Antibacterial Activity of Proteinaceous Extracts of Higher Basidiomycetes Mushrooms against Plant Pathogenic Bacteria

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Higher Basidiomycetes mushrooms have been shown to contain substances with potential inhibitory influence on harmful organism. A screening test to assess influence of proteinaceous mushroom extracts on the growth of major plant pathogenic bacteria in microtiter plates has been designed and evaluated in comparison with determination of inhibition on agar plates. Preliminary testing was performed with mushroom extracts and some purified plant and fungal protein inhibitors against major plant pathogenic bacteria.

Extraction of proteinaceous substances from mushrooms yielded 0.1 to 5 mg of protein mL^{-1}. Different parts of mushrooms were used for preparation of extracts, and it was not always possible to choose sterile tissue. Extracts were thus diluted to allow filter sterilization.

Growth of plant pathogenic bacteria on agar plates was as expected, depending on growth rates of different species. Slimy growth could mask inhibition zones in some cases. Growth of bacteria in microtiter plates (liquid media) depended largely on the temperature of incubation and shaking. With slow-growing pathogens the microtiter plate assay is technically challenging because of evaporation and, in case of tested slow-growing plant pathogens, high-oxygen demand. The choice of test, therefore, largely depends on the characteristics of the bacteria tested.

Significant differences in sensitivity were observed between determination of minimum inhibitory concentration (MIC) on agar plates and microtiter plate assay, with the former being more sensitive.

Extracts can be grouped into four groups depending on their influence on growth:

- Extracts not showing any inhibitory effect. These belonged to different species of both edible and non-edible mushrooms.
- Extracts exhibiting small zones of inhibition, up to 5 mm. Despite low efficacy these extracts often influenced taxonomically different bacteria. Extract from *Clitocybe geotropa* has shown the broadest range with low level inhibition against *Ralstonia solanacearum*, *Erwinia carotovora* subsp. *carotovora*, *Pseudomonas syringae* pv. *syringae*, *Xanthomonas campestris* pv. *Vesicatoria,* and *Clavibacter michiganensis* subsp. *sepedonicus.*
- Extracts with prominent inhibition. Zones of inhibition on agar plates were in the range of 45 mm. These extracts included some from non-edible mushrooms with known toxins presumably affecting RNA polymerase enzyme, while others were from non-edible or edible mushrooms of different species.
- In some cases, growth was improved by addition of extracts, possibly because they provide additional nutrients to bacteria. In the case of *Ralstonia solanacearum* one extract caused more intensive production of the characteristic brown pigment.

Of the purified proteins tested, clitocypin showed low levels of inhibition against *Clavibacter michiganensis* subsp. *sepedonicus* when tested on agar plates. Proteinase inhibitors from potato showed low-level inhibition against *Ralstonia solanacearum*, another potato pathogen.

Preliminary testing of proteinaceous extracts of higher Basidiomycetes mushrooms against plant pathogenic bacteria show promising results with several extracts exhibiting inhibitory influence on their growth. Both varieties of screening tests for growth inhibition, testing on agar plates and in microtiter plates using liquid media, have proved useful and the choice of which to use depends largely on the characteristics of bacteria.

Extracts showed a range of activities, from improving growth, possibly due to additional nutrients, to strong inhibition.

Although microplate assay is generally preferable for similar screening test as it is amenable to automatization and high-throughput analysis, it has been shown as less sensitive in our study. Low-level inhibition of some extracts against a range of bacteria could thus be only observed on agar plates.

Of the purified proteins tested clitocypin and proteinase inhibitors from potato showed only very low levels of inhibition against bacteria pathogenic to potato.

Further studies are underway and include testing of extracts regarding to:

- type of influence on growth (bacteriostatic versus bactericidal)
- possible influence of extracts on bacterial pathogenicity and disease development through pathogenicity tests on host plants
- titration and determination of minimum inhibitory concentration of inhibitory extracts

Further investigation of active substances and investigation of their use in protection of plants against bacterial diseases is forseen.

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Antifungal Secondary Metabolites from Fungal Fruiting Bodies

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The incidence of fungal infections has increased dramatically in the past 30 years. Uncommon fungal infections have become common, the lesser fungal pathogens have become stronger, and newly recognized fungal pathogens are appearing. The increased occurrence and strength of fungal pathogens parallel with AIDS, organ transplants, and steroid treatments. So what do all of these have in common? The answer is a compromised immune system. An individual with a healthy immune system will normally subdue a fungal pathogen before the pathogen can take root. However, when a person lacks the responsiveness of a good immune system, a fungal pathogen will begin to grow. Once a fungal pathogen is established even a fully functional immune system is normally not enough to resolve the infection, and persistent fungal infections will require the use of antifungal drugs. There are few antifungal agents available for treatment of fungal pathogens, and most of these drugs have adverse side effects. The number of antifungal drugs is dwarfed by the number of antibacterial treatments available. The reason for this vast difference is based solely on the lack of targets available for antifungal drugs to work against. This distinction is also responsible for a majority of adverse side effects from antifungal drugs since there tends to be significant overlap in the physiology of human and fungal cells.

In the past, antifungal drugs have been discovered from various sources, including other fungi. Although it seems paradoxical, fungi produce secondary metabolites that are able to kill other fungi, perhaps by accident, or this may be part of an innate defense system developed from years of competition. Whatever the case, secondary metabolites have lead to the discovery of many useful antifungal and antibacterial drugs, most notably penicillin. Both filamentous fungi and fungal fruiting bodies have produced useful secondary metabolites. The beneficial secondary metabolites of filamentous fungi are many times discovered by accident, since filamentous fungi tend to be ubiquitous, commonly showing up as contaminates in the lab. On the one hand, due to the non-fastidious growth of most filamentous fungi they often have worldwide distribution. On the other hand, macrofungi tend to be restricted to geographical locations due to substrate limitations or, in the case of mycorrhizal fungi, host limitations. Each geographical location represents a prospective reservoir for biosynthesized compounds that can have a multitude of bioactive

characteristics. However, these compounds are not usually found by accident – they are discovered through comprehensive and methodical screenings of hundreds of species.

The aim of this study was to employ natural products chemistry to discover new antifungal drugs from fungal fruiting bodies. Extracts from fungal fruiting bodies have led to the discovery of secondary metabolites that are inhibitory to the fungal pathogens. Using disc diffusion and minimum inhibitory concentration assays, crude extracts were applied to six tester strains in their pathogenic state: *Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans, Emmonsia crescens, Sporothrix schenckii,* and *Trichophyton* species. These comprehensive screenings of more than 200 species of fungi have lead to some possible candidates for new antifungal drugs. Samples showing inhibitory activity were subjected to chemical purifications using thin layer chromatography and high-pressure liquid chromatography. These agents once characterized may benefit patients and have a significant economic impact.

Reactive Oxidants Generating Characteristics of Mushroom Polysaccharides in Human Cell Lines

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Mushroom β-glucans are highly conserved structural components of the fungal cell wall and possess several characteristics of pattern-associated molecular patterns (PAMPs). Mushroom β-glucans activate phagocytes by binding to various receptors on the cell. This process stimulates the release of inflammatory mediators as TNF-α, interleukin1 (IL-1), and reactive oxidants (ROS). Upon receptor binding, (proteo)glucans can be internalized and as a result signalling pathways are switched on, leading to activation of the transcription factor NF-κB, upon which adaptive immunity is initiated.

We studied the capacity of hot water extracted polysaccharides (PS) of *Agaricus brasiliensis*, *A. bisporus*, *Ganoderma lucidum,* and *Phellinus linteus* to generate intracellular ROS in human PBMCs, in a T cell lymphoblast line (Jurkat), and in the myelogenous leukaemia line K562. The test was performed using dichloro-dihydrofluorescein-diacetate, which fluoresces when intracellularly oxidized. PS of all mushrooms tested induce immediate generation of ROS in those three lines; strong competition by laminarin, a small beta 1-3, 1-6 glucan from the sea weed *Laminaria digitata*, indicating binding to the cellular receptor Dect-1 was, however, only found in PBMCs and Jurkat. Preincubation of the cells with PS for 6 hours, followed by washing, kept the ROSs intact in PBMCs and Jurkat but showed no previously generated ROS in K562, suggesting that internalization of PS is not required for ROS generation. Phorbol 12-myristate, 13-acetate (PMA) had no effect on ROS generation.

When small amounts of ethanol extracts of *Agaricus brasiliensis* and of *Phellinus linteus* were added to incubation mixtures, the ROS generation was strongly inhibited although no cytopathic effects were found.

The PS extracts of all mushrooms, except of *Ph. linteus,* induced IFN-γ in PMA stimulated human PBMCs. PS of *Ph. linteus* induced a concentration-dependent decrease of IFN-γ synthesis and of TNF-α not due to cytotoxicity. This suggests possible anti-inflammatory characteristics of the *Ph. linteus* polysaccharide.

As all commercially available polysaccharide extracts of mushrooms and also ours contain variable amounts of protein and possibly of polyphenols, we have not been able to attribute the characteristics described here to single compounds. Moreover, the completely different secondary structure and sizes of the β-glucans in the mushroom extracts make this even more difficult.

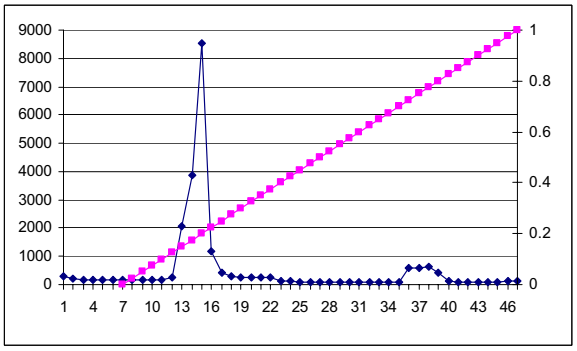

FIGURE 1. DEAE-cellulose elution profile of *Phellinus linteus* polysaccharide. Up: Carbohydrate and protein contents of fractions. Down: ROS generating activity in K562 cells. Fractions eluted by continuous 0–1.0 M NaCl gradient.

Innate Immunity Gene Expressions by β-Glucan of *Ganoderma lucidum* through Dectin-1 and Toll-like Receptor

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β-Glucan is a glucose polymer that has linkages of β-(1,3), -(1,4), and -(1,6). As they are exclusively found in fungal and bacterial cell walls, not in animal, β-glucans are recognized by innate immune systems. Dendritic cells (DC), or macrophages, possess pattern recognition molecules (PRM) for binding β-glucan as pathogen-associated molecular pattern (PAMP). Recently, the β-glucan receptor was cloned and reported from DC as dectin-1, which belongs to type II and C-type lectin family.

In our macrophage cell line culture system, dectin-1 mRNA was detected in RAW264.7 cells by the reverse transcription-polymerase chain reaction (RT-PCR). For elucidation of the mechanism of immunopotentiation by mimicking the intestinal environment, β-glucans of *Ganoderma lucidum* (GLG) were used for the stimulation of the macrophage cell line in the absence and/or presence of lipopolysaccharides (LPS). Treatment of RAW264.7 cells with GLG resulted in increased expression of dectin-1 as well as inflammatory cytokines such as IL-1, IL-6, IL-10, and TNF-α in the presence of LPS. The maximum mRNA expressions of IL-1, IL-6, and TNF-α were reached after 12 h of treatment of GLG, returning to normal levels after 48 h. The induction of the protein level of IL-6 and TNF-α was re-confirmed on membrane array assay. In addition to those cytokines, GLG also induced nitric oxide (NO) and reactive oxygen species (ROS) as inflammatory mediators both in the presence or absence of LPS. Toll-like receptors (TLRs) were also induced by GLG both in the presence or absence of LPS as a time-dependent manner, reaching a maximum after 48 h.

From these results, β-glucan of *Ganoderma lucidum* could exert its stimulation of innate immunity by binding to its receptor, dectin-1, resulting in induction of inflammatory cytokines as well as inflammatory mediators. The results also suggest that receptor dectin-1 cooperates with TLRs and CD14 to activate signal transduction, which is very critical in immunostimulation.

The Role of Oxizymes in Detection of Very Low Doses of Phenolics by Fungi

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Fungi belong to a group of microorganisms that are able to secrete several oxizymes as secondary metabolites with different potentialities in the degradation of lignin, a natural polymer of low-molecular phenolics. The amount of phenolic or non-phenolic (rich in methoxyl groups) aromatic particles determined the quality of lignin and their distribution in plant tissue. It also influences the progress of delignification of wood material.

The white-rot Basidomycetes provide the best enzymes for catalysis of *in vivo* and *in vitro* process of lignin biodeterioration. Laccases and various peroxidases are known for their activity in releasing simple phenolic particles from this natural biopolymer. *In vitro,* however, particularly, the process of nonphenolic lignin depolymerization needs the presence of radical mediators, such as HBT or ABTS, which as enzymatic co-substrates facilitate the oxidative cleavage of this natural polymer. In our lab we found strong evidence for the possibility to change the enzymatic activity of laccase and HR-peroxidase in the presence of very low doses of chosen phenolics and mediators, known as co-substrates of these enzymes. The oscillating characteristic changes in activity allowed dilutions of the tested aromatic substances with maximal and minimal effect on activity using the spectrophotometric and luminometric assays as well as the PAGE electrophoretic technique. The practical aspects of the activation of fungal oxizymes by homeopathic doses of chosen phenolics and aromas are discussed.

The Influence of Magnetic Fields and IR Laser Light on Mycelial Growth of Higher Fungi

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Currently, there are several known effects of electromagnetic fields and laser treatments on different organisms. Substantial experimental evidence indicates that electromagnetic fields influence many metabolic activities, such as changes in enzyme efficiency, protein-ligand interactions, gene expression, protein synthesis, and cell proliferation. Markov (Proceedings, 3rd International Workshop, Kos, Greece, 2004) reported the existence of window effects at various amplitudes and frequencies on myosin phosphorylation. An electromagnetic field had significant effects on spruce seedlings – both normal intact and those with their roots cut – especially on the growth of the roots; the electromagnetic field inhibited the growth of the intact main roots and enhanced the ramification and development of the lateral ones (Jerman et al., 1989, J Slovene Biol, 37(1):45–56). Toshihiko and co-workers (Bioelectromagnetics, 2006, 27:98–104) reported that the exposure of single suspension-cultured plant cells (*Catharanthus roseus*) to a magnetic field strengthens the cell wall structure, thereby increasing the Young's modulus of the regenerated cell wall. When cells are irradiated with visible and near infrared wavelengths, a variety of stimulatory effects are observed in their metabolism. Other researchers studied repair effects of laser on mutants of filamentous fungi (Yansheng et al., 1999, Proc. SPIE Vol. 3863, 455–459), and changes in the biochemical behavior of adenosine triphosphate (ATP) molecules (Amat et al., 2004, J Photochem Photobiol, 168:59–65).

For our study, we examined the effects of extremely low-frequency (ELF) magnetic fields and

infrared (IR) laser light exposure on fungi *Pleurotus ostreatus, Trametes versicolor,* and *Grifola frondosa*. The main purpose was to determine if those effects are also applicable to fungi and what kind of consequences they have. Therefore, we built a growth chamber that was divided into two parts – one for treated and the other one for controlled Petri dishes. The internal environment was controlled – dark, constant humidity and stable temperature at 26°C. One group of fungal mycelia was treated in ELF magnetic field in militesla (mT) range. Samples were exposed twice a day for one hour intermittently every 15 min. The other group was irradiated daily in a non-contact mode (distance of 7 cm from irradiated area) with a diode laser light shower with different exposure time and power.

We observed the growth area of mycelium that was inoculated on potato dextrose agar (PDA) in Petri dishes. The mycelial growth rate was measured daily by scanning and visually analyzing the surface area. Growth was measured until the whole surface area of growth medium was overgrown. After that the increment in growth was measured by absorption of light. The treated and controlled samples were compared and specific differences were noted in growth form, intensity, and density of mycelium. The influence was species specific; with *T. versicolor* regeneration of inoculum was improved, with *P. ostreatus* downy mycelial growth was detected, and with *G. frondosa* stimulated and condensed mycelium was observed. However, for determination of specific activities of treatment further studies are needed.

Uniformity of Proteolytic Diversity in Basidiomycetes

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Information about proteolytic activity in Basidiomycetes is limited to proteases involved in nutrient utilization in saprophytic and mycorrhizal Basidiomycetes and to virulence factors in pathogenic species. To investigate the presence of different types of proteases in Basidiomycetes, we analyzed aqueous extracts of 43 Basidiomycetes by gelatin zymography combined with assays with class-specific inhibitors.

The search, which was primarily aimed at disclosing the multiplicity of activities rather than their complete identification, revealed the prevalence of serine proteases among the proteolytic activities of all four catalytic classes in nearly all the mushroom samples tested, many of which may be exclusive to Basidiomycetes fungi. All four catalytic classes of proteases were present, with 4% of all activities classified as aspartic, 5% as cysteine, 6% as metallo, and 22% as serine proteases, while the remaining activities could not be assigned unambiguously. The majority of the latter were not inhibited by any of the inhibitors used and were termed insensitive. These may be standard proteases with known folds but with unique features accounting for their atypical properties. Alternatively, they may contain new and unusual folds, and provide new catalytic mechanisms, specificity, and ways of regulation. Different proteolytic activities are evenly distributed among members of all orders of Basidiomycetes, although some taxa are a richer source of proteases than others. A significant number of the cysteine protease activities shown here have not previously been reported in Basidiomycetes. The number and diversity of proteases found under specific conditions used in this study for basidiocarps, together with data on proteases characterized from different developmental stages described in literature, imply that the proteolytic potential of Basidiomycetes is immense.

In addition, we have focused on the endogenous proteolytic system of our model mushroom, *Clitocybe nebularis,* comprising proteases and their inhibitors. Crude protein extract from *C. nebularis* basidiocarps applied to gel exclusion chromatography revealed the highest proteolytic activity at acidic pH that shows inhibition by pepstatin A. Using two different affinity chromatographies, we have partially purified and characterized seven active putative aspartic proteases differing in their binding to specific lectin and protease inhibitor ligands, with different molecular masses, N-termini and measurable activity.

Clitocypin (*Clitocybe nebularis,* a cysteine protease inhibitor), a new type of inhibitor of cysteine proteinases was, due to its unique characteristics, assigned as the only member of a new family of cysteine protease inhibitors I48 of the MEROPS inhibitor classification. It is a 16.8 kDa protein lacking cysteine and methionine residues. The gene encoding clitocypin belongs to a small gene family of closely related genes that show sequence variability, which is limited to 18 discreet positions throughout the protein sequence. The variability is not reflected to function in spite of the differences in primary structure, structural, functional, and immunological equivalence was established for recombinant and natural clitocypins. Kinetic analyses revealed that natural and recombinant clitocypins exhibit the same pattern of inhibition as both inhibit the tested cysteine proteinases to a similar extent, demonstrating an unusually broad inhibitory spectrum including such distantly related proteinases as papain and mammalian legumain.

The above-mentioned study using gelatine zymography on aqueous extracts of Basidiomycetes included the use of fungal cysteine and serine protease inhibitors, clitocypin, and CNSPI (*Clitocybe nebularis,* a serine protease inhibitor) in the inhibitor assays. Both inhibited a number of activities in gelatine zymography and even a few activities that were otherwise insensitive to all other inhibitors used. Therefore, a regulatory role in endogenous proteolytic systems may be considered for clitocypin and CNSPI for the first time, in addition to the postulated defensive role against predator insects and parasitic microorganisms.

The number and diversity of proteases and their inhibitors in Basidiomycetes are remarkable and encourage further investigation. The observed heterogeneity of the cysteine protease inhibitor clitocypin and the diversity of proteolytic activities in different basidiocarps of different Basidiomycetes, together with the surprising number of different putative aspartic proteases from *C. nebularis,* seem to represent the rule rather than the exception, introducing a common theme of variability in fungal proteins that confer the diversity and adaptability of fungal physiology and metabolism. In addition, the diversity of fungal proteolytic systems represents another source of unique proteases and their inhibitors for use in drug discovery and design.

A Mushroom Melanin-Glucan Complex Counteracts the Development of Inducible Genome Instability

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There are many environmental factors (ultra-violet and ionizing radiation (IR), emotional stress, infections, hyperthermia, strong medical products, etc.) that can exert toxic influence on genomes. All of them can induce oxidative stress through activation of early regulatory and late structural genes. As a result, in posterity of certain parts of the cells, which have survived after intensive influence (in particular irradiation), chromosome aberrations and genetic mutations arise *de novo* with a high frequency during many generations. In some cases, this is the reason for increased cell death rate through apoptosis. These deferred effects caused by the radiating influence are known as radiation-induced instability of the genome (RIIG). It is established that 80% of DNA damage due to IR is caused by active radicals. Structural DNA damages are caused by nitrogen oxides and products of lipids, peroxide oxidations as well as by active forms of oxygen (AFO). At the RIIG, epigenetic changes take place of an oxidation-reduction metabolism inherited from irradiated cells with strengthened generation of AFO and raised of DNA sensitivity to oxyradicals and other toxins for genome. Through cytokines and other proteins the irradiated cells cause typical changes to not damaged cells-neighbours. Such changes typical for irradiated cells are: increase of AFO maintenance, frequencies sisterly chromatid exchanges, etc. («Bystander effect», ByEf). A long preservation of posterity of the irradiated cells in this condition increases the risk of tumor transformation. Therefore, the need to overcome induced instability of genome became one of the most actual problems of modern radiobiology.

The purpose of this research was to investigate the influence of melanin-glucan complex (MGC) from *Fomes fomentarius* (L.: Fr.) Fr. (Basidiomycota, Polyporales) on the RIIG and on the expressiveness of BySt at mice Balb/c (LD 50/305,85 Sv), irradiated at a doze 5 Sv for 16 hours.

We studied single strand break levels of DNA (SSB DNA) and the ability of cells from irradiated animals (taken from various tissues and organs, such as peripheral blood, spleen, liver) to induce an increased quantity of SSB DNA in cells obtained from 46 not irradiated animals. The analysis was done during the first few hours, in one week, in four weeks, and in four months of irradiation by DNA, labeled by fluorescent dye Picogreen technique. Subsequently, the velocity of its unwinding was estimated. The same group of animals, one day prior to irradiation, received intra peritoneum 0.5 ml of 0.9% NaCl solution with 0.5 mg MGC. Animals of this group received daily the same quantity of MGC with water and food after irradiation. The damaging effect was studied after addition of culture medium from irradiated cells to normal cells. After the first day of irradiation, the levels of SSB DNA in all kinds of cells increased. Regarding the lymphocytes and splenocytes of not irradiated animals, the addition of culture medium of irradiated cells caused 70% and 50% increase of SSF DNA, respectively.

In hepatocytes, this parameter increased on the second week after irradiation, and ByEf was precisely observed four months after irradiation. In the cells from irradiated animals, receiving MGC, the level of SSF DNA was much lower. The culture medium obtained from irradiated cells practically did not increase the SSB DNA in not irradiated cells. Conclusion is made about the presence of anty-Rays properties of MGC from *Fomes fomentarius*.

Study on Selenium Accumulation in *Trametes versicolor* Using Instrumental Neutron Activation Analysis

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A strain of *Trametes versicolor* (L.: Fr.)Lloyd (Turkey Tail Fungus) was obtained from Chiba University, Japan, and fermented in stationary Erlenmayer flasks 250 ml, containing PG media with Se (10 ppm as selenate) and without Se (control), incubated at 32–34°C. Selenium was supplemented as dissolved selenate, Na_2Se0_4, in which Se was enriched with ^{74}Se. Under neutron fluxes in nuclear reactor, ^{74}Se was activated into ^{75}Se emitted gamma rays (n,γ reaction), recorded for calculations of Se contents in the samples. *T. versicolor* was cultivated on mixed sawdust (based on rubber tree sawdust) supplemented with Se by injecting directly into center region of the substrate. Analysis of Se contents in fungal biomass harvested by using INAA with neutron flux at $10^{12}.cm^{-2}.s^{-1}$ in Nuclear Reactor at Dalat City, Vietnam.

The results showed that Se levels in *T. versicolor* mycelial biomass were up to 600–1500 ppm, while in the control (without Se supplement) only a trace of Se (approximately 1 ppm) was measured (Fig. 1). It would be related to the researches on configurations of a complex of polysaccharides and proteins in biomass with high bioactivities. Se would substitute S for other structures, particularly, complexes of polysaccharides and peptides, including diselenite bridges -Se-Se-, and enhance their bioactivities.

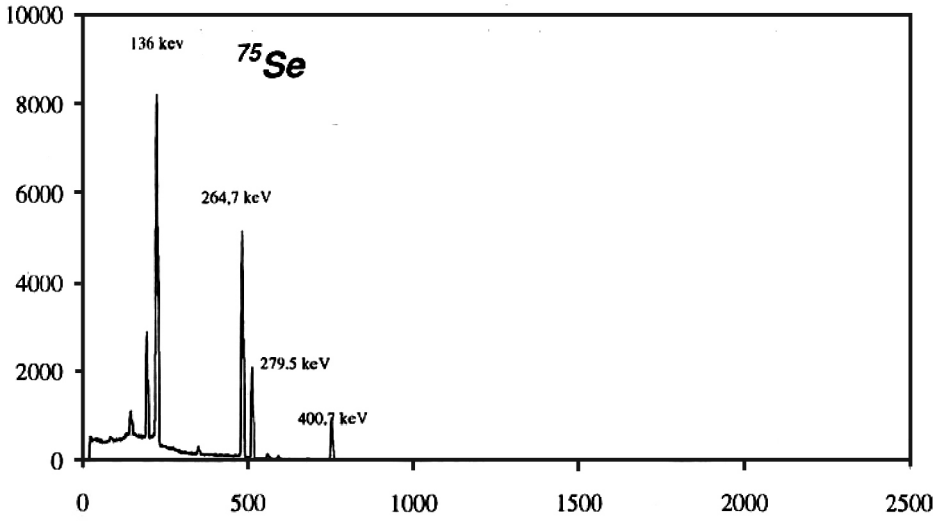

FIGURE 1. Spectrum of gamma ray energy emitted from Se-enriched mycelial biomass of *Trametes versicolor* fermented for 10 days with 10 ppm Se supplement.

The Importance of Arbuscular Mycorrhizal Fungi in Heavy Metal Pollution in Soils of the Upper Mezica Valley

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Soils of Upper Mežica Valley (Slovenia) are highly contaminated with Pb, Cd, and Zn as a consequence of lead mining and smelting activities from the 15th century. Similarly, the garden soil in this area is highly polluted and contains, on average, 34 mg Cd kg^{-1}, 5584 mg Pb kg^{-1}, and 2765 mg Zn kg^{-1}, which is significantly higher than the permissible boundary set by Slovenian laws (Ur. l. 68/96). Excessive heavy metals are easily taken up by plants and consequently incorporated into the human food chain. Local residents regularly plant vegetables for food in their gardens. To overcome heavy metal stress, plants have evolved strategies to protect against toxic concentrations; one of these strategies is arbuscular mycorrhiza (AM). It is a mutualistic association between plant roots (host) and fungi from the phylum Glomeromycota.

In order to test the ability of AM fungi to reduce the uptake of heavy metals into vegetables the most commonly used edible plants: green pepper (*Capsicum annuum*), tomato (*Lycopersicon esculentum*), parsley (*Petroselinum crispum*), carrot (*Daucus carrota*), and lettuce (*Lactuca sativa*) were inoculated with AM fungi in the field. An indigenous inoculum mixture containing *Glomus fasciculatum, G. mosseae,* and *G. intraradices* prepared from the original soil was applied when the plants were transplanted/seeded to a vegetable garden in Mežica. The protocol for the application of AM fungal biotechnology in horticulture (Regvar et al., 2003) was tested in practice. Mycorrhizal colonization was estimated according to Trouvelot et al. (1986), and heavy metal contents of soil and plants were determined using flame and electro-thermal atomic absorption spectrometric methods (Perkin Elmer SIMA 6000) after appropriate digestion of samples (Regvar et al., 2006).

Successful inoculation significantly increased root fresh weights of pepper, carrot, and lettuce, whereas shoot and fruit weights were not significantly affected.

Inoculated plants showed significantly reduced contents of Cd in lettuce (roots and shoots), of Pb in carrot roots and tomato (stems and fruits) and the contents of Zn in lettuce (roots and shoots), tomato (roots, shoots, and fruits), and parsley roots. The results confirm the potential of AM fungi biotechnology for the reduction of heavy metal uptake in edible parts of selected vegetables.

Recently, a Cd and Zn hyperaccumulating plants species *Thlaspi praecox* was reported to thrive in this heavy metal polluted area (Vogel-Mikuš et al., 2005). Hyperaccumulation is a phenomenon in which plants are able to take up and accumulate toxic concentration of heavy metals in leaves (Baker, 1981) and has attracted attention due to their potential use in one of the phytoremediation technologies, i.e., phytoextraction, which attempts to extract toxic elements from soils by these specifically adapted plants (Brooks, 1998). Heavy metal hyperaccumulating plants from the genus *Thlaspi* (Brassicaceae) are generally considered non-mycorrhizal (Pawlowska et al., 1996). However, AM was found in field-collected *Thlaspi praecox* Wulfen (Regvar et al., 2003; Vogel-Mikuš et al., 2005) and successful inoculation of with AM fungi under greenhouse conditions resulted in improved mineral status and reduced heavy metal uptake of the plant (Vogel-Mikuš et al., 2006). Field experiments on the potential of *T. praecox* to remove heavy metals are under way. The aim of the study is to reduce the content of heavy metals in garden soil and lower toxicity of these elements into the food chain via vegetables.

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Synthesis and Secretion of Endoglucanase in the Edible Mushroom, *Volvariella volvacea*

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The edible straw mushroom, *Volvariella volvacea* (Bull.: Fr.) Singer, is cultivated on an industrial scale in many tropical and sub-tropical regions using cotton waste "composts" as a growth substrate. In order to convert these high cellulose-containing "composts" to glucose, the fungus produces a multicomponent enzyme system consisting of endo-1,4-β-glucanases (EG), cellobiohydrolases, and β-glucosidases. Biodegradation of a cellulose polymer is initiated by endoglucanases that hydrolyze β-1,4–glucosidic bonds within amorphous regions of cellulose and, since insoluble cellulose cannot enter the fungal cell, the enzyme has to be exported through the hyphal wall and into the external medium.

Biochemical analyses of different culture fractions following growth of *V. volvacea* in submerged culture on crystalline cellulose have shown that most of the endoglucanase activity was present either in the culture filtrate (45.8%) or associated with the insoluble pellet fraction remaining after centrifugation of homogenized mycelia (32.6%). Confocal laser scanning microscopy combined with immunolabeling confirmed this distribution pattern and revealed the endoglucanase to be largely cell wall- associated or located extracellularly.

We have now adopted a combined molecular biological and immunocytochemical approach aimed at elucidating the dynamics of endoglucanase secretion *in*

vivo in *V. volvacea* over a 24-hour period during which the enzyme is regulated both by induction and catabolite repression. Using RT-PCR, expression of an endoglucanase isoform (EG1) was first detected after 3 h following addition of α-lactose to *V. volvacea* mycelium pre-grown for 72 h in basal medium containing 1% sorbitol. Expression levels peaked at 6 h but decreased thereafter. Distribution of the enzyme within fungal hyphae during the induction/repression cycle was determined using primary antibody, raised in rabbits against heterologous EG (expressed in *Pichia pastoris*) and rhodamine-labeled anti-rabbit secondary antibody. The purity and specificity of the primary antibody was confirmed by Western blot. Confocal laser microscopy revealed weak immunofluorescence distributed uniformly throughout the fungal hypha after 3 h induction. After 6–8 h induction, a stronger, continuous band of immunofluorescence was evident in the region of the hyphal wall. Labeling of hyphae 9–12 h after induction (onset of repression) was less intense and more dispersed. Control hyphae grown on sorbitol alone were unlabeled.

The long-term significance of this research lies in the potential to enhance the bioconversion of cellulosic wastes by *V. volvacea*, thereby increasing the biological efficiency and improving growth yields. The knowledge gained will also be of relevance to protein secretion by filamentous fungi in general. Potentially, this is of immense significance since many of the enzymes secreted by filamentous fungi have been developed for use in various industrial processes (e.g., in food and textile processing, and in pulp and paper production). Production of fungal enzymes on an industrial scale is now an important and growing sector of the fermentation industry, particularly since many of the producing organisms are regarded as "safe" by regulatory authorities. The capacity of filamentous fungi for high-level protein secretion is also one of the key features in considering them as potential hosts for producing high-value recombinant therapeutic proteins.

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Use of *Trametes versicolor* in Control of Aflatoxins Produced by *Aspergillus parasiticus*

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Aflatoxins are secondary metabolites produced by some fungi which are toxic, carcinogenic, teratogenic, and mutagenic for animal and human cells. The consumption of food and feed contaminated by aflatoxins presents a serious health hazard: their continuous intake, also at minimal doses, increases the risk of liver cancers and, in addition, they have a suppressing effect on the animal (human) immune system. The main fungi producers of aflatoxins belong to the genus *Aspergillus,* section *Flavi.* They can grow in the wide range of temperature and humidity. It has been proved that the synthesis of aflatoxins is closely related to the oxidative stress inside and outside of the cell. In fact, some antioxidants are in a position to control their production by toxigenic molds.

Some higher Basidiomycetes are known for their therapeutic effects in curing of a number of human and animal pathologies. Their action, often connected with polysaccharides and glycoproteins produced by these mushrooms, includes the strengthening of the immunological response, control of cholesterol, anti-tumoral action, antioxidative effects, and the protection of the liver against aflatoxins. More recently, the ability of some mushrooms to control the synthesis of aflatoxins by *A. parasiticus* has been shown. In particular, the capacity of β-glucans produced by *Lentinus edodes* to inhibit toxin production and stimulate antioxidant responses in *A. parasiticus* has been reported in our previous work. In this research the evaluation of the

use of compounds produced by *Trametes versicolor* (L.: Fr.) Pilat in control of the aflatoxins biosynthesis by *Aspergillus parasiticus* is reported. *T. versicolor* is a medicinal mushroom well-known for the production of some glycoproteins with antitumoral, antiviral, and antioxidant properties. Six different isolates of this mushroom were studied for their ability to produce compounds able to control the aflatoxins synthesis. Both lyophilized culture filtrates (LF) and lyophilized mycelial extracts (EM) were tested, and the inhibition of the aflatoxins synthesis from 60% to 90% was observed. There was no significant differences in inhibitory effects between LF and EM of the same strain. The inhibition of toxin production seems to be connected both with the quantity of β-glucans ($r2 = 0.90$; $p < 0.001$) present in LF and EM and with their antioxidant capacity ($r2 = 0.88$; $p < 0.01$). Further research on the mechanisms of inhibition effectuated by LF and EM has demonstrated that their presence in substrates of cultures induces the activity of some antioxidant enzymes. In particular, in the presence of LF the activity of superoxide dismutase (SOD) and glutathion peroxidase (GPX) in the mycelia of *A. parasiticus* was stimulated between 36 and 72 hours of incubation at 30°C, while the activity of catalase (CAT) had less influence. At the same time, the maximal production of aflatoxins was registrated. In addition, the quantity of reactive species, such as lipoperoxides (LOOH), in the mycelium of the toxigenic fungus was significantly reduced in the presence of LF during the whole period of incubation. In yeast, the antioxidant response of the cell is regulated by transcription factors (*yap*-1, *skn*-7, *hsf*-2, and others). These factors have been cloned, for the first time, in *A. parasiticus,* and the effect of LF on their expression has been studied.

The study has shown their anticipated activation and an inhibition of expression of some genes responsible for the synthesis of aflatoxins. Following these results that clarify some aspects of the mechanism of inhibition of synthesis of aflatoxins by LF of *T. versicolor*, experimental trials on wheat and maize seeds inoculated with conidia of *A. parasiticus,* incubated at temperature and humidity (30°C, $a_w = 0.90$) optimal for the development of toxigenic strains of *A. parasiticus,* were performed. The presence of LF (2% w/w) and lyophilized mycelia of *T. versicolor* inhibited aflatoxin production between 70% and 95% up to 30 days of incubation. These results are very promising.