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# ADAPTATION OF SELECTED ECTOMYCORRHIZAL FUNGI TO INCREASED CONCENTRATION OF CADMIUM AND LEAD

## ADAPTACJA WYBRANYCH GRZYBÓW EKTOMIKORYZOWYCH DO ZWIĘKSZONYCH STĘŻEŃ KADMU I OŁOWIU

**Abstract:** Plants together with water and minerals actively take from the soil heavy metals such as cadmium and lead. The negative role of ions of these metals on plant growth and development depends not only on their concentration in the soil, but also on a number of factors that may affect the transport of minerals from the soil to the roots. The harmful effects of xenobiotics getting from the soil to the plants are limited by the organic compounds contained in the soil, soil structure and pH. Particularly noteworthy are biotic factors, such as bacteria and fungi which greatly limit the translocation of heavy metals. Stream of new scientific reports show that the symbiotic combination of fungi with plant roots so called mycorrhizae is a factor that may be important in reducing the impact of soil contamination by heavy metals. Mycorrhiza by filtering solutions of water and mineral salts stop a considerable amount of heavy metals in the internal mycelium or on its surface. It was proved that plants with properly formed mycorrhiza grow better in hard to renew lands, such as salty, sterile soils contaminated with industrial waste. Questions to which answer was sought in this study are: 1) whether mycorrhizal fungi for many years growing in the contaminated areas have managed to adapt to these adverse conditions and 2) do the same species derived from clean areas are less resistant to contamination by heavy metals? Stated problems tried to be solved based on the fruiting bodies of fungi collected from ectomycorrhizal fungi picked from the areas contaminated by industrial emissions and areas free of contamination. The interaction of cadmium and lead ions on the growth of mycelium was examined by plate method and binding of heavy metals in fruiting structures of fungi were done by colorimetric method with use of methylene blue. It has been shown that the fungal resistance, even of the same species, to high concentration of heavy metals varies depending on the origin of symbiont. Isolated fungi from contaminated areas are better adapted to high concentrations of xenobiotics. Ability to bind cadmium and lead to fruiting bodies of fungi varies.

**Keywords:** ectomycorrhiza, ectomycorrhizal fungi, heavy metals

# Introduction

Steadily increasing soil contamination is a serious threat to the growth and development of plants. The development of industry, motorization and agricultural intensification involve a rising concentration of pollutants, especially very dangerous for living organisms heavy metals. Authors in numerous reports [1, 2] indicate their toxic effect

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on the number of basic metabolic and physiological processes in plants. The negative effects of their actions result mainly from the interaction of the functional groups of molecules belonging to the cell, in particular proteins (SH groups) and polynucleotides [3]. In wooden plants inhibition of roots growth [3, 4], reduction in biological membrane integrity and distortion of the key enzymes, such as nitrate reductase were observed [5]. The effect of this may be weaker growth and development of plants, and even their death.

However, the toxicity of the pollutant is determined mainly by its accessibility, defined as the ability to move from the soil solution to the living organisms [6]. It becomes harmful to plants only crosses the plasma membrane barrier and its concentration is determined as a toxic. Valuable, therefore, for the protection of ecosystems are all factors which bind heavy metal ions bind and stop their penetration of the soil and plant structures. Heavy metal bioavailability in plants depends on many factors, for example: pH, organic content in the soil, bacterial flora activity, red-ox potential and soil aeration [3, 7, 8]. Increasingly, attention is also paid to fungi as natural factors that may be important in reducing the impact of plant and soil contamination by heavy metals. This is well documented in wooden plants that go into the mutualistic mycorrhizal symbiosis and produce ectomycorrizal roots which preserve, store and exchange mineral salts and water [9]. It has been shown that this type of plant association with fungi significantly enhance their tolerance to toxic concentration of heavy metals in soil [10-13].

In the course of evolution, a number of mechanisms appeared to minimize the negative effect of the stressor on plant organisms, and thus allow its growth and development in contaminated ecosystems [14]. The positive role of mycorrhizal systems in this respect has been clearly demonstrated. Given the positive aspects of plant symbiosis, increasingly the artificial mycorrhization of plants is carried out. The use of licensed production or finished imported vaccines signifies the view promoted by commercialism that the origin of fungi used for artificial mycorrhization does not matter. The working assumption of this study is to verify the validity of this claim for symbiotic plants growing in areas degraded by heavy metals emitting industry. During preliminary observations in areas heavily contaminated by heavy metals emissions, only selective presence of certain species of mycorrhizal fungi was observed. These findings prompted the question: whether besides the selection of mycorrhizal fungi in the polluted environment can be observed its adaptation to the adverse conditions of life, which would be of great importance in the selection of fungi for artificial mycorrhization.

The aim of this study was to determine whether the selected fungi forming ectomycorrhiza and occurring in areas contaminated with excessive levels of heavy metals are adapted to high concentrations of cadmium and lead, and whether their sensitivity to heavy metals ions is similar to that which is observed in mycorrhizal fungi coming from uncontaminated environments.

## Materials and methods

### Selection of research stations

The material for the study: mycorrhizal roots and fungi fruiting bodies were collected from experimental stations. In order to determine their susceptibility to contamination with heavy metals, isolated fungi were collected from two sites located near industrial center. The area defined in this work as “Miasteczko Slaskie” is located in the immediate vicinity of lead smelters of the same name. Area named as Huta Czestochowa is located in the poor fresh coniferous forest, northwest of steelworks "Huta Stali Czestochowa". Designated control areas were in the village "Kokotek" about 25 km to the north - west from a larger emitter of pollutants "Miasteczko Slaskie". It lies within the forest circle Kosmidry in the forest division Lubliniec and is outside the scope of impact of large industrial plants. Habitat in which research stations were situated is the fresh coniferous forest, where the main component of the stand is pine with addition of birch. The second research station outside the influence of industrial emissions was "Przedborz", located in Wyzyna Malopolska in mesoregion called Wzgorza Opoczanskie. Material was collected about 2 km from the Przedborz towards Konskie. Within a radius of approx. 30-40 km away from the designated area of research, there are no heavy industry, which would be both a source of emissions of heavy metals, gases and metal dust carriers.

Habitats of all research areas were similar to the poor fresh coniferous forest.

**Selection of mycorrhizal fungi species**

The appearance of fruiting bodies of fungi on research areas were observed between late summer until late autumn 2014.

The most common species being found in the vicinity of pines and spruces were: *Xerocomus badius* (Fr.), *Paxillus involutus* (Batsch.) (Fr.), *Xerocomus submentosus* (Fr.), *Laccaria laccata* (Scop.) Cooke, *Russula xerampelina* (Schaeff.) Fr. In the vicinity of birch trees on all surfaces appearance of the fruiting bodies of *Amanita muscaria* and *Amanita citrina* was observed.

Fungal species that were found in all experimental plots, both contaminated and clean were: *Paxillus involutus*, *Xerocomus badius*, *Amanita muscaria*, *Amanita citrina*, *Hebeloma crustuliniforme*. They were selected as starting material for further research.

From all research stations healthy fruiting bodies with dimensions of 10-14 cm in height and approx 5 cm cap diameter were harvested and pure cultures of mycelium were derived in accordance to tissue method developed by Pachlewski and modified by Kozdroj [11] what involves cutting in sterile condition sections of plectenchyma from the centre of the cap of approx. 0.5 cm width, length and thickness. The isolation and surface inoculation of fungi was carried out on solid agar medium of the following composition: agar 15 g; glucose 20 g; maltose 5 g; ammonium tartrate ($[NH_4]C_4H_4O_6$) 0.5 g; potassium dihydrogen phosphate ($KH_2PO_4$) 1 g; magnesium sulfate ($MgSO_4 \cdot 7H_2O$) 0.5 g; iron citrate ($FeC_6H_5O_7$) (1%) 0.5 $cm^3$; zinc sulfate ($ZnSO_4$) (1:500 solution) 0.5 $cm^3$; thiamine 50 μg; distilled $H_2O$ up to 1000 $cm^3$; medium pH 5.5. Isolates were transferred to fresh agar slants until a pure culture were obtained.

**Determination of strains sensitivity on selected heavy metals**

To determine the sensitivity of individual strains on heavy metals, the increasing doses of cadmium and lead ions were introduced into the agar medium. That medium was placed on petri dishes with culture of vegetative mycelium derived from isolation. Doses of individual metal ions in the medium were gradually increased (cadmium: 25, 50, 100, 150, 200, 400, 800, 1500, 1800, 2000 and 2200 μg/$cm^3$; lead: 25, 50, 100, 150, 200, 400, 800, 1500, 1800, 2000 and 3000 μg/$cm^3$; Cd 2200 μg/$cm^3$ and Pb 3000 μg/$cm^3$ were maximum doses at which mycelia were able to survive) to determine the growth characteristics, the degree of adaptation and the end of mycelium life. In order to estimate growth rate, estimation scale was applied on two-week-old cultures in comparison to the control group.

**Adsorption measurement of heavy metal cations by mycelium using colorimetric method**

The method utilizes the adsorption capacity of heavy metal cations in mycelium cell walls by the displacement of methylene blue - dye of cationic character, which saturates the binding sites located in the fungal cell wall. Incubation of saturated mycelium with metal salt solutions causes the displacement of the dye by proper heavy metals cations. Using the equation of the standard curve, the number of moles of the dye repressed from dry unit weight of the mycelium by each of the cations (taking into the account the concentrations of metals) was calculated.

Fungi were weighted (3 g of dry weight) and placed into flasks with 10 $cm^3$ of 0.5 mM methylene blue solution. Flasks were stirred for half an hour and then transferred mycelium onto wet filter placed in a Buchner funnel supplied with vacuum suction kit. Material was extensively washed with deionized water. After washing the mycelium were placed in new flasks. To one flask 10 $cm^3$ of deionized water was added, and to the remaining 10 $cm^3$ of 10 mM solutions of cadmium and lead chloride. Incubation was carried out for half an hour at the 25°C. Then the liquid was decanted from the mycelium to the tubes and the absorbance of the supernatants was measured. It was performed at a wavelength of 540 nm using HELIOS spectrotrophotometer (Thermo Electron Corporation).

**Statistics**

The Shapiro-Wilk test was used to evaluate the distribution. Non-parametric tests were used because of the non-Gaussian distribution. Differences between cultures contaminated with cadmium were tested using Mann-Whitney U test. Differences between the accumulation of methylene blue in cultures from various locations were tested using Cochran-Cox test. The value of $p < 0.05$ was considered to be statistically significant. The statistical analysis was performed with STATISTICA 10.0 (Stat-Soft Poland Sp. z o.o., Krakow, Poland).

## Results

Growth of two-week-old cultures on medium contaminated with cadmium is shown on Figure 1. It was determined statistically significant differences between fungi originating from contaminated sites and uncontaminated Miasteczko Slaskie and Przedborz for *Amanita muscaria* ($p = 0.041$), *Hebeloma crusuliniformis* ($p = 0.031$), *Xeroccomus badius* ($p = 0.045$) and *Amanita citrina* ($p = 0.041$). Also statistically significant differences were between fungi from Miasteczko Slaskie and Kokotek for all examined species of fungi: *Paxillus involutus* ($p = 0.030$), *Amanita muscaria* ($p = 0.048$), *Hebeloma crusuliniformis* ($p = 0.030$), *Xeroccomus badius* ($p = 0.049$), and *Amanita citrina* ($p = 0.045$). In other cases, the differences were not statistically significant.

Growth of two-week-old cultures on medium contaminated with lead is shown in Figure 2. In case of lead contamination statistically significant differences were found between fungi coming from the towns of Miasteczko Slaskie and Przedborz for all examined fungi: *Paxillus involutus* ($p = 0.030$), *Amanita muscaria* ($p = 0.007$), *Hebeloma crusuliniformis* ($p = 0.018$), *Xeroccomus badius* ($p = 0.041$), and *Amanita citrina*

($p$ = 0.021). Also statistically significant differences were between fungi from the Silesian towns and Kokotek for all examined species of fungi: *Paxillus involutus* ($p$ = 0.016), *Amanita muscaria* ($p$ = 0.003), *Hebeloma crusuliniformis* ($p$ = 0.010), *Xeroccomus badius* ($p$ = 0.035), and *Amanita citrina* ($p$ = 0.016) and between fungi from Czestochowa and Przedborz such as *Amanita muscaria* ($p$ = 0.027) and *Hebeloma crusuliniformis* ($p$ = 0.045). In other cases, the differences were not statistically significant.

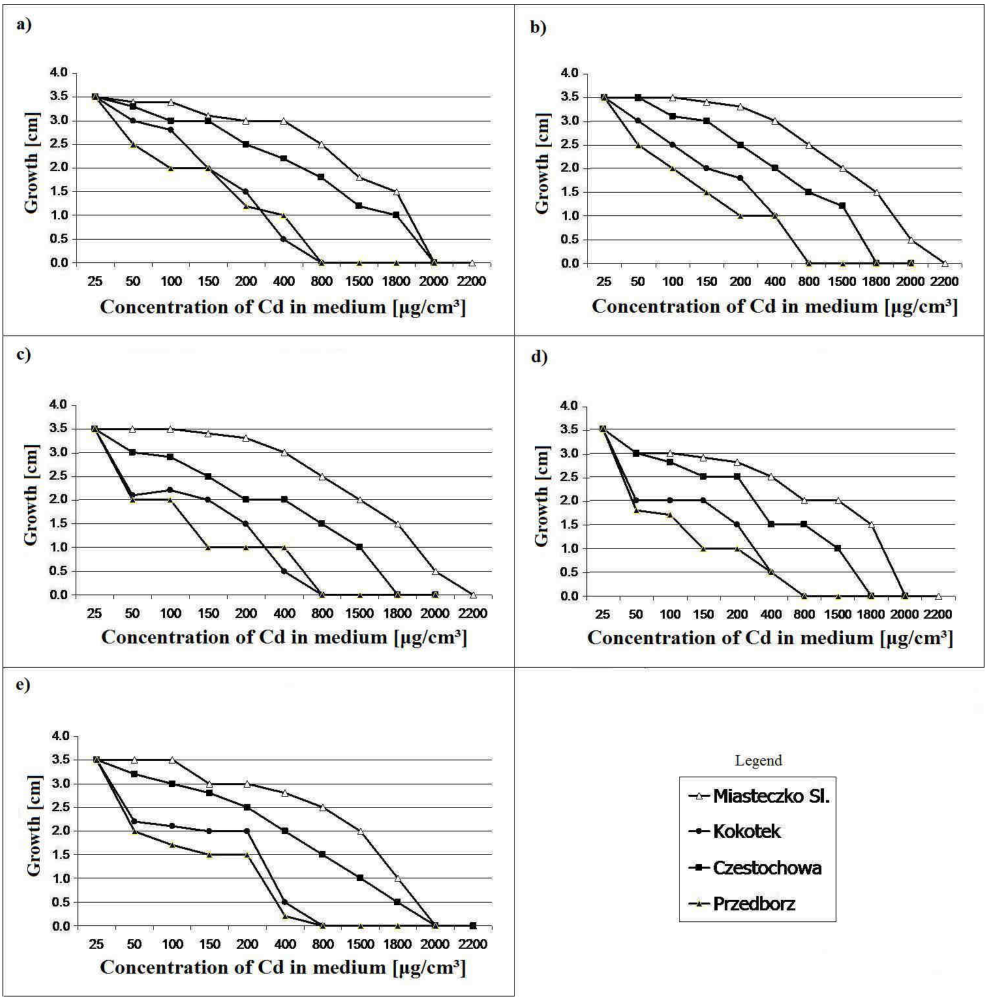


Fig. 1. Growth of two-week-old selected mycelium cultures: a) *Paxillus involutus*, b) *Amanita muscaria*, c) *Hebeloma crustuliniforme*, d) *Xeroccomus badius*, e) *Amanita citrina* on medium contaminated with cadmium

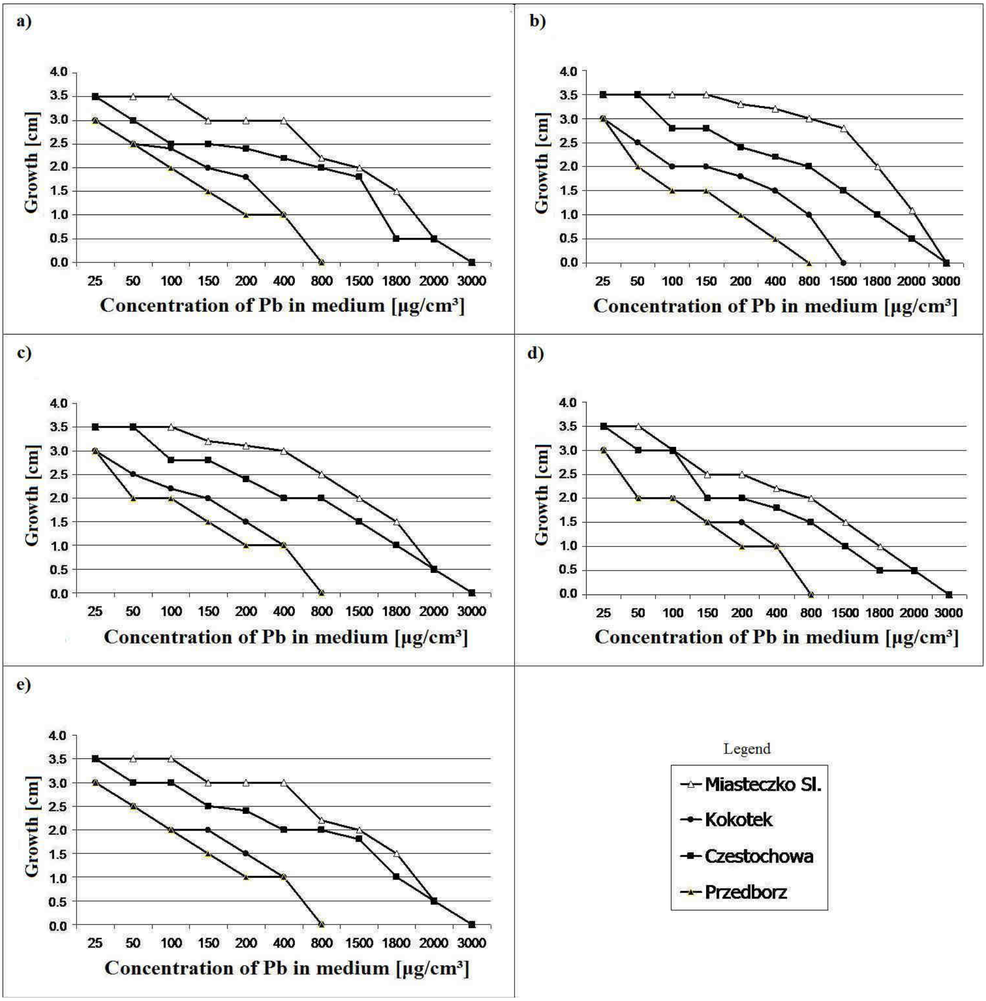


Fig. 2. Growth of two-week-old selected mycelium cultures: a) *Paxillus involutus*, b) *Amanita muscaria*, c) *Hebeloma crustuliniforme*, d) *Xeroccomus badius*, e) *Amanita citrina* on medium contaminated with lead

The results of the accumulation of methylene blue in case of contamination with lead and cadmium are shown in Tables 1 and 2, respectively. There is a statistically significant difference between the accumulations of metals in fungi derived from different locations in almost all cases with the exception of:

1. for lead contamination: *Paxillus involutus, Xeroccomus badius* and *Amanita citrina* collected from Przedborz and Kokotek (two places considered to be uncontaminated);
2. for cadmium contamination: *Amanita muscaria, Hebeloma crusuliniformis* and *Amanita citrina* collected from Przedborz and Kokotek (two places considered to be uncontaminated).

Table 1

Accumulation of methylene blue in case of contamination with lead (average)

| Fungi | Czestochowa | Miasteczko Sl. | Przedborz | Kokotek |
|---|---|---|---|---|
| *Paxillus involutus* | 0.275 | 0.248 | 0.450 | 0.462 |
| *Amanita muscaria* | 0.425 | 0.174 | 0.634 | 0.667 |
| *Hebeloma crusuliniformis* | 0.416 | 0.234 | 0.418 | 0.427 |
| *Xeroccomus badius* | 0.267 | 0.236 | 0.688 | 0.685 |
| *Amanita citrina* | 0.518 | 0.629 | 0.726 | 0.743 |

Table 2

Accumulation of methylene blue in case of contamination with cadmium (average)

| Fungi | Czestochowa | Miasteczko Sl. | Przedborz | Kokotek |
|---|---|---|---|---|
| *Paxillus involutus* | 0.335 | 0.252 | 0.283 | 0.309 |
| *Amanita muscaria* | 0.347 | 0.187 | 0.391 | 0.404 |
| *Hebeloma crusuliniformis* | 0.489 | 0.192 | 0.743 | 0.748 |
| *Xeroccomus badius* | 0.247 | 0.272 | 0.413 | 0.427 |
| *Amanita citrina* | 0.651 | 0.688 | 0.553 | 0.546 |

## Discussion

The technique of isolation and cultivation of ectomycorrhizal mycelium in pure cultures is a common model to study the physiology of growth and development of fungi. It is also a way to verify in the laboratory observations made in the field. This method is commonly used to study the effect of heavy metals on fungi. Colpaert et al, studied the effect of cadmium on the *Suillus luteus* and *Suillus bovinus* strains and found that one which were derived from contaminated soil were more tolerant to cadmium than one that came from the uncontaminated soil [15].

The results of their work are in agreement with data obtained in our research. Sembratowicz and Rusinek-Prystupa showed that fungi derived from areas contaminated with heavy metals were more resistant to higher concentration of lead and cadmium ions in the substrate compared with mycorrhizal fungi that came from uncontaminated areas [16]. It is worth to notice that in this kind of research it should be extremely careful to draw conclusions because the lab eliminates many factors that may play a role in natural biocenoses and *in vitro* tests as evidenced by Jones and Hutchinson [17] can be downright confusing. Their research showed that *in vitro* studies on ectomyccorhizal fungi are little connected with the real behavior of these fungi in the natural symbiosis. They isolated the ectomycorrhizal fungus *Betula papyrifera* from contaminated sites near Sudbury, as well as from “uncontaminated” areas. However, further studies on isolates - *Laccaria laccata*, *Lactarius rufus*, *Lactarius hibbardae* and *Scleroderma flavidum* in artificial cultures found no correlation between the increase and the tolerance of these fungi to heavy metals, and the place of origin.

Mushroom *Scleroderma flavidum* was unable to grow *in vitro* as a vegetative mycelium with even low concentrations of nickel, although it managed to produce fruiting bodies in a very high concentration of nickel in the soil [17]. The contradictions between the results of different authors may be only apparent, because the ability to adapt can be a feature of the species and even strains of the mycorrhizal fungus. A prerequisite to clarify this issue may be surprising results of the absorption of methylene blue. Generalizing, the fungi coming from uncontaminated areas retain more of heavy metals in their structures. Ostensibly this is contrary to the results of these fungi resistance to contamination. However, one should note that in the first case we examine the growth of vegetative mycelium and in the second case fragments of fruiting bodies. On this basis, we can even hypothesize that mycorrhizal fungi from areas contaminated with heavy metals have adapted to these conditions by saturation or partial blockade of functional groups catching cadmium and lead from the soil. It is certainly not only one of many functioning mechanisms. It is also important to find out if increased tolerance to heavy metals has genotypic or phenotypic cause in the particular species. Also it is significant to know in what form the metal is present in the soil and what form it was used in *in vitro* condition. Thus, although the subject includs a number of questions yet not fully explained, it appears that accurate analysis of the different fungi derived from contaminated areas controlled by *in vitro* tests will allow the use of certain fungal species as vaccines to improve the mechanisms of plant resistance to increased levels of heavy metals in the soil.

## Conclusions

The strains of studied mycorrhizal fungi derived from areas heavily polluted by heavy metals are more resistant to contamination than strains obtained from uncontaminated places, free from industrial pollution. It can be concluded that the following strains: *Paxillus involutus*, *Xerocomus badius*, *Amanita muscaria*, *Amanita citrina*, *Hebeloma crustuliniforme*, originated from contaminated sites were adapted to high concentrations of Cd and Pb. The degree of adaptation in most cases is directly proportional to the degree of contamination of the site of origin of the myccorhizal fungus strain. The obtained results of the accumulation of methylene blue in the mycelium suggest that one of the mechanisms of adaptation may be partially restricted sorption of metals by blocking the functional groups of anionic character.

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