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THE FUNGISTATIC ACTIVITY OF ORGANIC SELENIUM AND ITS APPLICATION TO THE PRODUCTION OF CULTIVATED MUSHROOMS *AGARICUS BISPORUS* AND *PLEUROTUS SPP.*

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Abstract – The activity of organic selenium against pathogenic molds and its use as a potential selenium source in the production of enriched mushrooms were examined. The effect of commercial selenized yeast on mycelia growth was examined using a method with mycelia disks and a well diffusion method. For mushroom enrichment, different concentrations of selenium were added to a growth substrate. The results presented in this paper suggest that the most suitable concentration of selenized yeast that inhibits the growth of the mycopathogenic molds is 70-100 mg/kg of selenium. With the addition of this concentration to the substrate, mushroom fruit bodies will uptake a high level of selenium, about 100 μg/g for *Pleurotus spp.*, and 200 μg/g for *Agaricus bisporus* in dry weight of the mushroom. Thereby a double effect in the cultivation of mushrooms is achieved.

Keywords: Agaricus bisporus, molds, organic selenium, *Pleurotus* spp.

INTRODUCTION

Selenium is an essential micronutrient required for the normal functioning of human and animal organisms. At concentrations above 800 μg/day, selenium is toxic (Amoako et al., 2009). The recommended dietary allowance for selenium is 55 μg/day and 70 μg/day for healthy men and women, respectively (Barceloux, 1999). The result of an insufficient intake of selenium is the appearance of a large number of disorders that may eventually lead to cardiovascular diseases, neurological, immune and muscular disorders and cancer occurrence (Ming et al., 2007). The bioavailability and toxicity of this trace element depends on its chemical form and concentration. In most soils all over the world, the concentration of selenium is low, ranging between 0.01 and 2 mg/kg. Therefore, the content of selenium in agricultural products is low (Hartikainen, 2005). Higher mushrooms and yeasts are able to assimilate inorganic selenium and transform it into an organic form. It is known that inorganic selenium is more toxic than organic selenium, such as seleno-amino acids contained in plants and fungi (Perez-Corona et al., 1997). These seleno-amino acids are not only less toxic, but they could be better absorbed than the inorganic form of selenium (Malinowska et al., 2009).

For thousands of years fungi have been very appreciated as food and medical resources worldwide. They possess very valuable nutrients that are necessary for the healthy functioning of the organism. Fungi are decomposers of organic and inorganic matter. During this process, they provide the necessary nutrients and accumulate them in fruit bodies. Besides the composition of the substrate, the accumula-

tion of selenium and other elements in mushrooms is dependent on species and their particular genetic characteristics. Almost 190 wild mushroom species, including *Agaricus bisporus, Pleurotus ostreatus* and *Lentinus edodes,* have usually less than 1 μg Se/g dw. (Costa-Silva et al., 2011). When cultivated on substrates with selenium, mushrooms can be enriched with this essential element. Consumption of selenium-enriched mushrooms could improve the selenium status and even enhance the fight against various diseases. Lucas in 1957 showed that Basidiomycetes mushrooms possess bioactive polysaccharides in the mycelium, fruiting body and sclerotium. *Pleurotus* mushrooms possess immunostimulatory effects and the consumption of *A. bisporus* mushrooms could lead to a reduction in chemically induced tumor growth (Da Silva, 2012; Huerta et al., 2006). Other authors have suggested that Se-enriched mushrooms have strong antioxidant activity (Zhao et al., 2008).

A number of disease-causing organisms, including molds, are found in compost and casing soil during mushroom cultivation (Hatvani et al., 2007). Williams et al. (2003) described the components of aggressiveness of *Trichoderma harzianum* groups toward the mushroom *Agaricus bisporus*. Apart from faster growth, they are capable of producing extracellular enzymes, volatile organic compounds and toxic secondary metabolites, which can result in a drastic decrease in mushroom production. *Cladobotryum spp.* is responsible for the cobweb disease of mushrooms, appearing first as small white patches on the casing soil, which then spread to the mushroom, finally causing rapid decay of the entire fruit body (Sharma et al., 2007). *Mycogone perniciosa* is known as wet bubble disease. As the mushroom develops, it becomes golden brown and starts to decay, giving off a foul odor. Symptoms of dry bubble, caused by *Verticillium fungicola* var. *fungicola* vary, depending on the time of infection. According to Fletcher and Yarham (1976), *V. fungicola* var. *fungicola* is resistant to benomyl, a fungicide frequently used to control *M. perniciosa*. By the mid-1980s, the first sign of resistance of *Cladobotryum* spp. to benomyl was recorded. Since the application of disinfectants and selected fungicides involves significant cost, leaves unwanted residues or is even forbidden, viable alternatives need to be examined (Sokovic and Van Griensven, 2006).

The objectives of this study were to investigate the antifungal activity of selenized yeast Sel-Plex against pathogenic molds, find the concentrations with the best effects and to use it as potential selenium source for the production of enriched mushrooms. The antibacterial properties of selenium-enriched probiotics are already known (Yang et al., 2009), but the efficacy of Sel-Plex against mycopathogenic fungi has not been well documented.

MATERIALS AND METHODS

Standards and reagents

Sel-Plex (Alltech) was used in the experiment as an organic non-toxic source of selenium (EC L330/11, 2006). The product is produced from yeast *Saccharomyces cerevisiae* CNCM I-3060, containing organically bound selenium in the form of selenomethionine (1.99 mg Se/g Sel-Plex). Suprapur® hydrogen peroxide and nitric acid (Carlo Erba, Italy) were used for sample digestions. Selenium ICP standard solution of 1000 mg/l of Se was purchased from Merck.

Sample growth

Samples of fresh mushrooms, *Pleurotus ostreatus* commercial strain P70, were produced at the Department of Industrial Microbiology, Faculty of Agriculture, University of Belgrade (Serbia) in a greenhouse as described earlier (Savić et al., 2009b). *Pleurotus ostreatus* strain P80 and *Pleurotus cornucopiae* were produced by Mycorex Mushroom Limited (Larnaca, Cyprus). The *manufacturer's instructions were followed. For selenium-enriched mushrooms, the* addition of selenium was carried out together with the watering of the substrate during preparation. Sel-Plex was added to reach Se concentrations of 25 and 125 mg/kg of dry substrate. To the substrate for P70 growth, a wider range of concentrations was used (25, 50, 75 and 100 mg/kg of dry weight). Three independent samples were used. The fruit bodies were cleaned, sliced, dried at 105°C

and milled. Chemical analyses were performed on the mushroom powder.

The quantitative determination of selenium

A total of 0.3 grams of sample were digested with 6 ml of concentrated HNO_3 and 2 ml of concentrated H_2O_2 in a Milestone Ethos microwave digestion system and diluted to 25 ml with deionized water. A blank digest was carried out in the same way (digestion conditions for the microwave system were applied as 10 min at 100°C, 7 min at 200°C, 8 min at 200°C, up to 500 W respectively). Distilled deionized water and Suprapur® chemicals were used to prepare all reagents, standards and samples. The filtrates were collected and analyzed by ICP-OES (Thermo Scientific ICAP 6500 DUO). The selenium content of the samples was quantified against standard solutions of known concentrations.

Assay of antifungal activity

The experiment was carried out with the fungal strains *Trichoderma harzianum* Ko_1T_6, *Trichoderma harzianum* P_1T_6, *Mycogone perniciosa* P_1M_2, *Cladobotryum dendroides* Kal_1C_6 and *Verticillium fungicola var. fungicola* V_1V_3 obtained from the *Institute* of *Pesticides* and Environmental Protection (Belgrade, Serbia), isolated from different Serbian farms. In order to prepare the inocula for testing, all organisms were cultured on Malt Extract Agar (Biolab, Hungary) and incubated for 1 week at 25±2°C and 20±2°C for *Verticillium fungicola var. fungicola.*

The antifungal activity of the selenized yeast was tested using a well diffusion method in 90x14 mm Petri dishes containing 10 ml of malt extract agar. After the agar had cooled down to room temperature, a small amount (5 mm × 5 mm) of mycelia was inoculated in each plate. After the mycelial colony appeared, the solution was added to the wells to 1 cm from the rim of the mycelial colony on the agar surface with a 9 mm cork borer. A new fungicide, Dezohem (Hemos, Serbia), was used as positive control. Distilled water without selenium was used as negative control. The yeast solution was prepared in distilled water and sterilized at 121°C for 15 min. The solution was poured into the well in concentrations 0, 25, 50, 100 and 150 μg/g Se, using a micropipette. The plates were incubated at 25±2°C and 20±2°C for *Verticillium fungicola var. fungicola* for 48 h until mycelial growth had enveloped the well containing the control and had formed crescents of inhibition around the wells containing samples with antifungal activity (Ngai et al., 2005).

To determine the IC50 value for the antifungal activity, three doses (50, 100 and 200 μg/g Se) of selenium source were added separately to Petri dishes. The yeast solution was prepared by mixing it in malt agar base. After sterilization, it was cooled at 45°C, mixed rapidly and poured into three separate Petri dishes, each containing 10 ml of the solution. After the agar had cooled down to room temperature, the same small amount (5 mm×5 mm) of mycelia was inoculated onto each plate. Only malt agar served as a negative control. After incubation for 72 h, the plates were examined visually and the zone of inhibition was calculated by measuring the diameter (in mm) of the mycelial colony. Readings were taken in three different fixed directions in all three replicates. The percentage of inhibition of fungal growth was determined by calculating the reduction in the area of the mycelial colony compared with the negative control. A graph plotting the percent reduction in the area of the mycelial colony against the log dose of antifungal selenized yeast expressed as selenium was used to determine the dose producing 50% inhibition (i.e. IC50). (Ngai et al., 2005)

Statistical analysis

All measurements were done in triplicate and data were expressed as mean ± standard deviation. The differences between the groups were examined by analysis of variance (ANOVA) and Student's t test. A P value of less than 0.05 was considered statistically significant. Linear regression analysis was used to calculate IC50. Statistical analyses were performed with the statistical program MS Excel (Microsoft Office 2007 Professional).

RESULTS AND DISCUSSION

The quantitative determination of selenium

The obtained results (Table 1) showed that the studied mushroom species have different abilities to absorb Se from substrate added in the form of Sel-Plex. The mycelia grew normally and the young primordial formed well. The first harvest of fruit bodies happened between 23 and 28 days after inoculation in the control and in samples grown in low concentrations of Se. When grown in the substrates with more than 75 mg/kg Se, harvesting was initiated 3-5 days later. A prolonged time for mushroom formation was observed in the *Agaricus bisporus* mushroom enriched with Sel-Plex during a previous experiment (Savić et al., 2011).

Results of earlier studies (Estrada et. al, 2009) indicated a significant correlation between the Se concentration in the substrate and the Se content in the fruit body. Tubes and gills are the most abundant in selenium, and the cap contains this element in greater concentrations than the stem (Falandysz, 2008), which indicates an unequal distribution of selenium in the fruit body during fructification. The average content of selenium in control samples of fungi was between 0.12 and 0.68 μg/g, which is very low and confirms that the substrate from nature and the mushrooms that grow on it have a low content of total selenium. Selenium distribution in the studied species showed that Se is present in a large quantity in the fruit bodies of enriched species, i.e. at levels of 10 mg/kg and above. The ability of the organism to absorb Se was proportionally lower to the amount of Sel-Plex added to the substrate. The lowest concentration (10 mg/kg of Se) tested on *P. ostreatus* P70 resulted in mushrooms with 22.41 μg/g of Se. The highest concentration (100 mg/kg of Se) tested on the same strain, resulted in mushrooms with 133.12 μg/g of Se on average. The toxic effect on mushroom growth began with the addition of an organic source containing 75 μg/g of selenium. There were significant differences ($P<0.05$) in the average content of selenium in industrially grown mushrooms (Mycorex, Cyprus). This Se content was higher in the *P. ostreatus* P80 fruit body than in *P. cornucopiae*. *Pleurotus cornucopiae* was an excellent, but weaker absorber than *Pleurotus ostreatus* P80. When a concentration 25 μg/g of Se was added to the substrate, results showed a similar accumulation in all three tested mushrooms, as these mushrooms absorbed between 42.44 μg/g and 50.28 μg/g of Se.

The capacity to accumulate Se from Sel-Plex was verified in *Agaricus bisporus* when the mushrooms were irrigated with water plus supplement. As noted, the amount of absorbed selenium in the fruit body depends on the moment when Sel-Plex is added to the substrate. Based on the results, it can be concluded that the fungus takes up a greater amount of selenium in the early stage of development. For a higher accumulation of Se the complete development of the mycelium is needed (Savić et al., 2011). The absorbed Se content in *A. bisporus* fruit bodies was two times higher than in *Pleurotus* strains. This value varied from 0.190 to 354 μg/g of Se, when similar concentrations of supplement were tested. The reason could be the way of Sel-Plex addition to the substrate. In *A. bisporus* production, it was added to the top of the compost prepared the day before, and in *Pleurotus* sp. growth, it was mixed with the substrate during preparation. In future experiments, what is the best way and which is the best moment to add selenium to the growth substrate should be established.

The form and concentration of selenium source is an important factor in the enrichment of fungi.

The preferred form of selenium used during the experiments is in the inorganic form of salt. *Ganoderma lucidum* absorbs 20-30% of inorganic selenium from the substrate, and biotransforms it into organic forms by integrating Se into proteins (50-70%) (Zhao et al., 2008). Comparisons between inorganic and organic sources of selenium were performed by adding known amounts of the inorganic salts Na_2SeO_4 and Na_2SeO_3, as well as organic complex compounds [Zn (dapsesc)] to the substrate for *Lentinus edodes* and *Pleurotus ostreatus* cultivation (Savić et al., 2009a; Savić. et al., 2011). The results indicated

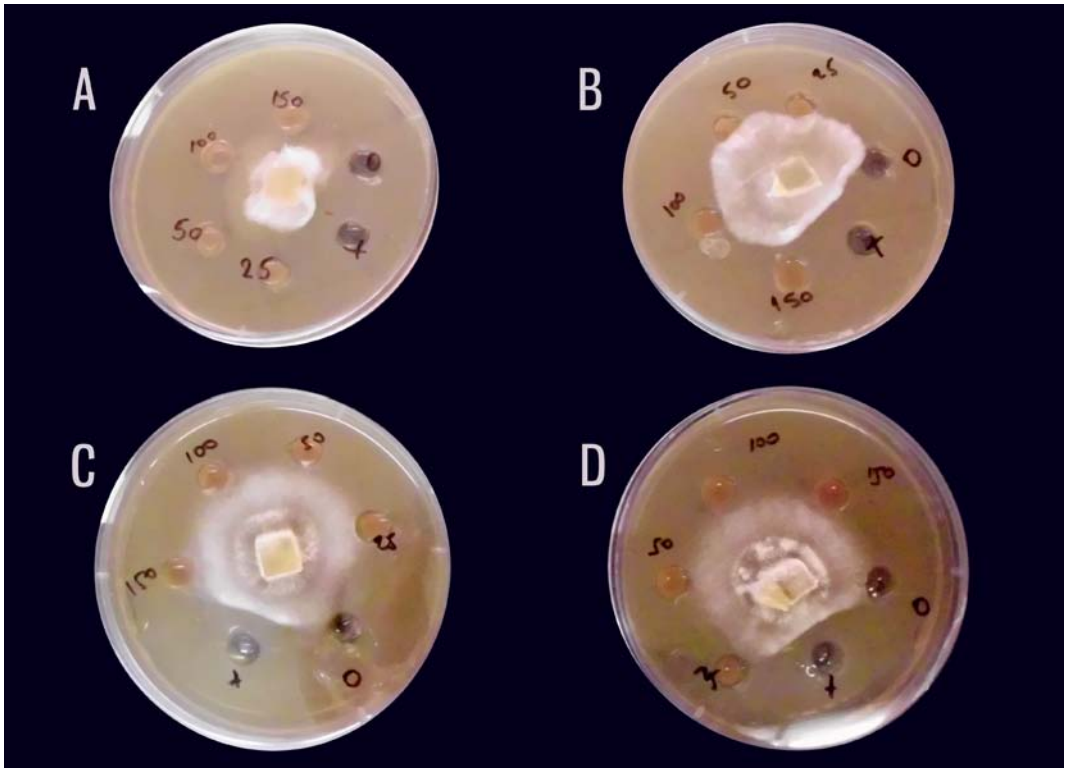

Fig. 1. Fungal growth inhibitory assay. The mycopathogens examined; (A) *Mycogone perniciosa* P_1M_2, (B) *Cladobotryum dendroides* Kal_1C_6, (C) *Trichoderma harzianum* Ko_1T_6, (D) *Trichoderma harzianum* P_1T_6 *with addition of Sel-Plex conc.* 0, 25, 50, 100 and 150 μg Se/g and positive control (+).

better accumulation of organic selenium in fungi for tested concentrations. Although the mushrooms are capable of adopting more selenium from these two groups of compounds, the use of selenized yeast as a potential new source of selenium has great advantages. Exposure to inorganic selenium can cause short- and long-term health problems to the human body. Selenized yeast is much safer for use. It is applied in animal nutrition and it achieves concentrations of selenium in the mushroom fruit body that meet the daily needs of humans. Likewise, selenized yeast was a more bioavailable Se form than Na_2SeO_3 for absorption and accumulation by *A. bisporus* fruiting bodies (Dernovics et al., 2002).

As regards the medicinal properties of the *Pleurotus and Agaricus* species and selenium antioxidative features, and considering the foregoing results, investigation of the effect of using Se-enriched fruit bodies in nutrition as well as their extracts can be done. The protein extracts from selenized mushrooms exhibit stronger antioxidant activities, which increase with the increase of Se content (Ming et al, 2007).

Assay of antifungal activity

The antifungal effect of Sel-Plex was tested against five mold species. Sel-Plex was found to have a strong fungistatic activity against molds that cause damage in mushroom cultivation (Fig. 1). Four of the five mold species were inhibited using a higher concentration of organic selenium (150ppm) when tested against the positive control. There was complete microbial confluence at the site of inoculation of the negative control and a lower concentration of selenium. Sel-Plex did not significantly inhibit the growth of *Verticillium fungicola var. fungicola* V_1V_3. The mycelial growth made by adding different con-

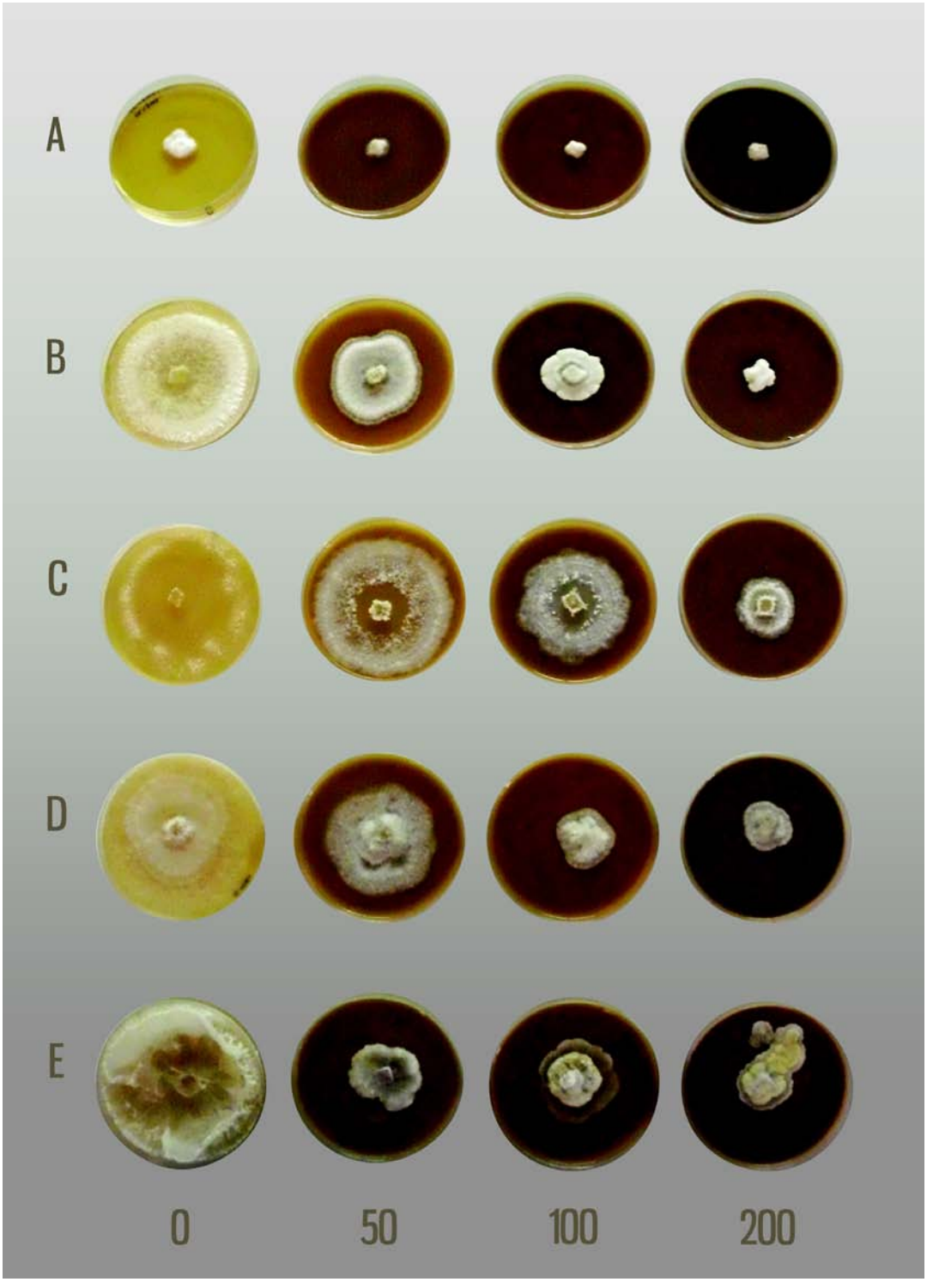

Figure 2. Percentage inhibition of Sel-Plex (50, 100, 200 μg Se/g) against examined mycopathogens (A) *Verticillium fungicola var. fungicola* V_1V_3, (B) *Trichoderma harzianum* P_1T_6, (C) *Trichoderma harzianum* Ko_1T_6, (D) *Mycogone perniciosa* P_1M_2, (E) *Cladobotryum dendroides* Kal_1C_6, as compared with plate without selenium.

Table 1: Uptake of Selenium by *Pleurotus* species[a]

Selenium treatment (mg/kg dw)	Se content in fruit bodies (μg/g dry weight)		
	P. ostreatus P70	*P. ostreatus* P80	*P. cornucopiae*
0	0.68 ± 0.02	0.12 ± 0.02	0.42 ± 0.03
10	22.41 ± 1.72	NA	NA
25	42.44 ± 3.39	50.28 ± 1.98	42.85 ± 1.41
50	88.24 ± 0.92	NA	NA
75	101.06 ± 3.96	NA	NA
100	133.12 ± 2.44	NA	NA
125	NA	137.84 ± 2.33	122.11 ± 2.03

[a] Values are the means ± standard deviation (n = 3). NA = Not Analyzed

Table 2. Antifungal activity of Sel-Plex against indicator organisms. The diameter of mycelial colony (in mm).

Indicator organisms	Se concentration (μg/g)			
	0	50	100	200
Trichoderma harzianum Ko_1T_6	55.33 ± 5.13	46.67 ± 1.15	41.03 ± 3.10	28.33 ± 6.66
Trichoderma harzianum P_1T_6	74.67 ± 0.58	43.33 ± 2.52	27.14 ± 4.58	15.67 ± 3.06
Mycogone perniciosa P_1M_2	49.33 ± 2.52	29.13 ± 6.56	19.45 ± 2.64	13.33 ± 1.53
Cladobotryum dendroides Kal_1C_6	69.01 ± 3.61	30.33 ± 2.08	23.67 ± 2.52	17.33 ± 1.53
Verticillium fungicola var. fungicola V_1V_3	19.33 ± 1.15	13.07 ± 1.09	11.12 ± 1.21	11.33 ± 0.58

All values are mean±SD (n=3).

Table 3. Antifungal activity of Sel-Plex against indicator organisms. IC50 values

Indicator organisms	IC50 (μg/g Se)
Trichoderma harzianum Ko_1T_6	229.0
Trichoderma harzianum P_1T_6	64.6
Mycogone perniciosa P_1M_2	67.6
Cladobotryum dendroides Kal_1C_6	31.7
Verticillium fungicola var. fungicola V_1V_3	574.4

centrations of selenium to the agar base, compared to the control, is shown in Fig. 2.

The results indicate that all molds could be suppressed in growth by using Sel-Plex for mushroom enrichment (Table 2). There was a significant difference ($P<0.05$) in the growth rate of the molds as a result of adding selenium to the agar base. A significant difference was noticed between strain V1V3 and other strains. The growth of V1V3 mycelium decreased with increasing concentrations above 50 ppm. The mycelial growth of other molds in all treatments were continuously and significantly suppressed as the selenium concentration increased ($P<0.05$).

The value of IC50 is listed in Table 3. According to the antifungal activity, the IC50 was calculated. The IC50 of Sel-Plex ranged between 31.63 μg/g and 574.4 μg/g expressed as selenium concentration. Sel-Plex has a strong fungistatic activity

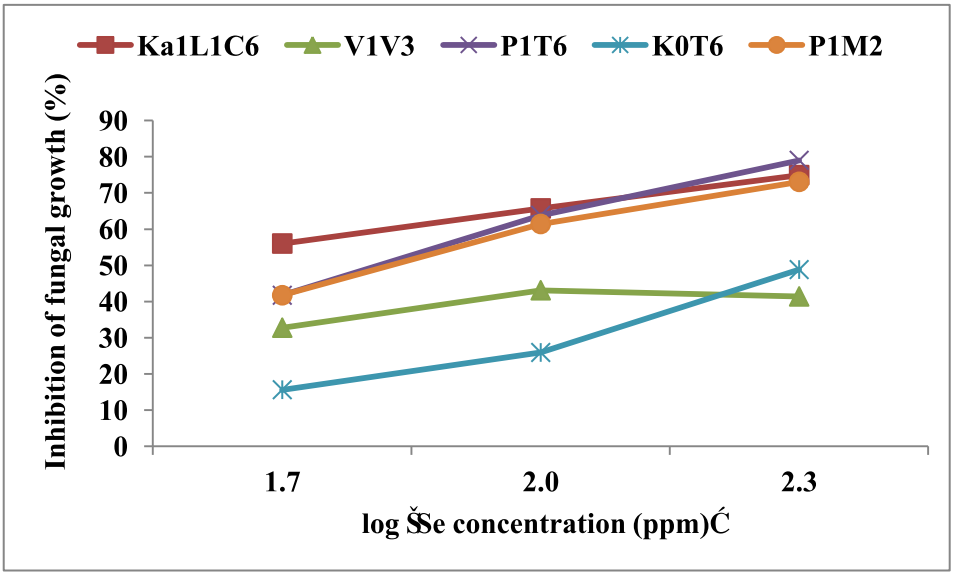

Figure 3. The percentage of inhibition against fungal growth determined by calculating the reduction in the area of mycelial colony compared with the negative control. Data represented mean ± SD (n=3)

against *Cladobotryum dendroides* (IC50 was 31.63 µg/g of Se).

A very low inhibitory effect against *Trichoderma harzianum* Ko_1T_6 and *Verticillium fungicola var. fungicola* V_1V_3 was observed. A 7-18 times greater concentration of organic selenium to inhibit growth by 50% was needed (Fig. 3).

CONCLUSION

The results of this investigation clearly show that the ability for absorption of organic selenium in the mushroom fruit body and its antifungal activity vary with the species of mushrooms and molds used. Hence, it has a potential of being used as a source of selenium for enriched mushroom production with strong fungistatic activity against a variety of fungal species. The results presented in this paper suggest that the most suitable concentrations of selenized yeast that inhibit the growth of the mycopathogenic molds are 70-100 µg/g of selenium. With the addition of these concentrations to the substrate, selenium will affect the growth rate of mushrooms in a few days, but the fruit bodies will uptake a high level of selenium, about 100 µg/g for *Pleurotus spp.* and 200 µg/g for *A. bisporus*, of the dry weight of the mushroom. In this way, a double effect in the cultivation of mushrooms is achieved. However, further work on the isolation and identification of active compounds from enriched mushrooms needs to be undertaken in the future. The activities of Sel-Plex against different strains of bacteria and other organisms known to be responsible for causing various diseases could also be tested in future studies. Selenium-enriched mushrooms could be of considerable interest for the development of new food supplements. The efficacy of any resulting treatment regimen should subsequently be proven with well-designed randomized control trials.

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