluted regions in Slovenia with high contents of V (100–200 μg/g) in soils (Byrne and Ravnik, 1976). Elevated contents of V were found both in the pilei and stipes of this mushroom (50–200 μg/g). Whereas, all other 25 species of higher fungi grown under the same conditions contained only trace levels of V (<1 μg/g). Uptake and translocate of V by *G. lucidum* is not high as well as other usual fungi but the effects at micromolar concentrations, at which V has some bioactivity, should be considered. Balfour et al. (1978) found V-stimulated natriuresis by inhibition of renal ATPases acting as regulators of sodium pump, even in red cells (Cantley et al., 1978). V was also found to function as an oxidation-reduction catalyst and was suggested to have an enzymatic role in lipid metabolism and as well as a catalytic or enzymatic function in bone metabolism or formation and in some DNA-metabolizing activities (Sabbioni et al., 1983). The pharmacological properties of V are to lower cholesterol in the blood by inhibiting squalene synthase as well as to lower the blood lipid level, and these activities are thought to relate to the effects of V on the force of contraction in cardiac preparations and coronary vasoconstriction (Sabbioni and Marfante, 1978; Sabbioni et al., 1989). Bioactivities of V-containing complexes in fungi and particularly in *G. lucidum* are thus of interest for further study.

The supplement of 5 mg of Se in Na_2SeO_4 labeled with ^{75}Se led to active uptake and translocation in growing fruitbodies of *G. lucidum* strain collected in Dalat. Table 3 shows the results of ^{75}Se measurements, calculated as the percentages to total activity of ^{75}Se (or total amount of Se), showing dynamic changes in Se accumulation in biomass during the development of *G. lucidum*.

Table 3. Se uptake and translocation in *G. lucidum* measured by using ^{75}Se tracer.

Time after inoculation (d)	Se content in fruitbodies (μg/g)[a]		
	Stipe	Pileus	Basidiospores
30	90±14	95±27	—
35	140±23	225±33	—
40	125±20	380±28	—
45	130±17	250±31	—
55	110±20	210±27	45±8
65	120±25	190±25	65±6
75	115±18	150±28	75±7

a) Amount of Se calculated from the total Se supplement into substrates (μg/g dry weight) ± standard deviation.

The pilei, particularly in the early stages of development, are clearly pivotal parts of accumulation of Se, with highest concentrations determined 10–20 d after injection of Se into substrates (>350 μg/g at 35 d after inoculation), when elongating primordia have just enlarged their tops to form initial pilei. Se accumulation and distribution decreased in differentiating and maturing pilei. We found an increase in ^{75}Se translocations into basidiospores of *G. lucidum* from 55 d after inoculation. From the yield of harvested fruitbodies, the efficiency of utilization of supplemented Se was calculated to be about 69%, and the average content of Se in biomass reached 140 μg/g. In *G. lucidum* cultivated without Se supplement, Se contents in stipes and pilei were less than 0.9 μg/g, corresponding to traces of Se in sawdust substrates (–0.8 μg/g) (Tham, 1996a, b). These data are similar to those for various wild mushrooms. Byrne and Ravnik (1976) also found distinguishable accumulations of Se (1–7 μg/g) in *Amanita* species, 3–6 times higher than that in the soils where they were collected. However, some wild edible mushrooms with 3–20 μg/g Se were considered as valuable foods, rich in Se, e.g., *B. edulis* (Bao and Thuy, 1983). Recently, yeast (*S. cerevisiae*) enriched with Se has been studied as a potentially effective antioxidant, radical scavenger and heavy metal eliminator by using conventional and nuclear techniques (Meng et al., 1990; Czauderna et al., 1994, 1996; Thuong et al., 1995). So, *G. lucidum* enriched with Se is a promising materia medica for such purposes.

Ganoderma lucidum enriched with Ge has been intensively investigated for a decade. We tested $GeCl_4$ for cultivation in plastic bags (1,000 ml). Ge in $GeCl_4$ labeled with ^{77}Ge (half-life 11 h) was injected into substrates when primordia had just elongated, and it was uptaken effectively and translocated into young tops of elongating fruitbodies during 3 d. The efficiency of enrichment with Ge was determined to be in the wide range of 60–140 μg/g in biomass harvested. Experiments with radioisotopes of Ge with longer half-lives should be performed to study the ability of *G. lucidum* to uptake and translocate Ge from substrates into fruitbodies in all stages of growth. The bioenrichment of Ge in *G. lucidum*, not only fermented in liquid media but also cultivated on woodlogs supplemented with GeO_2 or bis-β-carboxyethyl germanium sesquioxide, $(GeCH_2CH_2COOH)_2O_3$, has been reported by Mizuno et al. (1988, 1989) and Chen et al. (1994a, b). Yields of enrichment of approximately 1–2% were obtained in harvested fruitbodies of *G. lucidum* containing 14 and 140 μg/g Ge in the first and second year's crop, respectively. It can be concluded that Ge is more effectively utilized in cation form ($GeCl_4$) than anion form (germanium dioxide or germanium sesquioxide).

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