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# Heavy metal tolerance of filamentous fungal strains isolated from soil irrigated with industrial wastewater

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In the present study the tolerance level of different fungi (*Aspergillus flavus, Aspergillus niger, Aspergillus versicolor, Scopulariopsis* sp., *Curvularia* sp., *Helminthosporium* sp., *Humicola grisea* sp., *Nannizzia* sp., *and Fusarium* sp.) were investigated towards heavy metals which were isolated from contaminated peri-urban agricultural soils of Faisalabad.

The concentration of heavy metals in soil is determined by using atomic absorption spectrophotometer. The degree of tolerance of fungi was measured by minimum inhibitory concentration in the presence of different concentrations of heavy metals (Cr and Pb) and compared to control sample. Tolerance analysis depicts that growth rate of fungal isolates decreased with increase in concentrations and few isolates are tolerant, some are moderately tolerant and some are sensitive towards metal concentrations of Cr and Pb. Most of the isolates were tolerant to metals. Among all tested fungal strains, few isolates of *Aspergillus flavus* and *Aspergillus niger, Fusarium* were tolerant to Cr and Pb. Thus these tolerant isolates can be used for the purpose of bioremediation in future.

**Key words:** fungi, heavy metal, tolerance, chromium, lead

## INTRODUCTION

Heavy metals are highly persistent pollutants in the environment and are known to alter soil ecosystem diversity, structure, and function (Baath, 1989; Garland, Mills, 1991). Like all other elements, heavy metals are required by different environmental and biological components in definite proportions. With the advance of industrial revolution, however, their quantities have been increased (Bonaventura, Johnson, 1997) which is a great threat to environment, public and soil health (Zafar et al., 2006). Excessive quantities of heavy metals should be removed from each and

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every component of the environment because they are extremely toxic for all life forms on earth (Kapoor et al., 1998). In recent years heavy metals have received attention because of their release into the environment from different industries like electroplating, leather tanning, wood preservation, pulp processing, steel manufacturing, etc (Congeevaram et al., 2006). Effluents from large industrial activities contaminate the water that is being utilized for the irrigation of the nearby agricultural soil, which is a common practice in arid and semiarid regions and it is used as a readily available and inexpensive option to fresh water. Rough estimates indicate that at least 20 million hectares in 50 countries are irrigated with raw or partially treated wastewater (Mahmood, Maqbool, 2006). Heavy metals do not decay naturally and thus pose a challenge for their remediation (Bai, Abraham, 2003). Microorganisms can survive in all environments because of their innate ability to take up the pollutants as nutrients like heavy metals due to absorptive / accumulative capability. Soil microorganisms are known to play a key role in the mobilization and immobilization of metal cations, thereby changing their availability to plants (Birch, Bachofen, 1990).

In natural ecosystems they often encounter not one but several heavy metals. Tolerance of microorganisms towards heavy metals is their basic quality which proves them to be utilized for the reclamation of heavy metal contaminated soils (Gadd, Griffiths, 1978). Fungi are reported to exhibit considerable tolerance towards heavy metals and become dominant organisms in some polluted habitats. Melanins which are dark pigments located in the fungal cell wall (but also exist as extracellular polymers) can reduce the toxic effect of heavy metals due to the presence of various groups (Fogarty, Tobin, 1996; Ledin, 2000). This property is of great importance to organisms growing in polluted habitats and for a possible binding of heavy metals in natural environments as well as for their removal from waste waters and other aqueous substrata. Therefore, fungal tolerance towards a mixture of metals is of high importance both for fungal survival and their application for industrial purposes (Levinskaite, 2001).

The tolerance of some fungi to a variety of heavy metals is well documented (Hashem, Bahkali, 1994). In some cases it produces intracellular / extracellular enzymes to resist the metal concentration or they possess the processes of valence transformation, active uptake, complexation, crystallization and biosorption to cell walls (Zafar et al., 2006). Species like *Penicillium, Aspergillus*, *Pseudomonas*, *Sporophyticus*, *Bacillus*, *Phanerochaete*, etc are found to be very useful for the removal of heavy metals such as chromium and nickel (Congeevaram et al., 2006). Different species of *Aspergillus* have been reported as efficient heavy metals reducers (Congeevaram et al., 2006). *A. niger* has been reported to efficiently remove uranium ions while *Aspergillus oryzae* has been used to remove cadmium and copper ions from aqueous solutions (Kapoor et al., 1998). The aim of the present study was to check the tolerance level of different fungi isolates against heavy metals. For this study, soil samples were collected from peri-urban agricultural areas of Chahkayra Chak, Faisalabad which are irrigated by the contaminated industrial water. Fungi were isolated from the contaminated soil and studied for their tolerance analysis.

## MATERIALS AND METHODS

### Sampling and sampling site

The main purpose of the present study was to evaluate the tolerance potential of different isolated fungal strain towards heavy metals (Pb, Cr). For this purpose, soil samples were collected from peri-urban agricultural areas of Chakera Chack, Faisalabad (Fig. 1). The agriculture land of Chakera Chack is contaminated by sewage and industrial effluents from the nearby industries and contains heavy metals and toxic chemicals. During 2008 fungi were isolated and preserved for further detail investigation of heavy metal tolerance.

### Sterilization of appliances

Petri plates, media bottles, distilled water, McCartney bottles and syringes were sterilized in autoclave. For sterilization purposes all appliances were autoclaved for 40 min at 121 °C. After autoclaving all sterilized materials were dried in oven at 95 °C.

### Media preparation and fungal isolation

Potato Dextrose Agar (PDA) media was used for the isolation of fungi. For the preparation of PDA, potatoes (200 g) were peeled, sliced and boiled,

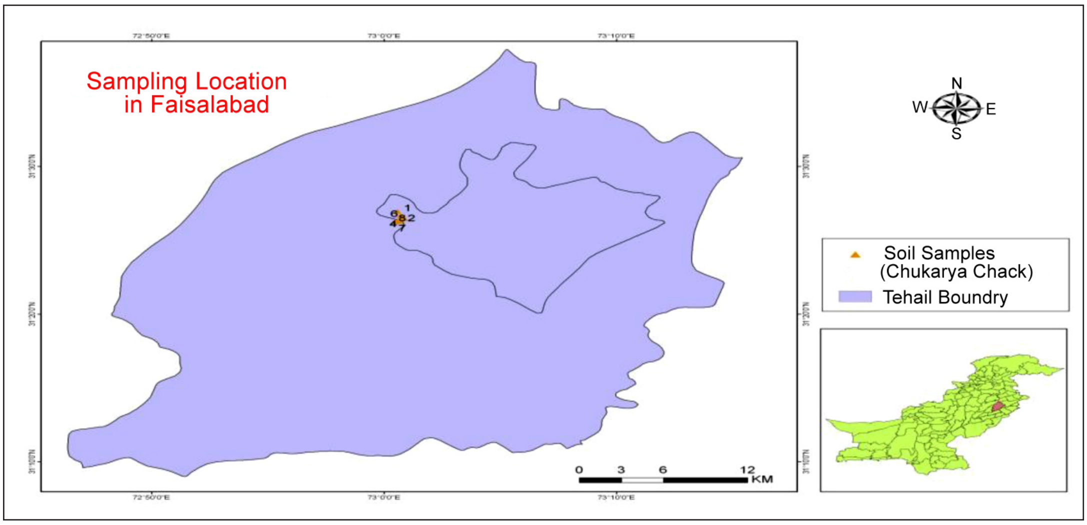


**Fig. 1.** Soil samples collection from the peri-urban areas of Faisalabad

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 min at 121 °C (Martin, 1995). Fungi were isolated on PDA by soil dilution method (Razak et al., 1999).

### Preparation of plates

Fungi were isolated on PDA by soil dilution method (Razak et al., 1999). The media was poured into Petri-dishes and allowed to solidify for 24 hours. To suppress the bacterial growth, 30 mg/lit of streptomycin was added to the medium (Martin, 1950). Once the agar solidified, plates were put in an inverted position for 24 hours at room temperature (Martin, 1995).

### Identification of fungi

The fungal cultures were identified on the basis of macroscopic (colonial morphology, color, texture, shape, diameter and appearance of colony) and microscopic (septation in mycelium, presence of specific reproductive structures, shape and structure of conidia, and presence of sterile mycelium) characteristics (Zafar et al., 2006). Pure cultures of fungi isolates were identified with the help of literature (Domsch et al., 1980; Barnett, Hunter, 1999).

### Heavy metal tolerance test experiment

For the evaluation of tolerance potential among isolated fungal strains, PDA medium was prepared and amended with various concentrations (0, 200, 400, 600, 800 and 1 000 mg/l) of heavy metal{$Cr(NO_3)_3 9H_2O$ and $Pb(NO_3)_2$} and 5.6 pH was maintained by adding 5 molar solution of NaOH. Media was autoclaved for 20 min at 121 °C and poured into Petri plates. The plates were incubated at 29 °C for 7 days. The growth of fungi was monitored from the point of inoculation or centre of the colony. Tolerance was measured by observing minimum inhibitory concentration (MIC) and tolerance index.

### Heavy metals analysis of soil

Each soil sample (1 g) was taken in the conical flask (50 ml) with addition of 10 ml of $HNO_3$:$HClO_4$ (1:2) solution (50 ml) and heated for half an hour. Solutions were filtered through Whatman 1 filter paper and volume was made up to 50 ml 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 $HNO_3$:$HClO_4$ (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 min. After the heating of cathode lamp, air acetylene flame was ignited and instrument was calibrated or standardized with different working standards (Vanloon, Lichwa, 1973).

### 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

In the present study, the tolerance potential of various filamentous fungi like *Aspergillus flavus, Aspergillus niger, Aspergillus versicolor, Scopulariopsis, Curvularia* sp., *Helmintho sporium, Humicola grisea, Nannizzia* sp., and *Fusarium* sp. were investigated against heavy metals (Cr and Pb).The fungal isolates (Table 1) were taken from the agricultural field of Faisalabad (Chakera Chack) irrigated / treated by industrial effluents. Application of domestic and industrial effluents to nearby agricultural fields is a common practice of irrigation and wastewater disposal in Pakistan (Lone et al., 2000; Ensink et al., 2004). When wastewater is applied to agricultural fields, heavy metals enter the soil and get fixed to the soil components. Thus continuous application of wastewater tends to accumulate large quantities of heavy metals in soil (Table 2), which persist there for an indefinite period to have long lasting effects in the soil. Fungi are natural inhabitants of soil and the occurrence of various fungi such as *Humicola grisea, Fusarium* sp., *Nannizzia* sp., *Curvularia* sp. and *Helementho sporium* in polluted soil with heavy metals (Cr, Pb, Zn, Cd) has been reported by other scientists from different parts of the world (Babich, Stozky, 1985). Soil fungi are able to grow in the presence of heavy metals due to physiological adaptation (Gadd, 1993) and such adaptation may be associated with increased metal sorption capacity. A range of fungi from all major taxonomic groups may be found in metal-polluted habitats and the ability to survive and grow in the presence of potentially toxic concentrations is frequently encountered (Ross, 1975; Gadd, Edwards, 1986). Describing the ability to grow at high metal concentrations, Wainwright and Gadd (1997) distinguished fungi tolerant to heavy metals.

The results of the present study demonstrated that different species of fungus show different tolerance pattern (Table 3). Some were sensitive, moderately tolerant and tolerant but they behaved differently against different metals. Few isolates of *Aspergillus flavus* (F7and F55) were more tolerant for both metals (Cr, Pb) while F43 and F47 were very sensitive to Cr but tolerant to Pb (Fig. 2).

**Table 1.** List of fungi isolated from contaminated soil of Faisalabad

| Fungi | Code No. |
|---|---|
| *Aspergillus flavus* | F2, F3, F4, F5, F7, F9, F10, F16, F17, F18, F19, F20, F21, F29, F32, F33, F34, F 36, F43, F44, F47, F55 |
| *Aspergillus niger* | F14, F23, F24, F25, F28, F31, F37, F38, F40, F41, F56, F57, F60 |
| *Aspergillus versicolor* | F11, F12, F15 |
| *Scopulariopsis* sp. | F49 |
| *Humicola grisea* | F1, F6, F22 |
| *Fusarium* sp. | F26, F46, F51, F53, F54, F61 |
| *Nannizzia* sp. | F13, F27, F42, F59 |
| *Curvularia* sp. | F45 |
| *Helementho sporium* | F58 |

**Table 2.** Concentration of heavy metals in soil (mg/kg)

| Heavy Metals | Concentration | | | |
|---|---|---|---|---|
| | Mean | S. D. | Min-Max | Median |
| Pb | 63.9 | ±12.3 | 54.0–87.9 | 60.1 |
| Cd | 2.7 | ±0.4 | 2.3–3.4 | 2.6 |
| Cr | 76.9 | ±28.0 | 46.9–122.4 | 71.1 |
| Cu | 70.3 | ±26.1 | 37.5–110.1 | 73.0 |
| Ni | 46.9 | ±13.7 | 31.6–66.9 | 45.0 |
| Zn | 105.6 | ±34.4 | 42.5–140.7 | 117.2 |

**Table 3.** Tolerance index of fungal isolates

| Fungal Isolates | Cr | Pb | Fungal Isolates | Cr | Pb |
|---|---|---|---|---|---|
| *Aspergillus flavus* (F2) | 0.59 | 0.11 | *Aspergillus niger* (F14) | 0.38 | 0.67 |
| *Aspergillus flavus* (F3) | 0.59 | 0.24 | *Aspergillus niger* (F23) | 0.19 | 0.55 |
| *Aspergillus flavus* (F4) | 0.51 | 0.27 | *Aspergillus niger* (F24) | 0.17 | 1.10 |
| *Aspergillus flavus* (F5) | 0.70 | 0.25 | *Aspergillus niger* (F25) | 0.34 | 0.47 |
| *Aspergillus flavus* (F7) | 1.05 | 0.80 | *Aspergillus niger* (F28) | 0.21 | 0.69 |
| *Aspergillus flavus* (F9) | 0.86 | 0.63 | *Aspergillus niger* (F31) | 0.12 | 0.83 |
| *Aspergillus flavus* (F10) | 0.73 | 0.64 | *Aspergillus niger* (F37) | 0.21 | 0.13 |
| *Aspergillus flavus* (F16) | 0.79 | 0.55 | *Aspergillus niger* (F38) | 0.18 | 0.79 |
| *Aspergillus flavus* (F17) | 0.80 | 0.68 | *Aspergillus niger* (F40) | 0.17 | 0.90 |
| *Aspergillus flavus* (F18) | 0.78 | 0.61 | *Aspergillus niger* (F41) | 0.69 | 0.59 |
| *Aspergillus flavus* (F19) | 0.70 | 0.60 | *Aspergillus niger* (F56) | 0.17 | 0.94 |
| *Aspergillus flavus* (F20) | 0.00 | 0.50 | *Aspergillus niger* (F57) | 0.28 | 0.62 |
| *Aspergillus flavus* (F21) | 0.38 | 0.36 | *Aspergillus niger* (F60) | 0.25 | 0.55 |
| *Aspergillus flavus* (F29) | 0.11 | 0.26 | *Nannizzia* sp. (F13) | 0.15 | 0.37 |
| *Aspergillus flavus* (F32) | 0.12 | 0.36 | *Nannizzia* sp. (F27) | 0.27 | 0.18 |
| *Aspergillus flavus* (F33) | 0.14 | 0.47 | *Nannizzia* sp. (F42) | 0.24 | 0.18 |
| *Aspergillus flavus* (F34) | 0.20 | 0.42 | *Nannizzia* sp. (F59) | 0.24 | 0.15 |
| *Aspergillus flavus* (F36) | 0.00 | 0.39 | *Scopularoisis* sp. (F49) | 0.01 | 0.25 |
| *Aspergillus flavus* (F43) | 0.00 | 0.81 | *Humicola grisea* (F1) | 0.51 | 0.15 |
| *Aspergillus flavus* (F44) | 0.00 | 0.42 | *Humicola grisea* (F6) | 0.37 | 0.25 |
| *Aspergillus flavus* (F47) | 0.00 | 0.73 | *Humicola grisea* (F22) | 0.14 | 0.25 |
| *Aspergillus flavus* (F55) | 1.01 | 0.60 | *Fusarium* sp. (F26) | 0.26 | 0.09 |
| *Aspergillus versicolor* (F11) | 0.04 | 0.00 | *Fusarium* sp. (46) | 0.48 | 0.10 |
| *Aspergillus versicolor* (F12) | 0.00 | 0.39 | *Fusarium* sp. (F51) | 0.85 | 0.10 |
| *Aspergillus versicolor* (F15) | 0.00 | 0.09 | *Fusarium* sp. (F53) | 0.41 | 0.08 |
| *Curvularia* sp. (F45) | 0.44 | 0.09 | *Fusarium* sp. (F54) | 0.45 | 0.18 |
| *Helementhosporium* (F58) | 0.42 | 0.25 | *Fusarium* sp. (F61) | 0.47 | 0.13 |

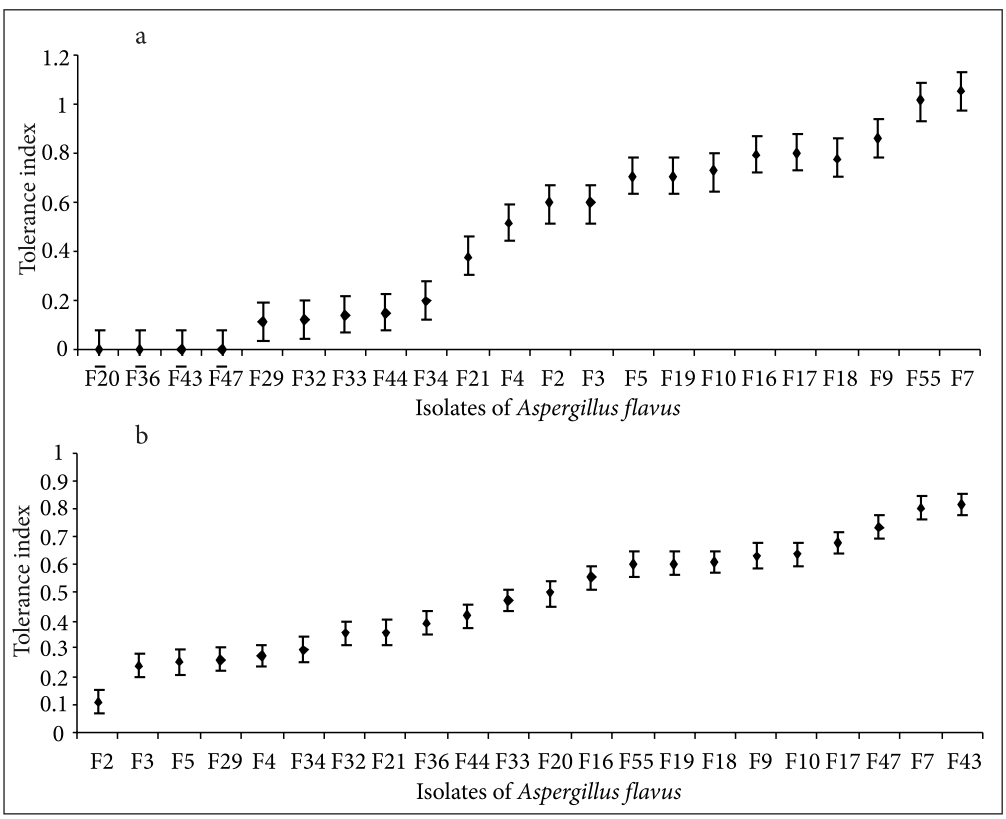


**Fig. 2.** Tolerance index of all isolates of *A. flavus* against chromium (a) and lead (b)

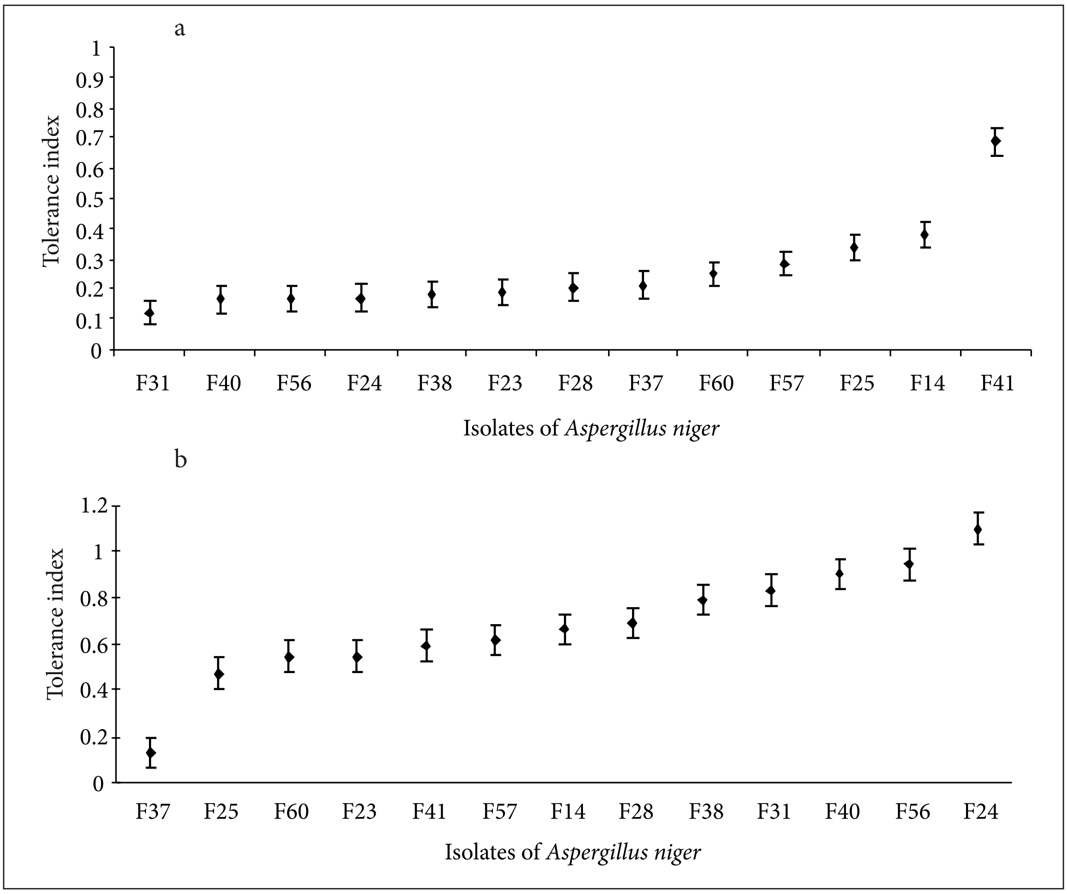


**Fig. 3.** Tolerance index of all isolates of *A. niger* against chromium (a) and lead (b)

In the same way the isolates of *Aspergillus niger* (F24, F40, and F56) were tolerant to Cr but sensitive to Pb and F41 are moderately tolerant to both metals (Cr, Pb) as shown in Fig. 3. *Humicola grisea* (F1) are moderately tolerant against both metals but other isolates are sensitive. *Curvularia* show moderate tolerance (0.51) against chromium and sensitive to lead (0.15) and *Helminthosporium* sp. show resistance towards chromium (F58 is 0.42 tolerant) than lead, while *Fusarium* sp. is not more tolerant, as only one isolate (F51) shows 0.85 against chromium while it is sensitive against lead and all other isolates were sensitive for both metals as shown in Table 3. *Nannizzia* sp., *Scopulariopsis* and *Aspergillus versicolor* sp. show 0.37, 0.25 and 0.39 sensitiveness against metals (Figs. 4 and 5).

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 not caused by a single metal and that the selection is probably driven either by the most toxic element or by more different metals acting synergistically (Baldrian, Gabriel, 2002).

Most of the isolates have high resistance against metal up to 1 000 mg/L belonging to genera *Aspergillus, Fusarium, Humicola* and *Nannizzia* sp. Lead is more tolerated by the isolates of the *Aspergillus* genus while chromium was tolerated by other fungal strains as reported by Ezzouhri et al. (2009) and Valix et al. (2000).

The determination of MIC suggests that the resistance level against individual metals which were dependent on different fungal isolates (Fig. 6). It indicated that *A. niger* and *A. flavus* show more resistance against metals with MIC 800–1 000 mg/l than all other tested fungal strains as shown in Fig. 6. The effects of the heavy metals on the growth of the fungi were assessed on the basis of their growth curve. Table 3 shows the growth curve of all isolates at their MIC which clearly point out that *A. niger* and *A. flavus* show more resistance.

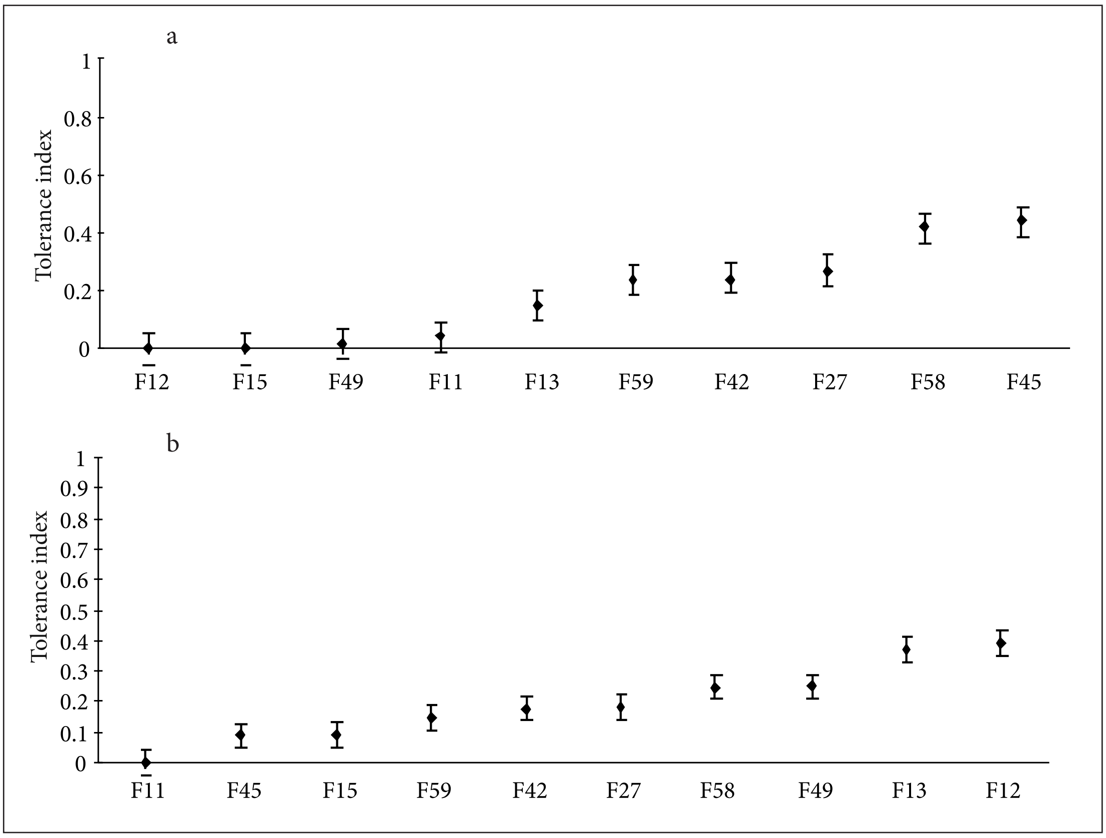


**Fig. 4.** Tolerance index of fungus of different isolates against chromium (a) and lead (b)

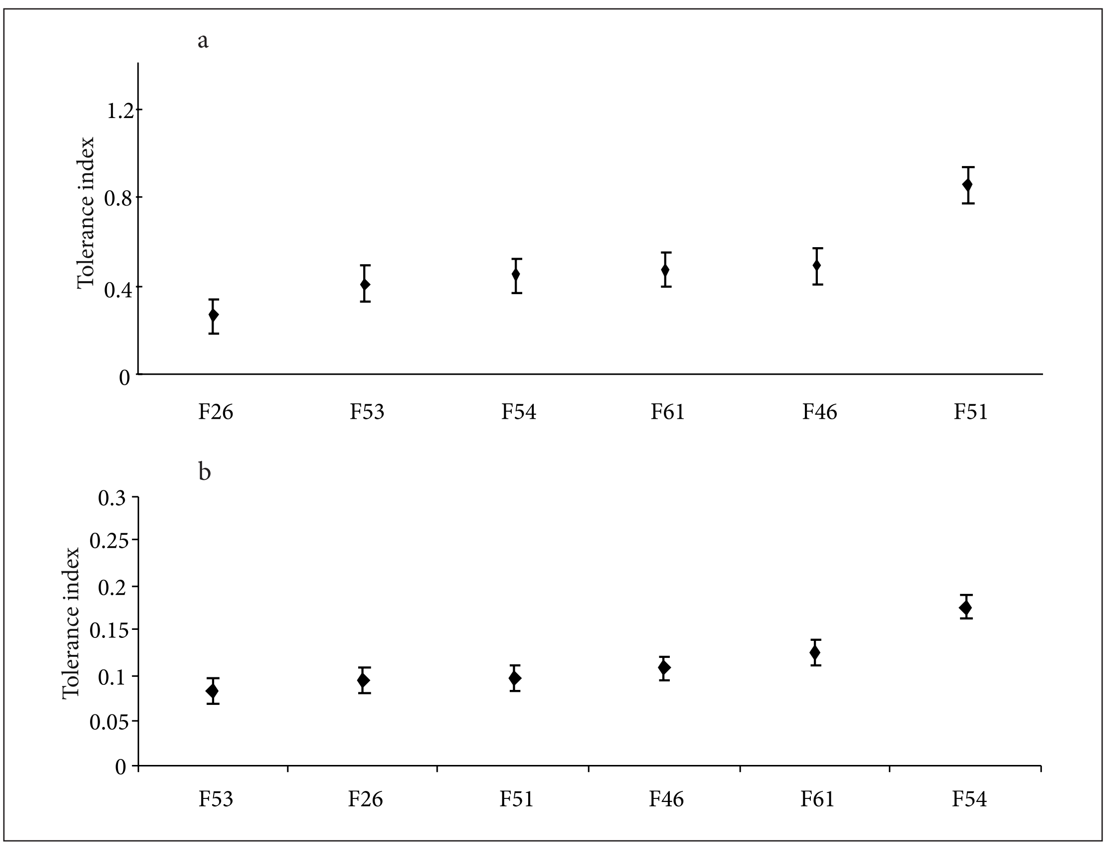


**Fig. 5.** Tolerance index of isolates of *Fusarium* against chromium (a) and lead (b)

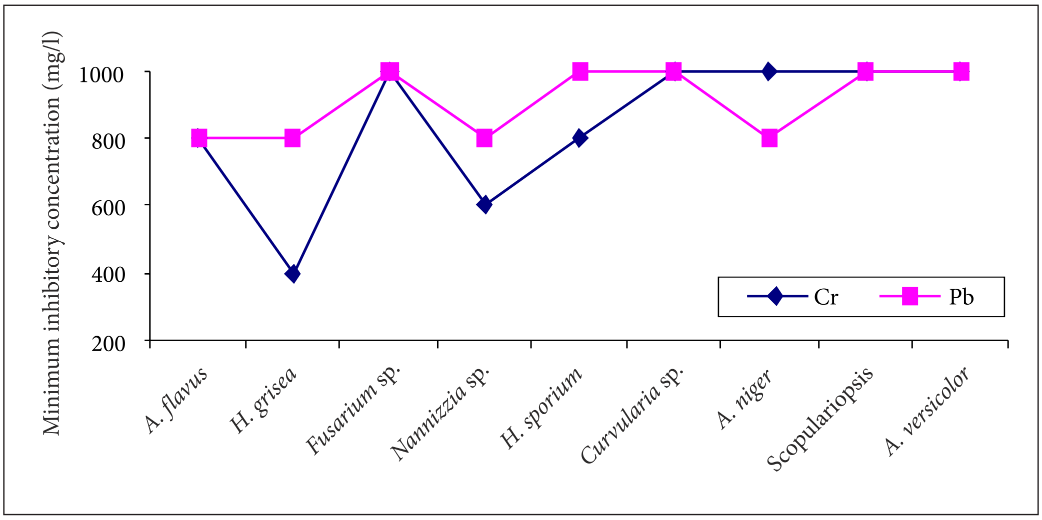


**Fig. 6.** MIC of all fungal isolates against metals

The growth phase of fungus was characterized by a lag, retarded, similar and enhanced rates compared to control which appear to reflect the tolerance development or adaptation of fungi in the presence of heavy metals (Hashem, Bahkali, 1994). All fungal strains exhibited growth at lower concentration of metals but it became reduced in the presence of higher concentration due to the increase in length of the lag phase as compared to control sample. The same increment and reduction in growth was noted during the study on filamentous fungi belonging to the genera *Aspergillus*, were more resistant to Cr at higher metal concentrations and suddenly the growth pattern changed (Valix et al., 2000). Species of fungi of the genera *Fusarium* and others have been isolated from contaminated soils, and their ability to tolerate the presence of different heavy metals has been analysed by Zafar et al. (2007). Ezzouhri et al. (2009) also screened fungi (*Fusarium* sp.) for their resistance to heavy metals. The results revealed that the majority of the isolates were resistant to Cr, Cu and Zn. The level of resistance depended on the isolate tested, as well as the site of its isolation. However, some authors found that microorganisms isolated from contaminated sites were more tolerant than those from natural environments (Massaccesi et al., 2002; Malik, 2004).

The results revealed that *A. niger* and *A. flavus* showed high level of resistance to both metals tested as compared to all other strains, which makes them more attractive potential candidates for further investigations regarding their ability to remove metals from contaminated sites. Our results were comparable with those reported by Badar et al., 2000; Verma et al., 2001; Bai, Abraham, 2003; Malik, 2004; Zouboulis et al., 2004; Yoshida et al., 2006.

## CONCLUSION

The present study concludes that soil irrigated with waste water contains tolerant fungi due to their physiological adaptation and they have greater potential for remediation by virtue of their aggressive growth; greater biomass, production and extensive hyphae reach in soil. Moreover, fungi have been widely used in bioremediation of industrially polluted soils and waters, specifically in the removal of hydrocarbons and heavy metals (Akhtar, Mohan, 1995; Khan, 2001; Potin et al., 2004). The results obtained confirmed that the response of isolates to heavy metals depended on the metal tested, its concentration in the medium and on the isolate under consideration.

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## SIŪLINIŲ GRYBŲ PADERMIŲ, IŠSKIRTŲ IŠ PRAMONINĖMIS NUOTEKOMIS DRĖKINAMO DIRVOŽEMIO, TOLERANCIJA SUNKIESIEMS METALAMS

*Santrauka*

Atlikti skirtingų grybų (*Aspergillus flavus, Aspergillus niger, Aspergillus versicolor, Scopulariopsis* sp., *Curvularia* sp., *Helminthosporium* sp., *Humicola grisea* sp., *Nannizzia* sp., *and Fusarium* sp.), išskirtų iš užteršto priemiesčio Faisalabad agronominio dirvožemio, tolerancijos sunkiesiems metalams tyrimai.

Sunkiųjų metalų koncentracija dirvožemyje buvo nustatyta atominės absorbcijos spektrofotometru. Matuojant tolerancijos lygį grybuose, pasinaudota minimalia inhibicijos koncentracija, esant skirtingoms sunkiųjų metalų koncentracijoms (Cr ir Pb), kuri buvo palyginta su kontroliniu pavyzdžiu. Tolerancijos analizė rodo, kad didesnės koncentracijos sumažino išskirtų grybų augimo greitį, kelios grybų padermės yra tolerantiškos, dalis jų yra vidutiniškai tolerantiški, o kai kurie yra jautrūs Cr ir Pb metalų koncentracijoms. Dauguma išskirtų grybų buvo tolerantiški metalams. Iš visų ištirtų grybų padermių *Aspergillus flavus* ir *Aspergillus niger, Fusarium* sp. buvo tolerantiški Cr ir Pb – taigi šie grybai gali būti panaudoti bioremediacijos tikslams ateityje.

**Raktažodžiai:** grybai, sunkieji metalai, tolerancija, chromas, švinas