

Heavy Metal Tolerance of Fungus Isolated from Soil Contaminated with Sewage and Industrial Wastewater

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Received: 24 January 2012

Accepted: 26 November 2012

Abstract

Fungi, including *Aspergillus flavus*, *Aspergillus niger*, *Fusarium solani*, and *Penicillium chrysogenum*, resistant to heavy metals like Cr and Pb were isolated after screening soil samples from peri-urban agricultural areas. The objective of soil sample screening was to investigate the status of heavy metals and to identify the heavy metal-tolerant fungi. The results revealed that the majority of the isolates were resistant to Pb and Cr, and only few of them were able to grow. Among the isolated fungal strains, *Aspergillus niger* was the most tolerant against Pb, with MIC of 600 mg/l, and *Aspergillus flavus* against Cr, with MIC of 400 mg/l, which makes them attractive potential candidates as bioremediation agents.

Keywords: fungi, heavy metals, tolerance, chromium, lead

Introduction

Heavy metal pollution is a serious environmental problem of global concern. Heavy metals are continuously released into the environment due to industrial and technological developments, and contamination of agricultural soil with heavy metals is a major problem at industrial and defense-related sites all over the world [1]. In developing countries industrial or municipal wastewater is mostly used for the irrigation of crops, mainly in peri urban ecosystems, due to its easy availability, disposal problems, and scarcity of fresh water [2]. In most parts of Pakistan, untreated city effluent is utilized for growing vegetables around large urban settlements. Farmers use it as a source of irrigation water and plant nutrients. However, its continuous use may have serious environmental implications, since it also contains heavy metals. Use of untreated city effluent for irrigation without risk assessment and management could be a serious hazard, impacting soil and crop quality and, ultimately, human health.

The industrial effluents are generated from hundreds of small and large manufacturing and plating industries such as metallurgical, electroplating, metal finishing, tanneries, chemical manufacturing, mine drainage, and battery manufacturing, and contain considerable amounts of heavy metals at elevated concentrations [3]. Long-term applications of such wastewater may result in the accumulation of heavy metals in soil and exert a selection pressure on soil microbiota and could pose a public health risk.

In naturally polluted environments the concentration and the availability of metals and the action of different factors such as the type of metal, the nature of medium, and microbial species govern the response of microbes to heavy metals toxicity [4]. Fungi and yeast biomasses are known to tolerate heavy metals [5, 6]. They can adapt and grow under various extreme conditions of pH, temperature, nutrient availability, and high metal concentrations; therefore, they are considered a versatile group [7].

Metal resistance is defined as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to metal species concerned. Heavy metals are indicated to be harmful pollutants in soils

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negatively affecting the species composition and function of the indigenous microorganisms, including fungi. Heavy metals can exert harmful effects in many ways, depending on environmental factors and metal species. Metals can variously influence soil fungi by changing fungal morphology and physiological activity, and affect the growth rate, reproduction process, enzyme production, etc. [8].

Fungi are known to tolerate and detoxify metals by several mechanisms, including valence transformation, extra and intracellular precipitation, and active uptake [9, 10]. Various biological mechanisms involved in fungal survival include extracellular precipitation, complexation and crystallization, transformation of metals, biosorption to cell wall and pigments, decreased transport or impermeability, efflux, intracellular compartmentation, and sequestration [10, 11].

Currently, scientists are exploring the bioremediation techniques by exploiting microbial and associated biota within the ecosystem, to degrade, accumulate and/or remove the pollutants [12], and strains isolated from contaminated sites have this excellent ability. El-Morsy [13] studied 32 fungal species isolated from polluted water in Egypt for their resistance to metals and found that *Cunninghamella echinulata* biomass could be employed as a biosorbent of metal ions in wastewater. Vadkertiova and Slavikova [14] have studied metal tolerance of yeasts isolated from polluted environments and found that there was an interspecific and intraspecific variation in the metal tolerance among tested strains. In the same way, Zafar et al. [15] reported promising biosorption for Cd and Cr by two filamentous fungi, *Aspergillus* sp. and *Rhizopus* sp., isolated from metal-contaminated agricultural soil.

Considering the above mechanisms of metal resistance in fungi, it was expected that screening of metal-tolerant fungi might provide strains with improved metal accumulation [16]. Only limited studies have been conducted in our country to systematically screen filamentous fungi from metal-polluted sites for their diversity and metal tolerance.

The present work reports the characterization of metal-resistant micro-organisms isolated from polluted environments and selection of more resistant strains. The reason for the selection of the fungi from contaminated fields is that the organisms that inhabit a certain environment usually adapt the conditions by developing survival mechanisms, and in future these fungi could be used as a bioremediation tool.

Materials and Methods

Study Area and Samples Collection

The main purpose of the present study was to see the tolerance of isolated strains of fungi (*Aspergillus flavus*, *Aspergillus niger*, *Fusarium solani*, and *Penicillium chrysogenum*) toward heavy metals. For present investigation soil samples were collected from peri-urban agricultural areas along the Hudiana drain, Lahore (Fig. 1) during 2008. The water of agricultural lands along the Hudiana drain, Lahore, was contaminated by sewage and industrial effluents and contained heavy metals and toxic chemicals. During 2008 fungi were isolated and preserved for further detailed investigation of heavy metal tolerance.

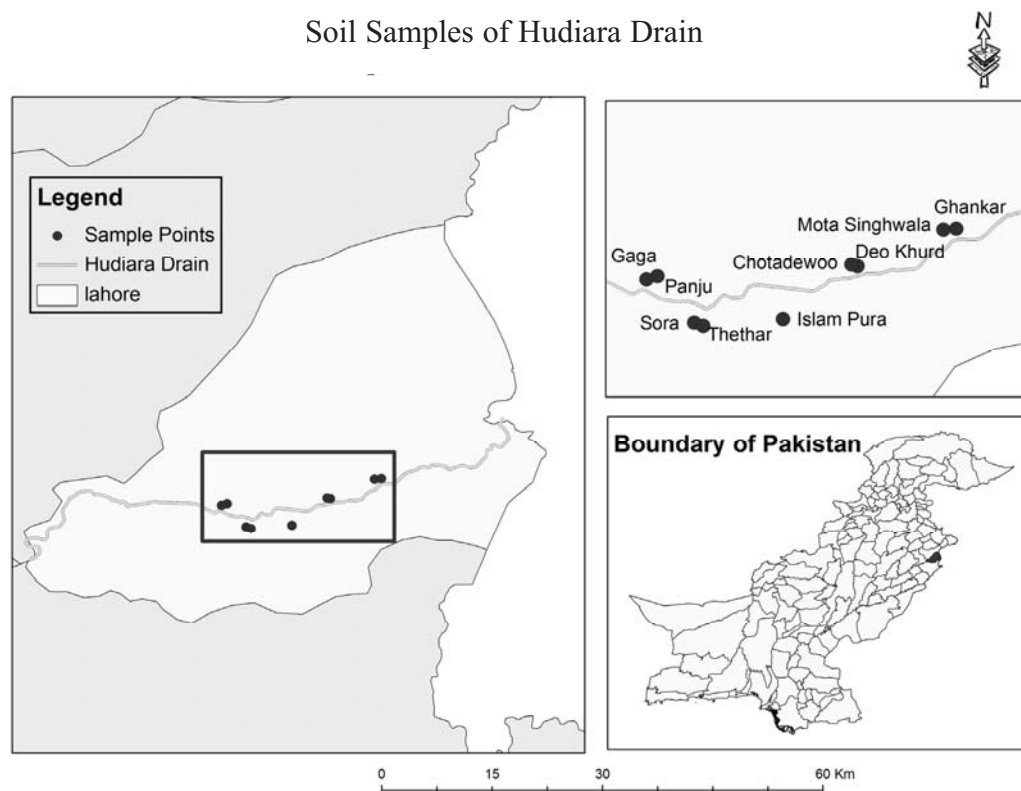


Fig. 1. Map showing the sites of soil samples along the Hudiana drain, Lahore.

Sterilization of Apparatus

Petri plates, media bottles, distilled water, McCartney bottles and syringes were sterilized in autoclave. For sterilization purpose all apparatus were autoclaved for 40 minutes at 121°C. After autoclaving all sterilized material were dried at 95°C.

Media Preparation

Potato dextrose agar (PDA) media was used for fungal cultures revival. Potatoes (200 g) were peeled, sliced, and boiled, and then sieved through a clean muslin cloth to get a broth to which agar (7.5 g) and dextrose sugar (7.5 g) was added. The media was then autoclaved for 30 minutes at 121°C [17].

Preparation of Plates

The media was poured in Petri-dishes and allowed to solidify for 24 hours. To suppress bacterial growth, 30 mg/l of streptomycin was added. Once the agar was solidified we then put plates in an inverted position for 24 hours at room temperature [18, 19].

Isolation and Identification of Fungal Isolates

Potato dextrose agar (PDA) media (1 liter) was used for the isolation of fungi [17]. The soil samples were processed with isolation procedure using the soil dilution plate method [18]. After incubation distinct colonies were counted and identified. The cultures were identified on the basis of macroscopic (colonial morphology, color, texture, shape, diameter and appearance of colony) and microscopic characteristics (septation in mycelium, presence of specific reproductive structures, shape and structure of conidia and presence of sterile mycelium). Pure cultures of fungi isolates were identified with the help of literature [20, 21].

Metal Tolerance Test for Fungi

Fungal strains, including *Aspergillus niger*, *Aspergillus flavus*, *Fusarium solani*, and *Penicillium chrysogenum*, were tested for their tolerance against different concentrations of heavy metals Cr(NO₃)₃ and Pb(NO₃)₂. Potato dextrose agar media was used for heavy metal resistance experiment. The different concentrations (0, 200, 400, 600, 800, and 1000 mg/l) of heavy metals Cr(NO₃)₃ and Pb(NO₃)₂ were used for the selection of fungi. Incubation was conducted at 29°C for one week [3]. The growth was monitored by measuring the culture from the point of inoculation or centre of the colony. Tolerance fungi were studied by the determination of tolerance index and minimum inhibitory concentration (MIC) [22].

Heavy Metals Analysis of Soil

Each soil sample (1 g) was taken in the conical flask (50 ml), added 10ml of HNO₃:HClO₄ (1:2) solution (50 ml),

and heated for half an hour. Solutions were filtered through Whatman 1 filter paper and volume was made to 50ml by adding distilled water. Soil samples were digested in triplicates and analyzed for Zn, Cd, Cr, Cu, Ni, and Pb. The blank was prepared for quality assurance of samples. The blank sample contained 10 ml of HNO₃:HClO₄ (1:2) solution and heated for half an hour, and volume was made 50 ml by adding distilled water. For the determination of heavy metals the atomic absorption spectrophotometer was powered on and warmed up for 30 minutes. After the heating of cathode lamp, the air acetylene flame was ignited and the instrument was calibrated or standardized with different working standards [23].

Statistical Analysis

The experiments were set up with three replicates. Analysis of variance was performed by using statistical software (SPSS 17) to compare resistance to metal among individual isolates.

Results and Discussion

Long-time exposure of soil fungi to heavy metals can lead to physiological adaptation or considerable modification of their microbial populations, reducing their activity and their number, and such changes may be associated with increased metal sorption capacity [24]. In the present study, various filamentous fungi were isolated from peri urban agriculture field soil, where heavy metals and other pollutants have been emitted in industrial effluents and sewage water for several years. The heavy metals content of soil samples is listed in Table 1.

Long-time reception of industrial effluents is the main reason for the high heavy metal content in the soil. Different species of fungi were isolated and identified from the collected 26 soil samples. Table 2 shows the diversity of fungi in the soil samples of Hudiara drain Lahore.

Fungi isolated belonged to the genera *Aspergillus*, *Penicillium*, and *Fusarium*. Species of the genus *Aspergillus* were the most abundant in all the sites. The occurrence of these genera in heavy metal-polluted soil has been reported in different parts of the world [10].

Table 1. Concentration of Heavy Metals in Soil (mg/kg).

Heavy Metals	Concentration			
	Mean	S.D	Min-Max	Median
Pb	68.4	±16.2	51.3-106.1	63.9
Cd	2.6	±0.9	1.1-4.0	2.5
Cr	90.6	±39.8	44.9-135.8	90.2
Cu	94.5	±21	67.1-127.9	92.2
Ni	55.8	±13.3	42.2-82	52.8
Zn	108.0	±42.8	51.4-169.6	104.7

Table 2. Diversity of Fungi in the soil samples of Hudiaara Drain Lahore.

Fungi	Sample Code
<i>Aspergillus flavus</i>	*S1, S2, S3, S4, S5,S6, S17, S18, S19, S20, S21, S23, S24
<i>Aspergillus niger</i>	S7, S8, S9, S10, S11, S12, S13, S14, S15, S16
<i>Penicillium chrysogenum</i>	S22
<i>Fusarium solani</i>	S25, S26

*S for Sample

The differences between the sampled sites regarding their richness on microbial isolates appear to be closely linked to the degree of heavy metal pollution. Generally, pollution of soil by heavy metals may lead to a decrease in microbial diversity due to the extinction of species sensitive to the stress imposed. Sometimes enhanced growth of some species take place, which means the presence of resistant species like *Aspergillus* isolates, which also was observed in the presence of chromium and lead (Table 3). Similarly, Levinskaite [25] studied the response of soil fungi to chromium and found the metal resistant and sensitive to isolates.

The table shows that *Aspergillus flavus* and *Aspergillus niger* isolates were most tolerant to metal concentrations of chromium, but different strains of *Aspergillus flavus* were sensitive to lead. On the other hand, different isolates of *Aspergillus niger* were tolerant against lead. Similar results were reported by Ezzouhri et al. [26] about different isolates of *Aspergillus niger* having a high tolerance index for lead and chromium.

As far as *Fusarium* isolates were concerned, they were moderately resistant to the presence of metal ions in the growth medium, except the one (S25) that was quite sensitive to concentrations of lead. Sanyal et al. [27] reported that lead ions are not toxic to the fungus *Fusarium oxysporium*, which readily grows after exposure to metal ions. Similarly, *Penicillium chrysogenum* was found resistant to chromium but sensitive to lead. This means the level of resistance differed among different isolates. Similar results were reported by Baldrian and Gabriel [28], who found that various strains of fungi, originating from metal-contaminated sites did not have the same level of tolerance. The most probable reason for the difference in resistance levels could be the variation in the mechanism of resistance [26, 29]. Statistical analysis showed the diversity in heavy metal tolerance of different isolates (Figs. 2 and 3). Similar results were reported by other researchers [15, 22, 26]. The resistance against individual metals was much more dependent on the isolate than on the sites of its isolation [30]. Mo et al. [31] also found comparable tolerance rates for isolates originating from metal-contaminated and uncontaminated sites regardless of the concentration of the contaminant in the medium.

Major differences in Cr and Pb tolerance have been found among different isolates. The variation in the metal tolerance may be due to the presence of different types of tolerance processes or resistance mechanisms exhibited by

Table 3. Tolerance index of isolated micro-organisms able to grow on PDA with 1000 mg/l of metal.

Isolates	Metallic Cations	
	Cr	Pb
<i>Aspergillus flavus</i> (S1)	0.91	0.18
<i>Aspergillus flavus</i> (S2)	0.76	0.16
<i>Aspergillus flavus</i> (S3)	1	0.25
<i>Aspergillus flavus</i> (S4)	1.3	0.21
<i>Aspergillus flavus</i> (S5)	1	0.35
<i>Aspergillus flavus</i> (S6)	0.76	0.17
<i>Aspergillus niger</i> (S7)	0.84	0.55
<i>Aspergillus niger</i> (S8)	0.89	0.67
<i>Aspergillus niger</i> (S9)	1.2	0.8
<i>Aspergillus niger</i> (S10)	0.93	0.25
<i>Aspergillus niger</i> (S11)	0.75	0.7
<i>Aspergillus niger</i> (S12)	1.5	0.8
<i>Aspergillus niger</i> (S13)	0.78	0.68
<i>Aspergillus niger</i> (S14)	1	0.7
<i>Aspergillus niger</i> (S15)	0.68	0.8
<i>Aspergillus niger</i> (S16)	1	0.82
<i>Aspergillus flavus</i> (S17)	0.6	0.17
<i>Aspergillus flavus</i> (S18)	0.7	0.35
<i>Aspergillus flavus</i> (S19)	0.7	0.25
<i>Aspergillus flavus</i> (S20)	0.6	0.31
<i>Aspergillus flavus</i> (S21)	0.8	0.33
<i>Penicillium chrysogenum</i> (S22)	0.8	0
<i>Aspergillus flavus</i> (S23)	0.7	0.3
<i>Aspergillus flavus</i> (S24)	0.63	0.73
<i>Fusarium solani</i> (S25)	0.6	0.35
<i>Fusarium solani</i> (S26)	0.5	0.51

different isolates. The detoxification of chromium by *Aspergillus niger* may be mediated by an enzymatic antioxidant system such as peroxidase, catalase, and ascorbate peroxidase [32]. From this preliminary test, heavy metal-resistant filamentous fungi were selected and the minimal inhibitory concentration (MIC) to Cr and Pb was determined.

The MICs of the Cr and Pb against the studied fungal isolates are shown in Fig. 4. In the presence of heavy metal relative to the control, the growth rate of the fungi exhibited a lag, retarded, similar, and enhanced rates of growth. The growth pattern appears to suggest tolerance development or adaptation of the fungi to the presence of heavy metals [33].

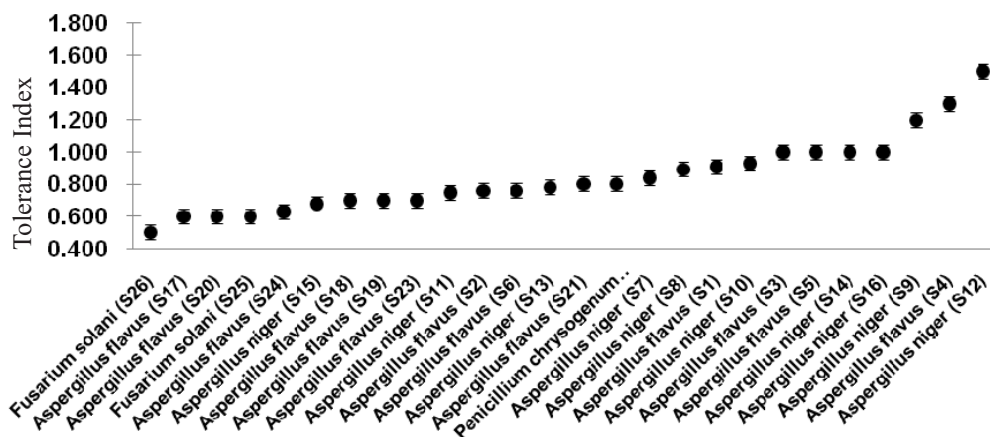


Fig. 2. Chromium tolerance index among isolates.

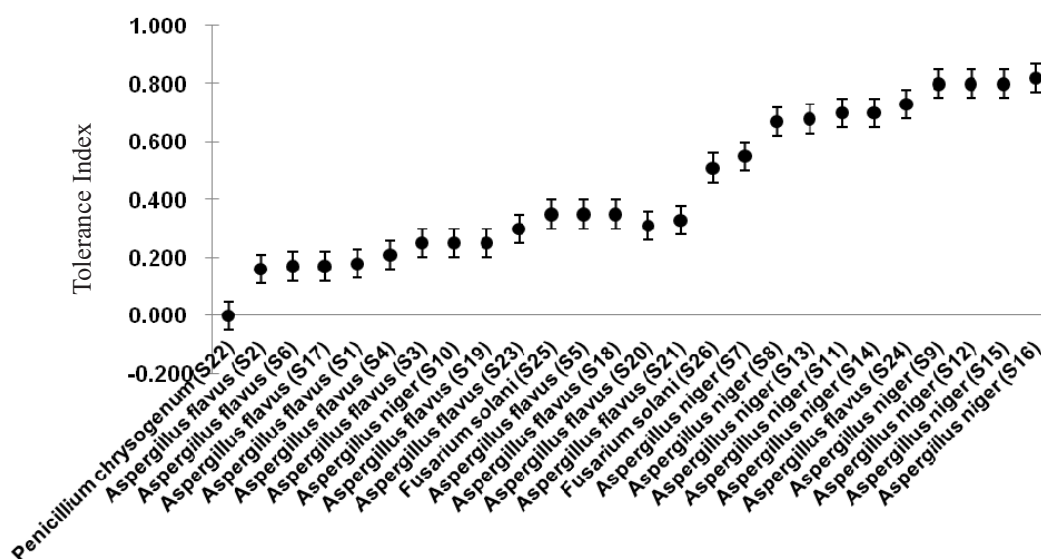


Fig. 3. Lead tolerance index among isolates.

At lower metal ions concentrations, the tested fungal isolates were very resistant and exhibited strong growth. Higher metal ion concentrations caused a reduction in growth and increased the length of the lag phase compared to the control. A reduction in the growth rate is a typical response of fungi to toxicants [10], whereas the lengthening of the lag phase is not always present. The same increment and reduction in growth was observed during study on filamentous fungi, which belonged to the genera *Aspergillus*, and were more resistant to Cr at higher metal concentrations, and suddenly the growth pattern changed [34].

Chromium is released during industrial processes such as leather tanning and pigment manufacture [35, 36]. Among all isolates studied, the most tolerant isolate belonged to the genus *Aspergillus*, with a MIC of 200 to 400 mg/l. Similar results were reported by Price et al., [37], who showed that *Aspergillus* was better to grow or tolerate heavy metals as compared to other fungi. *Penicillium* and *Fusarium* isolates were less tolerant to chromium (up to 100 mg/l). Bader [38] found that *Monilia* sp. and *Penicillium* sp. showed high resistance to Cr (up to 520 mg/l). The growth rate of fungi tested was reduced. A similar result was reported

in the study of Levinskaite [25], where growth and conidiogenesis of *T. viride* and *P. chrysogenum* were slowed down at 600 mg/l Cr in the medium.

Lead ions appeared more toxic in comparison with the chromium. Different isolates of genera *Aspergillus flavus*, *Penicillium*, and *Fusarium* showed less MIC. Atuanya and Oseghe [39] found that higher concentrations of lead were

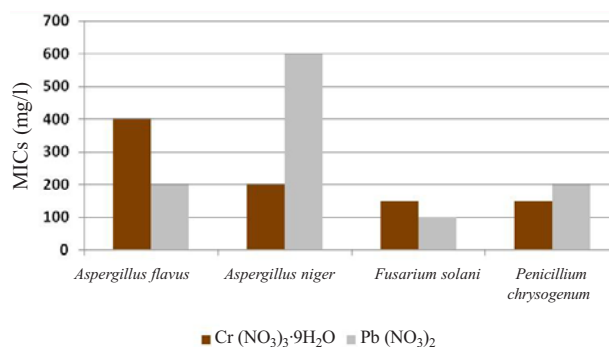


Fig. 4. Minimal inhibitory concentrations (MICs) of fungal isolates.

toxic for bacteria and fungi. Isolates of *Aspergillus niger* showed a difference in their tolerance to metals; however, the growth of *Aspergillus niger* isolates on agar media containing a high lead concentration was higher as compared to other isolates. This is probably due to a period of adaptation where cells of the *Aspergillus niger* isolate synthesized some enzymes essential for the uptake of lead [40].

The results obtained affirmed that the response of the isolates to heavy metals depend on the metal tested, its concentration in the medium, and on the isolate considered. The results of the present study were comparable with those reported by [15, 16, 41-46]. The toxicity may be presented differently, depending on the isolate and its site of isolation. Some isolates were tolerant, while others reacted negatively even at low metal ion concentrations. This could be explained by the heterogeneity of pollution in the location from which the tested isolates originated. However, although some authors found that micro-organisms isolated from contaminated sites were more tolerant than those from natural environments [43], some studies did not confirm this [31, 47, 48]. They reported very little differences in metal tolerance between strains from polluted and unpolluted sites. The resistance of isolates appeared could be correlated with the sites of their isolation. Heavy metal analysis of the soil showed higher concentration levels of chromium and lead present in the soil and the Cr and Pb resistant species as calculated by MIC were abundantly present in the soil. Various genera and also isolates of the same genus did not necessarily have the same heavy metal tolerance. The variation in the metal tolerance may be due to the presence of one or more strategies of tolerance or resistance mechanisms exhibited by fungi. It must also be taken into account that the contamination at the polluted sites is usually caused by a combination of metals and that the selection is probably driven either by the most toxic element or by more different metals acting synergistically [28]. Gadd and Sayer [49] reported that the microbiota isolated from co-contaminated environments could exhibit resistance to more than one ion and, consequently, co-tolerance may be a common natural response.

Findings of the present study indicate that fungal populations isolated from heavy metal-contaminated sites have the ability to resist higher concentrations of metals. The tolerance and the resistance of the isolates depended much more on the fungus tested than on the sites of its isolation. This variation may be explained by the development of tolerance or adaptation of the fungi to heavy metals. *Aspergillus* isolates were the most resistant to the metals tested, which may make them promising candidates for further investigations regarding their ability to remove metals from contaminated environments.

Conclusion

In this study, chromium- and lead-resistant fungi were isolated from contaminated environments with high metal content. The results showed that the fungal population iso-

lated from heavy metal-contaminated sites has the ability to resist higher concentrations of metals. The tolerance and resistance of the isolates depended much more on the fungus tested than on the site of its isolation. This variation may be explained by the development of tolerance and adaptation of the fungi to heavy metals. Among different fungal isolates, *Aspergillus niger* was the most resistant to all the metals tested, which make them promising candidates for further investigations regarding their ability to remove metals from a contaminated environment and they can be used as bioremediation agents.

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