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FULL COMMUNICATION

Copper and Zinc Bioaccumulation and Bioavailability of *Ganoderma lucidum*

Ramiro González Matute,[1,2] Analia Serra,[1] Debora Figlas,[1,2] and Néstor Curvetto[1,3]

[1]*Laboratory of Biotechnology of Edible and Medicinal Mushrooms, CERZOS (CONICET), Bahía Blanca, Argentina.*
[2]*Commission of Scientific Investigations of the Province of Buenos Aires, Buenos Aires, Argentina.*
[3]*Department of Agronomy, National Southern University, Bahía Blanca, Argentina.*

ABSTRACT *Ganoderma lucidum* is a widely recognized medicinal mushroom. The bioaccumulation and potential bioavailability of copper (Cu) and zinc (Zn), which are essentials for human health, were analyzed in *G. lucidum* mycelium and fruit bodies grown in the presence of these metals to test their potential utility as a food dietary supplement. Mycelia grown in culture medium with non-mycotoxic doses of Cu or Zn (25 and 50 mg/kg) were selected for evaluation of the bioavailability of these metals in the gastrointestinal tract by using an *in vitro* simulated digestion system. One gram of dried mycelium grown in the presence of 50 mg/kg Cu or Zn showed a bioavailability of 19% for Cu and 2% for Zn of the recommended daily intake (RDI). When production of fruit bodies was evaluated, the highest biological efficiency (23%) was reached when the substrate was enriched with 100 mg/kg Cu. Cu and Zn contents obtained either before or after digestion of fruit bodies from all metal-enriched treatments were substantially lower than those from metal-enriched mycelia. The metal bioavailability was also low: 1.5% of the Cu RDI and almost negligible for Zn. The results are discussed in relation to the RDI values exhibited by two commercial supplements. The potential incorporation of these mineral-enriched mycelia/fruit bodies in capsules, infusions, and dietary supplements is evaluated.

KEY WORDS: • ***food dietary supplement*** • **Ling Zhi** • ***oligoelements*** • **Reishi** • ***sunflower seed hulls***

INTRODUCTION

Copper (Cu) and zinc (Zn) are essential minerals for human health.[1,2] The recommended daily intake (RDI) is 1.5–3.0 mg of Cu and 15 mg of Zn, and the maximum intake not associated with adverse effects is 9 mg and 30 mg, respectively.[3,4] The excess may have toxic effects, deleterious for health, whereas deficiencies or scarcities produce illness or physiological disorders.[5]

Bioavailability is the proportion of an ingested element that is absorbed, transported, and transformed into its active form(s). The ingested mineral level, its chemical form, solubility, interactions with other minerals and nutrients, chelator agents, inhibitors, physiological state and age of the person or animal, and the previous processing of dietary components affect the bioavailability.[6]

Zn intake can be inadequate for covering the daily requirements because it may be of low bioavailability in the diet. It was suggested that mineral supplements from organic sources should have higher bioavailability than those from inorganic ones. When Cu is supplemented in an organic form, like those chelated with lysine and methionine or associated with proteins, it is absorbed in similar or higher quantity than Cu sulfate.[6,7] The same result was reported for Zn, which was provided in the same or higher bioavailability from organic sources as the best inorganic one ($ZnSO_4$).[7,8]

Many assays have been done with mineral-enriched mushrooms in order to provide humans with essential minerals at higher bioavailability, allowing an increased absorption during its growth.

Edible mushrooms are known to possess health beneficial effects because of their high nutritional value. Some of them also produce secondary metabolites that are effective in prevention, treatment, and recovery from illnesses.[9]

Wild and cultivated mushrooms can accumulate heavy metals and can be used as bioindicators of environmental contamination or as a potential bioremediation tool. Independently from their growth environment, wild species can accumulate metals like cadmium, Cu, lead, Zn, iron, mercury, and manganese.[10] Cu and other heavy metal levels found in accumulating mushrooms are higher than those occurring in cumulative plant species, whereas for other elements, like Zn, the concentrations are comparable.[11,12] This suggests the presence of an effective mechanism that allows mineral absorption from the ecosystem and the substrate.[13–15]

The concentration in wild mushrooms varies according to the species. For example, the greatest Cu accumulation in mushrooms was found in *Tricholoma terreum*, reaching 51 mg/kg, and *Fommes fomentarius*, 54 mg/kg (dry weight

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Address correspondence to: Ramiro González-Matute, Laboratory of Biotechnology of Edible and Medicinal Mushrooms, CERZOS, C.C. 738, 8000, Bahía Blanca, Argentina, E-mail: rmatute@criba.edu.ar

basis); the lowest levels corresponded to *Pleurotus ostreatus*, 5 mg/kg, and *Boletus appendiculatus*, 18 mg/kg.[10,13] Cu concentration in fruit bodies of cumulative mushroom species varies from 100 to 300 mg/kg of dry matter, which is not considered a risk for human health.[12] As regards Zn accumulation, the greatest value was found in *Polyporus frondosus* (= *Grifola frondosa*), 122 mg/kg, and the least in *Hydnum repandum*, 17 mg/kg.[10,13]

Cu and Zn contents in 28 species of edible mushrooms were studied, and it was concluded that their concentrations are species-dependent: some are bioaccumulators (some *Agaricus* spp.), whereas others are bioexcluders.[16] Cu and Zn accumulation in *Agaricus blazei* mycelium was recently studied in the presence of different metal contents in liquid nutritive medium: at 400 mg/kg, the mycelium accumulated 449 and 163 times the basal content of Cu and Zn, respectively.[17] After a sequential chemical extraction and simulated gastrointestinal digestion of mycelia obtained from a liquid culture with Cu or Zn non-mycotoxic concentrations (100 and 200 mg/kg), it was observed that close to 90% of the metals accumulated in the available nonresidual fraction, which was similar to or better than the values found in two commercial supplements.[17]

It was demonstrated that *G. frondosa* mycelia cultivated in a liquid medium with non-mycotoxic concentrations of 100 and 200 mg/kg Cu or 25 and 50 mg/kg Zn could accumulate these metals, and when they were subjected to a simulated gastrointestinal digestion *in vitro*, solubility in these digestive fluids from 1 g of dried mycelium was 642–669 mg/kg Cu and 102–530 mg/kg Zn, which represent 32–33% and 0.7–3.5% for the Cu and Zn RDI, respectively.[18]

Ganoderma lucidum, a basidiomycete of the family Ganodermataceae, is a well-known medicinal mushroom with strong health benefits, including antitumor, hypopressor, antioxidant, immunomodulating, immunostimulating, hypocholesterolemic, and hypoglycemic activities.[19–25]

The aim of this work was to evaluate the ability of the *G. lucidum* mycelium/fruit body to accumulate Cu or Zn separately when cultivated in a metal-enriched liquid medium/substrate and to estimate the potential bioavailability of these minerals. This information should be useful to rationally support the potential utility of the *G. lucidum* metal-enriched dry mycelium/fruit body as a dietary supplement or ingredient.

MATERIALS AND METHODS

Mushroom strain

G. lucidum E47 strain (University of Guelph, Guelph, Canada) was used. Mushroom mycelium was first cultivated in 20 g/L malt extract, 2 g/L yeast extract, 10 g/L glucose, and 20 g/L agar medium (MYGA), pH 6, in the dark at 25°C for 7–8 days. We used only one *G. lucidum* strain to test Cu and Zn bioaccumulation and bioavailability. Previous results showed that the 34-D (Fungi Perfecti) strain of *G. lucidum* bioaccumulated these metals in a different way (data not shown).

Liquid culture and mycelium growth

Fifty milliliters of MYG nutritive medium (same as MYGA but containing no agar) and with addition of 65 g/L sunflower seeds (*Helianthus annus*)[26] was placed in an 250-mL Erlenmeyer flask, allowing an air–medium relationship of 5:1 (vol/vol).[27] Five pieces (0.6 cm in diameter) of young mycelium grown in MYGA medium were inoculated, under aseptic conditions, in each Erlenmeyer flask. Cultures were incubated with agitation using an orbital horizontal shaker at 90 rpm and 25°C in the dark for 12 days.

Dried mycelium mass was daily determined starting from the fourth day of growth and up to day 12. The mycelium was separated from liquid medium by centrifugation (1,500 *g* for 20 minutes) and washed three times with a 10 g/L dextrose solution after each centrifugation. The mycelium pellet was dried at 70°C until constant weight ($n = 5$). Once the mycelium growth curve was obtained, Cu and Zn mycelium accumulation was evaluated using liquid cultivation for the period of time that resulted in optimal mycelial growth. For this purpose, sulfate salts of these metals were added to the liquid medium at concentrations of 0, 25, 50, 100, 200, and 400 mg/kg. The inoculation and incubation procedures were as previously described.

Spawn production

Spawn was prepared as described by Curvetto *et al*.[28] In brief, wheat (*Triticum durum*) grains were mixed with 0.1% $CaCO_3$, 0.8% $CaSO_4$, and 40% water (by weight), put into 1-L bottles, and sterilized at 15 psi for 1.5 hours. Then, they were inoculated with the MYGA medium mycelium, incubated at 25°C in the dark for 10–15 days, and periodically shaken.

Substrate preparation

The substrate final composition was 32.5% sunflower seed hulls, 5.0% barley (*Hordeum vulgare*), 2.0% $CaSO_4$, 0.5% $CaCO_3$ (dry weight), and 60% water with or without Cu or Zn addition as sulfate salts at 25, 50, and 100 m/kg concentrations. The substrate was soaked overnight and used to fill autoclavable high-density polyethylene (pore size, 100 μm) bags 17 cm in diameter and 33 cm long ($n = 10$). About 1 kg of substrate was packed to two-thirds of each bag, which was sealed with a cotton plug inside polyvinyl chloride pipe rings on the bag necks. Bags were sterilized by autoclaving at 121°C and 15 psi for 120 minutes and were inoculated 24 hours later at a spawn ratio of approximately 8% (by complete mixing) into the substrate bags under laminar flow. Then, an incubation period for run spawning followed in an environmentally controlled chamber at 25°C in the dark.

Fruiting and mushroom yield

Once the substrate was completely colonized by mycelium, bags were transferred to a fruiting room. The cotton plugs were removed, leaving the polyvinyl chloride rings as an air chamber, and the bags were stacked like a wall.

Mushroom yield (first and second flush and total yield) was expressed as biological efficiency (*i.e.*, [kg of fresh mushrooms/kg of dry substrate] × 100); productivity (biological efficiency [in %]/days from inoculation to end of second flush) was also obtained.

Mineral analysis

Dried mycelium or powdered dried fruit bodies (250 mg) (18 mesh, Butt ground mill model AM-48, 60177, Ionomex, Buenos Aires, Argentina) were digested with 1.5 mL of an HNO_3:$HClO_4$ (2:1 vol/vol) mixture for 2 hours at 280°C and diluted with double distilled water. Mineral contents were measured by inductively coupled plasma optical emission spectrometry (model 1000:III, Shimadzu, Kyoto, Japan).

Cu and Zn bioavailability

The potential bioavailability (measured as degree of solubility) of the Cu and Zn accumulated by the enriched mycelium and fruit body was estimated using the *in vitro* simulated gastrointestinal digestion method.[29]

Mycelium grown in liquid culture medium with the addition of either 25 and 50 mg/kg Cu or Zn and in control medium (no metal addition) was evaluated in comparison with two commercial mineral supplements: supplement II, Long Acting Multivitamin and Mineral Supplement (15 mg of $ZnSO_4$ and 2 mg of $CuSO_4$ per 1.31-g pill; Good 'N Natural, Bohemia, NY, USA); and supplement III, Berocca Plus (10 mg of Zn citrate trihydrate per 1.46-g pill; Bayer, Leverkeusen, Germany).

In addition, mycelia of fruit bodies grown on substrate with or without the addition of 25, 50, and 100 mg/kg Cu or Zn were also evaluated before and after gastrointestinal digestion.

Samples of dried mycelium, fruit body, or mineral supplements (250 mg each, $n = 3$) were digested with simulated gastric and intestinal fluids. For gastric digestion, 15 mL of distilled water was added to each sample. Also, 250-mg mushroom powder samples in 10 mL of water were preheated at 100°C for 5 minutes and were subjected to *in vitro* digestion to evaluate possible heat metal solubility enhancement. Each mix was homogenized and adjusted to pH 2 with 5 *M* HCl, and 0.75 mL of pepsin solution (1 g of pepsin dissolved in 50 mL of 0.1 *M* HCl) was added and incubated at 37°C with agitation (190–200 rpm) for 1 hour. For intestinal digestion, partially digested samples were adjusted to pH 6 with 1 *M* $NaHCO_3$, and 3.75 mL of biliary extract containing pancreatic enzyme (0.30 g of biliary extract dissolved in 35 mL of 0.1 *M* $NaHCO_3$ with 0.05 g of pancreatin) was added. Then the pH was adjusted to 7 with 1 *N* NaOH, and 5 mL of 120 m*M* NaCl and 5 m*M* KCl was added. Samples were incubated at 37°C with agitation (190–200 rpm) for 1 hour. Extracts were centrifuged at 1,500 *g* for 10 minutes, filtered, and analyzed by inductively coupled plasma optical emission spectrometry, as previously described.

Statistical analysis

Biomass, Cu and Zn bioaccumulation, and biological efficiency data were analyzed by one-way analysis of variance. The mean values separation was done by Tukey's test at the .01 or .05 significance level.

RESULTS

Mycelium growth in liquid culture medium

G. lucidum mycelium growth was measured from days 4 to 12. Highest biomass concentration was obtained by day 12, which did not differ significantly from the values obtained from days 9 to 11; thus, the ninth cultivation day was chosen for the Cu and Zn bioaccumulation and bioavailability assays to provide enough biomass to carry out these experiments.

Mineral content

Mineral content of mycelium grown in liquid culture is shown in Table 1.

Effect of Cu and Zn on G. lucidum mycelial growth

Cu and Zn sulfate salts in the liquid culture medium influenced the mycelial mass evolution as shown in Figures 1 and 2. Addition of 25 and 50 mg/kg Cu showed a tendency to stimulate mycelial growth, but at higher levels ($\geq$ 200 mg/kg Cu) a significant growth inhibition was produced (Fig. 1A) in MYG liquid medium.

Addition of 25 and 50 mg/kg Zn in the medium produced light mycelial growth stimulation. At higher Zn sulfate concentrations $\geq$ 200 mg/kg, a highly significant growth inhibition occurred, as shown in Figure 2A.

Cu and Zn bioaccumulation

Accumulation of Cu in the mycelium increased significantly with the increase of the oligoelement concentration in the growth liquid medium. Maximum accumulation was obtained at 200 and 400 mg/kg Cu in the medium (1,578 and 3,020 mg/kg, respectively), but these concentrations also produced a strong mycelium growth inhibition. Finally, 25 and 50 mg/kg Cu doses, with 331 and 762 mg/kg accumulation, respectively (Fig. 1B), were selected for *in vitro* bioavailability assays.

Table 1. Mycelium Mineral Content of *G. lucidum* Grown in Malt Extract/Yeast Extract/Glucose Liquid Culture After 9 Days

Mineral	*Content (mg/kg)*
P	3,640.0 ± 299.00
Na	790.0 ± 150.00
K	10,190.0 ± 949.00
Mg	1,380.0 ± 145.00
S	2,180.0 ± 58.00
Ca	4,330.0 ± 289.00
Mn	1.6 ± 0.11
Zn	47.0 ± 3.00
Cu	40.0 ± 1.00

Data are mean values ± 1 SD ($n = 3$) in mg/kg, on a dry weight basis.

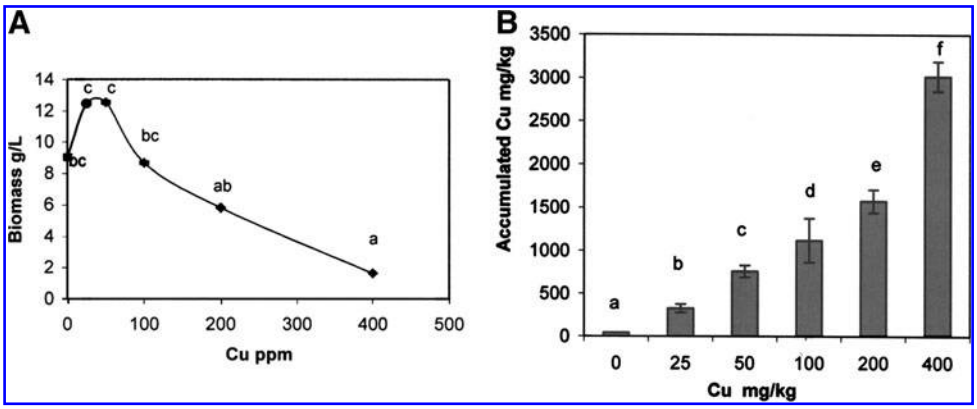

FIG. 1. (**A**) Mycelial growth (g/L) and (**B**) copper (Cu) accumulation (mg/kg) in *G. lucidum* cultivated on malt extract/yeast extract/glucose (MYG) liquid culture medium containing $CuSO_4$ (0–400 ppm Cu^{2+}). Data are mean values ± 1 SD ($n = 5$). ^{abcdef}Values with different letters are highly significantly different ($P < .01$), according to Tukey's test.

G. lucidum mycelium also accumulated Zn in a dose-dependent manner (Fig. 2B). Metal incorporation was maximum at 200 mg/kg (76,801 mg/kg), but, again, this Zn concentration was strongly inhibitory for mycelial growth. Concentrations chosen for bioavailability assays were 25 and 50 mg/kg Zn in the medium, which produced mycelium accumulations of 652 and 7,500 mg/kg, respectively.

Cu and Zn bioavailability

Figure 3 shows the total Cu and Zn solubilized when 1 g of mineral-enriched mycelium or commercial dietary supplements was subjected to a gastric digestion followed by an intestinal digestion with fluids simulating the whole digestive tract.

Cu solubilized from enriched mycelium grown with 25 and 50 mg/kg Cu was considerable higher (177 and 376 μg/g, respectively) than that obtained from control mycelium (12.25 μg/g). These values represent, respectively, 25% and 54% of the Cu extracted from commercial supplement II (697 μg/g), as shown in Figure 3, and correspond to approximately 9% and 19% of the RDI for Cu.

Zn extracted by simulated gastrointestinal digestion from enriched mycelium was 157 and 343 μg/g in the presence of 25 and 50 mg/kg Zn, respectively, which was substantially higher than the Zn content in the control mycelium (7.9 μg/g). However, values obtained with dietary supplements were much higher, 2,958 and 1,948 μg/g for supplements II and III, respectively (Fig. 3). Highest solubility obtained for zinc was equivalent to approximately 2% of the RDI per gram of dry mycelium. Supplement II showed a high SD value for Zn solubility after simulated gastrointestinal digestion (Fig. 3). Similar deviations were observed in previous analysis of this digested supplement when compared with Zn-enriched mycelium of both *A. blazei* Murrill and *G. frondosa*, probably because of a high variability in the metal composition of the tablets.[17,18]

Fruit body mineral content and bioavailability

Mineral content of *G. lucidum* fruitbodies before and after simulated gastrointestinal digestion is shown in Table 2. Only a small proportion of the metals is solubilized, and, in the case of minor minerals, the bioavailable content is negligible.

Cu and Zn bioavailability on enriched fruit bodies

When *G. lucidum* strain E47 is cultured under solid-state fermentation conditions and in the presence of different concentrations of Cu and Zn (25, 50, and 100 mg/kg), fruit bodies bioaccumulated only 51%, 82%, and 107% more Cu, respectively, and 0%, 78%, and 55% more Zn, respectively, with respect to control fruit bodies. With regard to the bioavailability, there was 40%, 107%, and 207% more Cu and 20%, 61%, and 3 % more Zn, respectively, than their controls after simulated gastrointestinal digestion (Table 3). These values represent a very low percentage of the RDI for

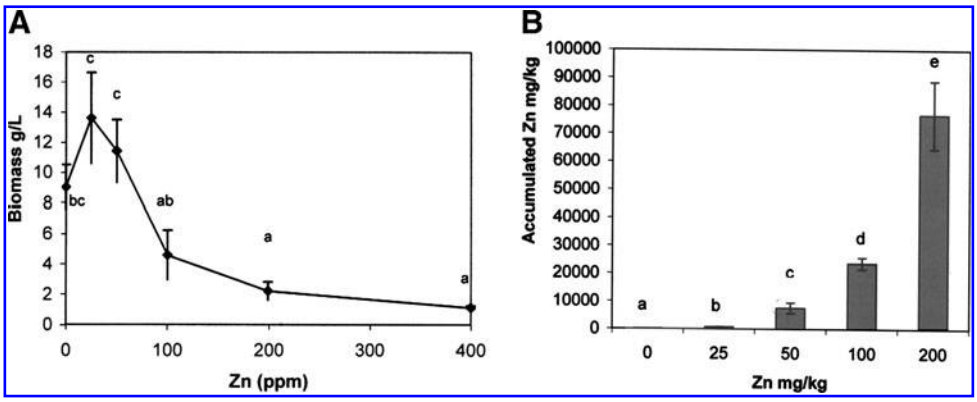

FIG. 2. (**A**) Mycelial growth (g/L) and (**B**) zinc (Zn) accumulation (mg/kg) in *G. lucidum* cultivated on MYG liquid culture medium containing $ZnSO_4$ (0–400 ppm Zn^{2+}). Data are mean values ± 1 SD ($n = 5$). ^{abcde}Values with different letters are highly significantly different ($P < .01$), according to Tukey's test.

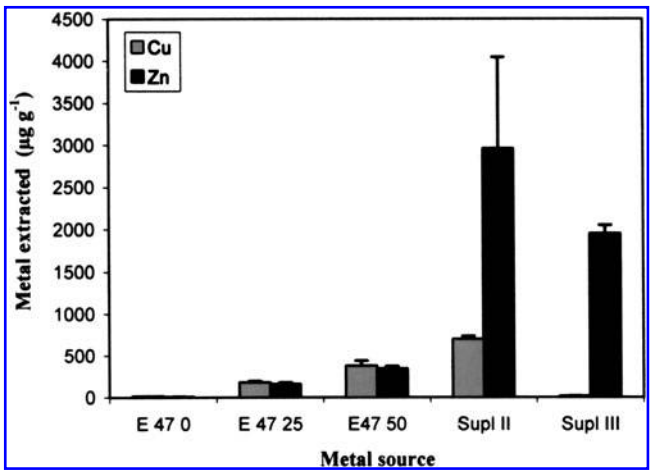

FIG. 3. Levels (μg/g) of Cu and Zn solubilized after simulated gastrointestinal digestion of *G. lucidum* mycelium and commercial dietary supplements. E47 25, mycelium grown in the presence of 25 mg/kg Cu or Zn; E47 50, mycelium grown in the presence of 50 mg/kg Cu or Zn; Supl, supplement. Data are solubilized mineral mean values +1 SD ($n=3$).

these elements, per gram of dry mycelium: 0.7–1.5% of the RDI for Cu and around 0.1% of the RDI for Zn.

Preheated enriched mushroom powder did not show any difference in terms of Cu and Zn solubility with respect to enriched mushroom powder without heat treatment, when both were subjected to gastrointestinal digestion (data not shown).

Mushroom yield on mineral-enriched substrate

Table 4 shows the mushroom yield in terms of biological efficiency and productivity following the solid-state cultivation using a substrate based on sunflower seed hulls and barley in the absence and presence of either Cu or Zn.

DISCUSSION

Mycelium growth of the E47 strain of *G. lucidum* on MYG medium required 9 days to reach the growth plateau of 9.5 g/L mycelial biomass. Once an adequate liquid culture time for mycelium growth was established, the bioaccumulation ability of Cu and Zn by mycelium of *G. lucidum* cultivated in liquid culture was evaluated, and the potential bioavailability of these minerals was studied in order to consider its possible dietary supplementation use. At low concentrations, Cu and Zn are two essential minerals for mushroom growth to complete its biological cycle. However, the optimal concentration range is narrow, and higher contents result in mycotoxicity.[30] This range in the case of *G. lucidum* mycelium growth in the absence and in the presence of Cu and Zn proved to be very narrow, and toxicity was absent up to 200 mg/kg for both elements.

There was a stimulating growth tendency, although not significant, up to 50 mg/kg Cu. It is known that Cu stimulates the production and activity of laccases, extracellular enzymes capable of degrading lignocellulosic materials. These enzymes have Cu as a prosthetic group and can be found in many edible and medicinal mushrooms, like *A. blazei*, *P. ostreatus*, *G. frondosa*, and *G. lucidum*.[31] The fact that the

Table 2. Mineral Content in Fruit Bodies of *G. lucidum* Before and After Simulated Gastrointestinal Digestion

Element	*Before digestion (mg/kg)*	*After digestion (mg/kg)*
Na	1,404.0	—*
Mg	1,330.0	675.0
P	3,376.0	461.0
S	1,966.0	170.0
K	9,150.0	—*
Ca	1,280.0	455.0
Mn	5.8	<0.1
Fe	90.0	2.2
Cu	46.2	9.8
Zn	60.0	15.0

Data are mean values ± 1 SD ($n=3$).
*The digestion reactants contain this element.

Table 3. Cu and Zn Content in *G. lucidum* Fruit Bodies Grown Under Solid-State Fermentation Conditions in the Absence and Presence of Different Concentrations of Cu and Zn in the Culture Medium

	Metal (mg/kg)			
	Cu		*Zn*	
Concentration (mg/kg)	*Content*	*Solubility*	*Content*	*Solubility*
0	46.2	9.8 (0.15)	60.0	15.0 (0.91)
25	70.0	13.7 (0.20)	58.5	18.0 (0.68)
50	84.0	20.3 (2.92)	106.5	24.1 (1.18)
100	95.5	30.1 (2.94)	93.0	20.3 (1.03)

The solubility (metal bioavailability [mg/kg]) after subjecting the fruit bodies to simulated gastrointestinal digestion is also shown (SD in parentheses).

Table 4. *G. lucidum* Yield Expressed as Biological Efficiency and Productivity

Treatment	*BE % first flush*	*BE % second flush*	*Accumulated BE %*	*Days*	*Productivity*
Control	9.1 ± 2.9[a]	7.0 ± 1.6	16.1[a]	63	0.26
Cu (mg/kg)					
25	13.1 ± 2.6[ab]	7.7 ± 2.4	20.8[bc]	76	0.27
50	10.9 ± 3.1[a]	6.5 ± 2.0	17.4[ab]	58	0.30
100	15.4 ± 2.3[b]	7.8 ± 1.6	23.2[c]	79	0.29
Zn (mg/kg)					
25	12.4 ± 3.5[ab]	6.6 ± 1.1	19.0[b]	82	0.23
50	10.1 ± 2.0[a]	6.7 ± 1.5	16.8[ab]	83	0.20
100	9.4 ± 2.9[a]	7.1 ± 2.2	16.5[a]	71	0.23

Mushrooms were cultivated on sunflower seed hulls/barley substrate in the absence and presence of Cu or Zn as sulfate salts at 25, 50, and 100 mg/kg concentrations ($n=10$). Data are mean ± SD values. Percentage biological efficiency (BE) (%) was defined as (kg of fresh mushrooms/kg of dry substrate) × 100. Productivity was defined as (BE %/days from inoculation).
[abc]Different letters represent significant differences ($P<.05$, Tukey's test).

mycelium growth was not significantly inhibited at concentrations up to 200 mg/kg Cu suggests the presence of some resistance/tolerance mechanism to an excess of this metal. As a matter of fact, different tolerance mechanisms have been suggested for mushrooms at high levels of heavy metals, such as Cu ion retention among the components of the cell wall, alteration in the Cu absorption, extracellular chelation or precipitation by mushroom-secreted metabolites, and the formation of intracellular complex with polypeptides, such as metallothioneins and phytochelatins.[32,33]

As an example, the lignocellulolytic mushroom *Poria placenta* is able to form a Cu complex, producing oxalic acid to form the corresponding Cu salt, which allows its growth in Cu-treated wood. On the other hand, mushrooms in culture medium are capable of producing huge amounts of polysaccharides, and it was suggested that they could act like biosorbents for heavy metals.[30]

In the present study, the *G. lucidum* mycelium exhibited an accumulation capacity of the metals added to the liquid culture medium. Cu or Zn levels in the mycelial biomass increased with increasing mineral concentrations in culture medium. Mushroom mycelium accumulated a maximum of 3,020 mg/kg Cu and 76,801 mg/kg Zn, at the highest concentration (400 mg/kg) of these elements in the medium, although this concentration was mycotoxic in both cases. With a non-mycotoxic content of these elements (50 mg/kg) in the nutrient medium, the respective accumulation values for Cu and Zn were 761 mg/kg and 7,500 mg/kg, which represented an increase of 18- and 160-fold compared with the control, respectively. Although we evaluated the capacity of *G. lucidum* mycelium to separately accumulate Cu and Zn, it would be interesting to study the effect of simultaneous addition in different concentration combinations in the culture medium in order to evaluate the interactions between absorption of both metals, such as facilitation and synergy or competence.

In a similar study carried out with *A. blazei*,[17] it was found that with Cu or Zn concentrations of 400 mg/kg in the culture medium, a maximum accumulation of the elements was reached, 11,130 and 14,369 mg/kg, respectively (*i.e.*, an increase of 449 times for Cu and 163 for Zn relative to the mycelium control [88 mg/kg]). However, 400 mg/kg Cu incorporation significantly inhibited *A. blazei* mycelial growth, whereas at the same Zn concentration, a mycelial biomass decrease was observed, but it was not statistically significant.

To evaluate the potential utility of *G. lucidum* mycelium enriched with Cu or Zn as a nutritional supplement, it is important to know not only the total concentrations of minerals in mycelium, but also their bioavailability degree in terms of solubility. Although solubility cannot be considered a synonym for bioavailability, it is an important factor affecting bioavailability. Cu or Zn chemical forms with the highest solubility are considered more potentially bioavailable than the less soluble ones. Moreover, for an element to be absorbed and possibly used by an organism, it must necessarily be in a soluble form in the intestinal fluid, as either free ion or chelated with another nutrient;[34] in the case of the accumulation of minerals by *Brassica juncea*, it was observed after a sequential extraction that the accumulated minerals reached a concentration in solution higher than the one provided by mineral supplements, meaning that the Cu organic forms presented a higher solubility than the inorganic ones.

Solubility of Cu and Zn in the digestive tract was already evaluated in terms of the bioavailability through *in vitro* simulation of gastrointestinal digestion of both dry mycelium and supplements. Cu and Zn accumulated by *A. blazei* mycelium after the simulated gastrointestinal digestion had solubility values that represent at least 57–98% and 9–11% of the RDI for Cu and Zn, respectively, per gram of dry mycelium.[17]

It should be noted that when the gastrointestinal digestive environment fluids, the pH, and temperature are simulated in the *in vitro* gastrointestinal digestion, the concentration of the material to digest in the solution of these fluids could be too high in comparison with actual conditions. The addition of drinking water, saliva, and other digestive juices would promote a continuous dilution of the gastrointestinal tract contents, which could generate a greater solubilization compared with *in vitro* conditions. Therefore, it could be possible that both solubility and RDI values obtained in the present study have been underestimated. We can conclude that *G. lucidum* mycelia, grown in Cu- or Zn-enriched liquid medium, have accumulated these metals, and their solubility values obtained after the simulated gastrointestinal digestion would represent at least 19% of the RDI for Cu and a Zn solubility value equivalent to approximately 2% of the RDI per gram of dry mycelium.

However, in the case of metal-enriched fruit bodies, the solubility values obtained for either Cu or Zn after the gastrointestinal digestion correspond to a very low percentage of the RDI, approximately 1.5% for Cu and 0.14% for Zn, per gram of dry fruit body. These values could not be increased by heat pretreatment of mineral-enriched mushroom powder.

In terms of yield values, we differentiated biological efficiency from productivity values. The best total biological efficiency results were reached with the substrate enriched with 100 mg/kg Cu (23.2%). However, it is interesting to note that the time to crop fruiting was markedly (although not significantly) shorter in the case of the substrate enriched with 50 mg/kg Cu (58 days), which is important from a productivity point of view to reduce mushroom production cycles.

Previous results indicate that the mycelial liquid culture of *G. lucidum* in MYG medium produces optimal levels of mycelium at 9 days and that the individual addition of Cu or Zn at 25 or 50 mg/kg to nutritive medium should effectively be used to obtain a nutritional supplement with the additional value of the nutraceutical properties of this mushroom.

Because of the differences found in the solubility values of either oligoelement, we can conclude that the enriched mycelium with Cu should be more effective to be used as a nutritional supplement because it provides an important percentage of the RDI per mass unit. We suggest that these enriched mycelia could be incorporated in capsules, infusions, and dietary supplements. In contrast, the metal-enriched fruit bodies obtained in the conditions assayed in this work do not contribute significantly to the RDI values for these metals; thus mycelium culture should be the election.

On the other hand, it was interesting to find that addition of 50 and 100 mg/kg Cu to the substrate could increase mushroom productivity, which is also an important contribution for the commercial production of this mushroom.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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