ZEOLITES AS POSSIBLE BIOFORTIFIERS IN MAITAKE CULTIVATION

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Abstract – The levels of Ni, Cu and Mg in *Grifola frondosa* (also known as Maitake mushroom) fruit body produced on zeolite Minazel Plus (MG)-supplemented substrate were measured with inductively coupled plasma optical emission spectrometry (ICP-OES). Two different concentrations of MG were added to the substrate for mushroom cultivation. Levels of selected metals were measured in cultivated dry carpophores. The content of Ni increased in fruit bodies produced on supplemented substrate, while in case of Cu, a pronounced decrease was observed. When two different concentrations of MG were implemented, the Mg level showed both positive and negative trend, depending on the applied concentration of zeolite. MG in a concentration of 1% showed the strongest influence on the observed elements in the cultivated fruiting body of Maitake mushroom.

Keywords: Grifola frondosa, Maitake mushroom, biofortification, zeolite, Minazel Plus; ICP-OES

INTRODUCTION

Trace elements occur in organisms in small quantities (less than 300 μg/g), and they are indispensable for proper physiological and biochemical processes. To maintain their optimal level in an organism it is necessary to provide an adequate intake through daily diet. It can be difficult to achieve an optimal intake of microelements considering the differences in food composition and quantity in different parts of the world. Generally, there are two scenarios in trace-element deficiency. In more developed countries, processed and refined food is the predominant form in which food is consumed. During food processing, most of the valuable microelements are lost and consumption of such foodstuffs does not provide an adequate intake of the trace elements (Masironi, 2000). In less developed countries, people generally consume fresh food, but their food lacks variety and quantity. Another problem with trace-element intake is the wide range of factors that affect its actual and available concentration. These factors are interactions among different elements, dose-response effects, oxidation state, chelation, immutability, bioavailability, absorption and excretion, cause-versus-effect, the health condition of an organism and pharmacologic effects (Bogden, 2000). It is estimated that about 40% of the population across the world is at high risk for one or more trace-element deficiency (Bogden, 2000). In these conditions, the consumption of biofortified food with optimally balanced amounts of microelements could be a potential solution.

Copper is regarded as one of the most deficient elements. Copper is contained in metalloproteins that operate as enzymes. Copper is included in energy utilization, oxidation-reduction reactions and other metabolic reactions. Its deficiency could

cause a number of health issues such as failure of pigmentation, ataxia, cardiac, vascular and skeletal defects. According to the Food and Nutrition Board of the US National Academy of Sciences, the safe and adequate daily intake of copper from food is in the range from 1.5 to 3.0 mg/day for adults. The WHO suggests that an amount of 0.5 mg/kg of body weight is safe, which is up to 10 mg/day. This value is marked as a No Observed Adverse Effect Level (NOAEL) (Hathcock, 2000). Meats, legumes, nuts and seafood are regarded as good sources of copper. (Kleavay, 1998).

Nickel could be considered as an essential element for as much as studies link it to the function of the vitamin B12, nickeloplasmin, folic acid and as an activator of numerous enzymes (Nielsen et al., 1993). It is essential for many enzymatic processes in microorganisms such as hydrogenation, desulfurization and carboxylation reactions (Hausinger, 1994). The suggested dietary requirement for nickel is 25-35 mg/day, while upper safe intake can be even 600 mg/day since there has not been observed a life threatening toxic effect. Usually the daily intake does not exceed 100-150 mg/day (Nielsen, 2000).

Magnesium is an essential element. Its recommended dietary allowance (RDA) is from 320-420 mg/day (for females and males, respectively). The human body contains about 21-28 g of magnesium, mostly concentrated in the bones, while in the cells it is the second most abundant element. It is a cofactor of more than 300 enzymes and its deficiency is correlated to osteoporosis, hypertension, diabetes, myocardial infarction, atherosclerosis, mood disorders during menstrual cycle, obstetrical problems, kidney disease, kidney stones, blood clots, bowel disease, cystitis, fatigue and psychiatric disorders (Segado et al., 2003; Singewald et al., 2004; Eisenberg, 1992). It is estimated that about 11% of the world's population have hypomagnesemia manifesting as different heart disorders, psychiatric conditions, neuromuscular hyperactivity and Ca/K abnormality (Singh, 1990). Whole grains and vegetables are good sources of this element, rather than processed foods.

Fungi are widespread in the human diet, especially in Europe and Asia where people have been collecting wild-growing species and using them as food and remedies. Although mushrooms are highly valuable and distinct in nutrient content, they could be a source of heavy metals like Hg or Cd since they are able to accumulate different elements from the growing substrate (Muller et al., 1997). This ability of mushrooms could represent a problem in areas with highly developed industries. Soil around these areas is usually very rich in the toxic elements that are then deposited in mushroom mycelia and fruiting body (Rudawska et al., 2005). The direct consequence of heavy metals accumulation is an excessive intake of these elements. Depending on the age, gender, immune system status and concentration of the specific element, various health conditions could develop. In the case of cultivated species, undesirable elements could be monitored through the choice of substrate, designed not just to keep toxic metals under the allowed limits but also to increase the content of microelements that could contribute to human health. Mushrooms are considered a good source of different nutrients such as proteins, polysaccharides, vitamin B, ergocalciferol, dietetic fibers and essential microelements sodium and potassium (Kalač, 2009). Mattila et al. (2001) state that the riboflavin content of common cultivated species like *Agaricus bisporus* and *Pleurotus ostreatus* is significantly higher than in vegetables. Numerous recent studies emphasize the medicinal value of mushrooms, especially their β-glucan content.

G. frondosa is one of the selected species with high medicinal potential. According to Roupas et al. (2012), *G. frondosa* exhibits anticancer, immune-modulation and bone health-promoting activities. It is well known in traditional Eastern medicine. Its name in Japanese is "Maitake – dancing mushroom" which describes its importance for the local population. Modern medicine also appreciates this mushroom and the increased demand for the fruiting bodies of *G. frondosa* is understandable. While most of the studies concern trace-element levels in wild mushroom species since they are recognized as microelement accumulators and potentially harm-

ful for consumers if picked near polluted areas and smelters (Sivrikaya et al., 2002), data about cultivated species are scarce.

Zeolite is the general name for a wide group of natural and chemically modified minerals. Zeolites are in the form of a 3-D framework constructed from tetrahedral of $[SiO_4]$ and $[AlO_4]$ (Lobo, 2003). They form a well-organized structure with holes and cavities that act as water and ion containers. Zeolites possess a charge excess that derives from Al and therefore they require external elements for structure stabilization. These external elements are usually Ca and Mg and they are relatively easily exchanged. Chemical characteristics of zeolites are the direct consequence of their basic structure with external ions and guest molecules. The most important characteristics of zeolites are ion-exchange ability and catalytic activity (Pickering et al., 2001). Parham (1984) stated that zeolites possess great potential in improving agriculture, especially as slow-release fertilizers.

The main objectives of this study were to determine the possibility of using zeolites, designated Minazel Plus, as biofortifiers in mushroom cultivation, and to investigate their influence on trace-element levels in the produced fruiting body.

MATERIALS AND METHODS

A pure culture of *G. frondosa* was obtained from the collection of the Department for Industrial Microbiology of Agriculture Faculty, University of Belgrade, Serbia. Mycelia were cultivated on malt extract agar purchased from Himedia. Wheat straw originated from a local wheat field near Belgrade and oak sawdust was procured from Mt. Goč (Serbia). Sacks for

mushroom fruiting body cultivation were obtained from saco2 (Belgium). The Institute for Technology, Nuclear and other Raw Materials (ITNMS, Belgrade, Serbia) kindly supplied us with Minazel Plus (a modified zeolite) as already prepared. HNO_3 (65%) and H_2O_2 (30%) were obtained from Merck (Germany). Trace-element level analysis was performed on ICP-OES (Spectro Genesis, Genesis FEE, Germany).

G. frondosa cultivation

After boiling, grains of wheat were cooled and mixed with $CaCO_3$. Glass jars (1 kg) were filled with grains and sterilized for 40 min at 121°C. The grains were inoculated with mycelia previously cultivated on malt agar. Inoculated jars were kept in the dark for two weeks at 25°C. The wheat grains were completely covered with white mycelium, which was used for substrate inoculation. For substrate preparation, wheat straw, oak sawdust and malt sprouts were mixed at 30:50:20 ratios, respectively, and soaked in tap water overnight. To regulate the pH, 0.5% $CaCO_3$ was added. Humidity in the mixture was 75%. Minazel Plus in concentrations of 0.25% and 1% was added into the mixture manually, in three repetitions for each concentration. The control did not contain zeolites. Microsacs were loaded with 3 kg of prepared substrate each and sterilized in autoclave for 2 h at 121°C. After cooling, substrate was inoculated with spawn. Cultivation conditions are presented in Table 1. The produced fruiting bodies were collected, brush cleaned, dried to a constant mass and kept in the dark prior to analysis.

Trace-element analysis

Dried samples were subjected to wet digestion in

Table 1. Cultivation conditions for the mushroom *G. frondosa*.

	Temperature (°C)	Relative humidity (%)	CO_2 (ppm)	Air exchanges	Light requirements
Spawn	21-24	95-100	2000-4000	0-1	NA
Primordia	10-15.6	95	2000-5000	4-8	100-500
Stem	10-15.6	95	2000-5000	4-8	100-500
Fruit body	13-18	85-90	<1000	4-8	500-1000

NA = not available

a closed vessel microwave system (Berghof, Speed Wave MSS-3+, Germany). Three hundred mg of fungi samples were placed in a Teflon vessel and digested with HNO_3 and H_2O_2 for 40 min. The digested sample was diluted with deionized water. A blank was prepared in the same manner. After sample preparation, ICP-OES was performed in order to measure Ni, Cu and Mg concentrations. Optimization of working parameters for ICP-OES was done before analysis (Table 2.) Tomato leaves SRM 1573a (National Institute for Standards and Technology) was used as a standard reference material.

Table 2. ICP-OES operating conditions.

Power	1400 W
Coolant flow	14 L/min
Auxiliary flow	0.8 L/min
Nebulizer flow	0.9 L/min
Sample aspiration rate	2.0 ml/min
Plasma Torch	Quartz, fixed, 2.5 mm injector tube
Spray Chamber	Double Pas
Nebulizer	Cross Flow
Replicate read time	50 s/replicate

Statistical analysis

All measurements were done in triplicate and represented as mean ± standard deviation (SD). SPSS program (version 12.0) was used for statistical analysis. The data were subjected to two factorial repeated ANOVA. If significance was established by ANOVA, differences in the means were determined using Duncan's multiple range tests ($p < 0.05$).

RESULTS AND DISCUSSION

Concentrations of Ni, Cu and Mg were measured in the cultivated fruiting body of Maitake and the data are presented in Table 3. Zeolite supplementation of the substrate resulted in a change the profile of trace elements in the mushroom, compared to the control. There was no uniform pattern in terms of the concentration change of the selected metals. Generally, trace elements have shown a high ability of translocation and implementation in the mushroom's fruit-

ing body (Jain et al., 1988). Bhatia et al. (2013) reported that Se-biofortification of mushrooms with Se-enriched agricultural residues was very efficient.

Concentration of nickel

Our investigation showed that the nickel content increased in the samples cultivated on supplemented substrate when compared to the control. The observed change was statistically significant. The greatest increase appeared in samples cultivated on substrate supplemented with 1% of zeolite. The measured value ranged from 1.92 to 3.58 mg/kg of dry weight (DW), whereas the content of Ni found in the control was below the limit of detection. Li et al. (2011) reported that the content of nickel was 1.68-3.01 mg/kg DW for *Boletus* species. In other studies, concentrations of this element in wild-growing species were 1.72-24.1 µg/g DW (Soyak et al, 2005), 0.7-4.2 mg/kg DW (Sarikurkcu et al., 2011) and 44.6-145 mg/kg DW (Demirbas, 2001), while for cultivated *Pleurotus sajor-caju* it was 16-18 µg/g DW (Sivrikaya et al., 2002). Clearly, our findings correspond to those in the literature. As regards the substrate, the addition of MG resulted in an increase in the nickel content of the fruiting body. The accumulation of nickel could be due to MG structure, which is a clinoptilolite, modified to adsorb and incorporate the NH_4^+ ion. This natural zeolite shows the most affinity for divalent cations (Lobo, 2003), while the most common oxidation state of Ni is +2 (Greenwood, 1997), which ranks it among the preferred elements for ion exchange inside a zeolite's framework. This probably made nickel more available to mycelium utilization. Although substrate supplementation proved to support nickel accumulation in the fruiting body, the achieved amounts are within allowed limits.

Concentration of copper

ICP-OES analysis showed that copper concentration decreased in samples cultivated on zeolite-supplemented substrate. There was no statistically significant difference between the applied concentrations of MG, although 1% of MG resulted in a greater decrease in copper. The content of Cu in the control

Table 3. Concentrations of Ni, Cu and Mg in cultivated fruit body of *G. frondosa*.

	Ni	Cu	Mg
control	0 ± 0^a	28.42 ± 0.39^a	1700.17 ± 65.04^a
Mg 0.25%	1.92 ± 0.00^b	18.17 ± 0.07^b	1620.08 ± 127.80^a
Mg 1%	3.58 ± 0.00^c	14.67 ± 0.17^b	2438.42 ± 64.14^b

All values are means±SD (n=3).
[a-c] Values with different superscripts within the same columns are significantly different ($p < 0.05$).

sample was 28.42 mg/kg DW, which complies with 20-100 mg/kg DW (Kalač, 2010). The same author stated that mushrooms are excellent copper accumulators and if they grow near Cu smelters, the level of this metal can reach up to 427-505 mg/kg DW. Other authors reported the following: 39-181.5 mg/kg DW (Chen et al., 2009), 5.11-92.5 mg/kg DW (Li et al., 2011), 6-187 mg/kg DW (Sarikurkcu, 2011) for wild growing species, while for the cultivated *Pleurotus* species it was 10.5-14 μg/g DW (Sivrikaya, 2002). Although the copper content in our samples was lower than for most of the wild species. *G. frondosa* is marked as a species with acidic peptide that increases the amount of soluble copper, reflexed in increased Cu-absorption in the intestine (Shimaoka et al., 1993). This is an advantage of Maitake because the availability of copper from mushrooms is low (Kalač and Svoboda, 2000). Human intoxication with copper from food is relatively rare due to its negligible adsorption in the intestine (40-60% according to the WHO, 1996). The bioavailability of this metal could be increased if the diet is rich in proteins (100-150 g/day), and knowing that mushrooms are rich sources of proteins, the stated amount could be achieved.

Concentration of magnesium

Zeolite supplementation of the substrate appeared beneficial in the case of magnesium level. According to the data from Table 3, Mg in a concentration of 1% provided a statistically significant increase in magnesium level, while for Mg applied in 0.25% concentration and the control there were no significant differences. Mg amount was in the interval 1620.08-

2438.42 mg/kg DW, which is in agreement with values reported in literature. Demirbas (2001) stated that amounts of magnesium in wild species from the eastern Black Sea region were 850-1320 mg/kg DW. These values are much lower than in our samples. Other authors reported values from 0.90-4.54 mg/g DW (Genccelep et al., 2009), while in species from Poland the magnesium concentration was about 0.7 g/kg DW (Rudawska, 2005). Ouzouni et al. (2009) measured a concentration of magnesium of 688.7-1150.7 mg/kg DW. We assume that the increase in Mg level in our samples was due to the presence of Mg as a zeolite external element and possibility of its ion exchange. In addition, the zeolite's ability to hold water in its cavities and pores might be beneficial in creating the proper environment for electrolyte activity in the substrate, making microelements more available to the mycelium and for accumulation in the fruiting body. It seems that diffusion of ions such as Mg, Ni and Cu were due to van der Waals forces between oxygen atoms in the zeolite structure and elements that diffuse (Lobo, 2003).

CONCLUSION

Results from our study clearly imply that zeolite application in the cultivation of mushrooms could be beneficial in increasing the concentration of microelements, especially of health-beneficial elements such as Mg. Although the absolute amount of observed elements increased in our samples, further studies into the bioavailability of specific elements from mushrooms are necessary. Supplementation of the substrate gave positive results, suggesting that

zeolites could be considered as possible biofortifiers in mushroom production.

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