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Metal tolerance potential of filamentous fungi isolated from soils irrigated with untreated municipal effluent

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Abstract

Considering the importance of filamentous fungi for bioremediation of wastewater and contaminated soils, this study was planned to investigate the metal tolerance potential of indigenous filamentous fungi. Nineteen fungal strains were isolated from soils irrigated with untreated municipal/industrial effluent using dilution technique and 10 prominent isolates were used for metal tolerance. The isolated fungal isolates were screened for metal tolerance index (MTI) at I mM cadmium (Cd), nickel (Ni) and copper (Cu) concentrations and for minimum inhibitory concentration (MIC) and metal tolerance by growing on potato dextrose agar plates amended with varying amounts of Cd, Cu and Ni. Seven out of 10 isolated fungi belonged to the genera Aspergillus and three belonged to Curvularia, Acrimonium and Pithyum. The results revealed that the order of tolerance of isolates for metals was Cd > Cu > Ni and Aspergillus sp. were more tolerant than other fungi. Tolerance ranged from 900 – 9218 mg L^{-1} for Cd, followed by 381 - 1780 mg L^{-1} for Cu and 293-1580 mg L^{-1} for Ni. The isolated fungi exhibiting great tolerance to metals (Cd, Cu and Ni) can be used successfully for bioremediation of metals from contaminated soil and wastewaters.

Key words: Heavy metal contamination, metal tolerance, minimum inhibitory concentration, tolerance index

Introduction

Release of untreated municipal/industrial effluent to arable lands and water bodies is a common practice in developing countries including Pakistan (Sigua *et al.,* 2005). The municipal/industrial effluent contains considerable amount of metals, i.e. cadmium (Cd), chromium (Cr), lead (Pb), nickel (Ni), copper (Cu) and contributes to the heavy metal content of soils (Mapanda *et al*., 2005; Nan *et al*. 2002; Ahmad *et al.,* 2013). Its long-term application can adversely affect soil health, fresh water resources and groundwater quality (Kahlown *et al.,* 2006).The heavy metals content of crops being irrigated with municipal/industrial effluent and shallow groundwater are high enough to cause clinical problems both to animals and human health (Ling *et al.*, 2007; McLaughlin *et al.*, 2000). Prolonged exposure to excessive metal concentration could pose a significant threat of carcinogenesis, neuralgia, encephalopathy, respiratory cancer, mutagenesis, and cardiovascular and gastrointestinal diseases (McCluggage 1991). Further surface soil is a rich habitat of all major groups of microorganisms, i.e., bacteria, actinomycetes, fungi, and algae and are natural recyclers. These microorganisms convert toxic organic and inorganic compounds to harmless products, often carbon dioxide and water.

Several chemical, biological and physical techniques are used for remediation of polluted soil and water. Conventionally, chemical (precipitation and neutralization) and physical (ion exchange, membrane separation, electro-dialysis and activated carbon adsorption) techniques are applied to remove heavy metals from wastewater and contaminated soils (Atkinson *et al.,* 1998). Currently, scientists are exploring the remediation potential of biological materials such as microbial and associated biota within the ecosystem and the technique is known as bio-remediation, i.e. degradation, accumulation and/or removal of pollutants (Khoo and Ting, 2000).

Bioremediation is an integrated management of a polluted ecosystem where different organisms are employed to catalyze the natural processes those decontaminate the environment. Bioremediation is defined as "the utilization of microorganisms to reduce or eliminate environmental hazards by mediating desired chemical reactions or physical processes" (Skladany and Metting 1993). Soil is a rich habitat of all major groups of microorganisms and continuous application of untreated wastewater to the agricultural soil elevates the metal concentrations in surface soil which are toxic to bacteria and fungi. However, a long-term exposure of microorganism to high metal concentration develops immunity in them. The metal tolerance of microorganisms has been studied in different areas not only for removing metals from polluted soils, but also to provide a tool for bioremediation of polluted wastewater by using these metal tolerant species. Parameswari *et al.* (2010) conducted a laboratory study and

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results showed that Minimum Inhibitory Concentrartion (MIC) of *Aspergillus niger, Phanerochaete chrysosporium* and *Trichoderma* were from 75 to 100 mg L^{-1} chromium and for nickel (50 to 100 mg L^{-1}), which depended on the fungal isolate. In Pakistan, less information is available, so this study is designed to explore the metal tolerance levels of fungal isolates present in metal polluted areas of Punjab.

Microorganism are of primary importance in bioremediation of contaminated soils and wastewater, essentially because of their ability to alter the chemical status of the metal ions and in turn metal ions mobility through processes such as reduction, bioaccumulation, mobilization and immobilization (Khan *et al.,* 2009). Among the microorganisms, fungi are very important for bioremediation due to their mycelial nature and well documented ability to accumulate metals of all kinds (Gadd, 1993). Fungal resistance to heavy metals results from various mechanisms, i.e., active transport of metal ions outside the cell, masking metals by chelating, enzymatic transformation of metal ions, creating vacuoles in which metal ions are gathered and immobilization in the form of polyphosphates, increased production of melanin and other pigments, and production of specific metal binding compounds inside the cell (Balamarugan and Schaffner, 2006; Hastrup *et al.,* 2005; Gonzalez-Chavez *et al.*, 2002). The removal of metals from soil and water bodies by fungi have also industrial relevance as this process not only cleans the environment and protects its biodiversity, but it also allows the recovery of the metals and their subsequent reuse (Brierley *et al.*, 1983; Gadd, 1993). Considering the importance of fungi in bioremediation, this study was designed to isolate indigenous filamentous fungi from heavy metals polluted sites of Gujranwala and Sialkot and assess their cadmium, nickel and copper tolerance potential.

Material and Methods

Study area and samples collection

Surface soil samples (not exceeding 15 cm depth) used in this study were collected from peri-urban areas of Gujranwala (32° 09' N and 74° 11' E) and Sialkot (32° 29' N and 74° 32 E) which were irrigated with untreated wastewater – a mixture of domestic and cottage industrial effluent. Irrigation samples were collected from randomly selected eight sites of Sialkot and six sites from Gujranwala. Soil samples were collected by randomly selecting at transect of 10×10 m at each site. Then from each site, a composite soil sample was collected from four carefully mixed subsamples taken at different random locations within that transect area. The collected samples were transported to Soil Environment Laboratory of National Agriculture Research Center (NARC), Islamabad for further investigation. A portion of the soil samples were prepared and stored at 4°C to ensure minimal biological activity. Isolation of fungi was carried out within 24 hours of sample collection.

Isolation and morphological characterization of fungi

The filamentous fungi were isolated by standard spread plat technique after necessary dilution (upto 10^{-4})using potato dextrose agar (PDA) media (Oxoid Ltd, England, UK) in three replicates. The plates were incubatied at 28±1°C for 72 hours and prominent colonies were picked and inoculated individually in PDA plates for further purification.

Slides were prepared from purified fungal isolates, grown on PDA plates, with Aniline Blue stain for morphological characterization and were examined under microscope (at 40x) to ascertain mycelia appearance, sporangiophore position, columella and spore shape. The fungal isolates were identified by comparing these morphological characteristics with those described by Barnett and Hunter (1999).

Screening of heavy metal-resistant fungi metal tolerance index

Purified prominent isolates (fast growing with large biomass) were screened for their cadmium (Cd^{1+}), nickel (Ni^{2+}) and copper (Cu^{2+}) tolerance. PDA plates supplemented with 1mM of heavy metal were inoculated aseptically with a drop of spores suspension (of selected isolates). The inoculated plates were incubated at 28±1 °C for 7 days. The effect of each heavy metal on the growth of the isolates was estimated individually by measuring the radial colony extension against the control (without metal). Metal Tolerance Index (T_i) was calculated as the ratio of the extended radius of the treated colony to that of the untreated colony.

$$T_i = \frac{D_t}{D_u}$$

Where D_t is the radial extension (cm) of treated colony and D_u is the radial extension (cm) of untreated colony.

Minimum Inhibitory Concentrations (MICs) and metal tolerance experiment

The minimum inhibitory concentration of the isolates was determined as the lowest concentrations of metals that inhibits visible growth of the isolates. Ten selected fungal strains were exposed to varying concentration of Cd^{+1}, Cu^{+2}, and Ni^{+2}. Metal ions were added separately to PDA

medium at concentration ranging from 1 mM to resistance level with interval of 1 mM. The metal ions treated plates were inoculated with 5mm agar plugs from young fungal colonies grown on normal PDA medium in three replicates and were incubated at 28±1 °C for at least 7 days.

Results and Discussion

Total heavy metals in Gujranwala and Sialkot soils

Surface soil samples used in this study were collected from peri-urban area of Gujranwala and Sialkot being irrigated with untreated wastewater. Soil organic matter content in Gujranwala was 0.83-2.03% and 0.52-1.69% in Sialkot (Table 1). The soils were alkaline in reaction (pH from 7.2 to 8.6), non-saline (electrical conductivity from 0.11 to 1.77 dS m^{-1}) and calcareous in nature (lime contents from 0.5 to 15.2%).The concentration of Cd (mg kg^{-1} of dry soil) in soil of the study areas ranged from 1.6 to 9.5 and Cu concentrations ranged from 38.5 to 379.3 mg kg^{-1}, Pb concentration was 208.3 to 241.26 mg kg^{-1} and Ni concentration was 97.2 to 135.7 mg kg^{-1}. The total soil Cd, Cu, Pb and Ni contents in almost all the soil samples were higher than the permissible limits, i.e., 3 mg kg^{-1},100 mg kg^{-1}, 100 mg kg^{-1} and 50 mg kg^{-1}, respectively, as proposed by FAO/WHO (2001). The elevated concentrations of heavy metals in the soils are most likely due to long-term continuous application of untreated municipal/industrial effluent containing these heavy metals (Mahmood-ul-Hassan *et al.,* 2011).

Table 1: Minimum inhibitory concentrations of tested fungi (MIC) (mg L^{-1})

Test organism	Cu	Cd	Ni
Aspergillus niger(*GF-1)	318	56	117
Pythyme (GF-2*)*	127	56	235
Aspergillus flavus(GF-3)	191	112	176
Aspergillus spp. (GF-4)	32	56	59
Aspergillus niger (GF-5)	63.5	56	59
Curvularia (GF-6*)*	318	56	59
Aspergillus bervipes (GF-7)	191	112	59
Aspergillus flavus (**SF-4*)*	64	112	117
Aspergillus niger (SF-5)	445	1461	117
Pythyme (SF-1)	32	56	59

*Gujranwala fungi **Sialkot fungi

Fungal diversity

Prolonged exposure of soil fungi to elevated heavy metals contents has developed resistance in them (Gadd, 1993). In the present study, a total of 19 soil fungi were isolated from the heavy metal contaminated soils. The isolates were characterized and identified by fine tuning their morphological characteristics with those described by Barnett and Hunter (1999). The isolated fungal isolates belonged to genera *Aspergillus*, *Pythyme sp*., *Acrimonium sp.* and *Curvularia monata* group. *Aspergillus sp.* were the most frequently encountered (12 out of 19) isolates from the soil samples of both sites followed by *Pythyme sp.* and others. The *Aspergillus sp*. appeared to be the most commonly occurring in the heavy metals contaminated soils as also reported elsewhere (Ahmad *et al.,* 2005; Zafar *et al.*, 2007).

Minimum Inhibitory Concentrations (MIC)

The MIC values, the lowest concentration of metals that inhibit visible growth, suggest that all the fungal isolates tested were qualified for MIC. However, the resistance level of each isolate was independent against individual metal ions (Table 1). The highest MIC of Cu was 444 mg L^{-1}*Aspergillus niger* (SF-5) and ranged from 32 to 444 mg L^{-1} Cu. Similarly, *Aspergillus niger* (SF-5) also showed the highest MIC of Cd (1461 mg L^{-1}) and for the rest of the isolates it ranged between 56 and 1461 mg L^{-1}. Whereas, *Pythyme* (GF-2) showed maximum MIC value (234 mg L^{-1} Ni) and ranged from 59–234 mg L^{-1}Ni.The MIC values of fungi observed in this study are very much higher than those reported by Zafar *et al.* (2007) and Parameswari *et al.* (2010). They reported that MIC of Cu ranged from 0.6 to 9 mg L^{-1}, Cd from 0.2 to 5 mg $L^{1}$and 0.1 to 4 mg L^{-1} Ni. Further, Parameswari *et al.* (2010) relatively reported high Cu MIC values ranging between 50 and 75 mg L^{-1}. However, the MIC values observed in this study are very much similar to those reported by Ahmad *et al.* (2005). They reported MIC values 1000 -2000, 100-600 and 400 mg L^{-1} for Cd, Cu and Ni, respectively.

Tolerance Index

Copper, cadmium and nickel tolerance indices of all the tested fungal isolates are presented in Figure 1, 2, and 3, respectively. Error bars represent variability among isolates. Enhanced growth (diameter) of 5 *Apergilus Aspergillus* isolates, i.e., *A. flavus* (SF-1), *A. niger* (SF-5), *A. flavus* (GF-5), *A. bervipes* (GF-7) and *A. flavus* (SF-4) ranging from 6.5 to 100% was observed in the presence of 1 mM Cu concentration (Figure 1).

The growth of *A. flavus* (SF-1) and *A. flavus* (SF-4) was increased by 6 and 38% at 1 mM Cd content, respectively, and remaining eight isolates showed 33-85% reduction in growth (Figure 2). A large disparity in Cu and Ni tolerance has been noticed among the genera and even the isolates of the same genus. This disparity in the metal tolerance may be attributed to the different types of

tolerance processes/resistance mechanisms possessed by different isolates (Iram *et al.,* 2009). Ezzouhri *et al*. (2009) reported that Isolates of the same genus could present a marked difference in the levels of metal resistance. For example, copper resistance in *Aspergillus niger* is due to an active process involving copper metallothionein synthesis (Kermasha *et al.,* 1993) and in *Penicillium* sp*.,* both intracellular bioaccumulation and extracellular biosorption which contributes to the high level of resistance (Sun and Shao, 2007).

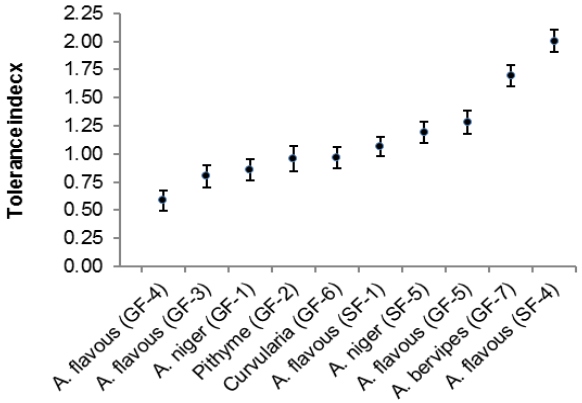

Figure 1: Fungal isolates' index of tolerance at 1mM Cu concentration (I = error bar)

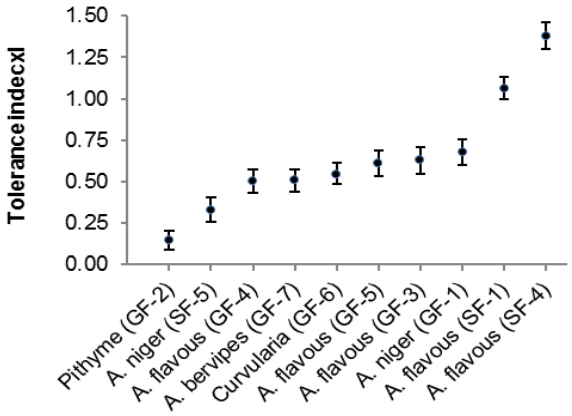

Figure 2: Fungal isolates' index of tolerance at 1mM Ni concentration

Growth of all the isolates was restrained at 1 mM Cd concentration (Figure 3) and growth inhibition was stronger in fungi isolated from Gujranwala soils than those isolated from the Sialkot soils. This may be due to the fact that Cd pollution was noticed more in Sialkot soils as compared to Gujranwala. This statement agrees well with Cernansky *et al*. (2007) who reported that microorganisms isolated from contaminated environments with heavy metals have adapted to such environments. These findings support the earlier observations of Ezzouhri *et al.* (2009) and Baldrian and Gabriel (2002) in showing that the Cd growth inhibition of isolates was independent of the metals contamination of the sites of isolation. Roane and Pepper (2000) reported that the variation in degree of resistance was most probably due to the potential variation in the mechanism of resistance. Severe Cd inhibition to physiological processes in micro-organisms, such as growth and photosynthesis at concentration less than 2 ppm has also been reported by Perfus-Barbeoch *et al.* (2002). Similarly, Lilly *et al.* (1992) have found that the addition of only 0.2 mM Cd led to severe inhibition of a *Schizophyllum* commune strain.

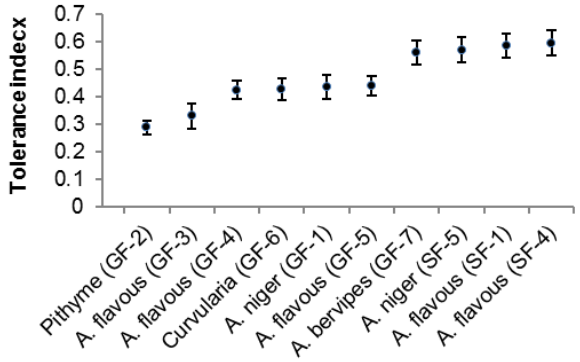

Figure 3: Fungal isolates' index of tolerance at 1mM Cd concentration

Metal tolerance

The effect of heavy metals on fungal growth was assessed on the basis of mycelia diameter and all the species of genus *Aspergillus, Pythyme* and *Curvularia* showed high metal tolerance. All the tested strains showed strong colony growth on Cu media at 30 mg L^{-1} in comparison to the control (Figure 4). Even at 60 mg L^{-1} Cu concentration half the strains exceeded the control. The stimulated growth at 30 and 60 mg L^{-1} Cu concentration may be due to the involvement of Cu in enzyme synthesis (Subramanian, 1956). As Cu is a co-factor in numerous enzymatic processes and represents the third most abundant transition metal found in living organisms (Brandolini *et al.* 2002).

Reduction in mycelia diameter of some of the tested fungi was observed at 63 mg L^{-1} Cu content and 136 mg L^{-1}Cu initiated inhibitory effect on mycelia growth of all the isolates. However, complete disappearance of all the isolates was observed at 1780 mg L^{-1} Cu concentration. *Aspergillus niger* (SF-5) showed higher degree of Cu tolerance which could show minimal growth in media with 1716 mg L^{-1}Cu concentration. The next highest level of resistance exhibited by another strain, *Pithym sp*. (SF-1), which could grow up to 700 mg L^{-1}Cu concentration. Other 8 species showed moderate to low resistance against Cu at the concentration ranges from 500 to 700 mg L^{-1}.

(GF-4) and ranged between 899 and 1124 mg L^{-1}. The high metal tolerant species most probably have developed the physiological adaptation mechanism for surviving in elevated Cd concentrations (Balamarugan, Schaffner 2006, Gonzalez-Chavez *et al.* 2002). In accordance with these findings, it was reported that the genera *Aspergillus* are of high capacity to biosorb cadmium and other heavy metals (Volesky 1990; Zafar *et al.* 2007; Lopez-Errasquin and Vasques 2003). Similar metal tolerance differences among the isolates of the same genus have also been observed in this study. Cernansky *et al.* (2007) reported that microorganisms

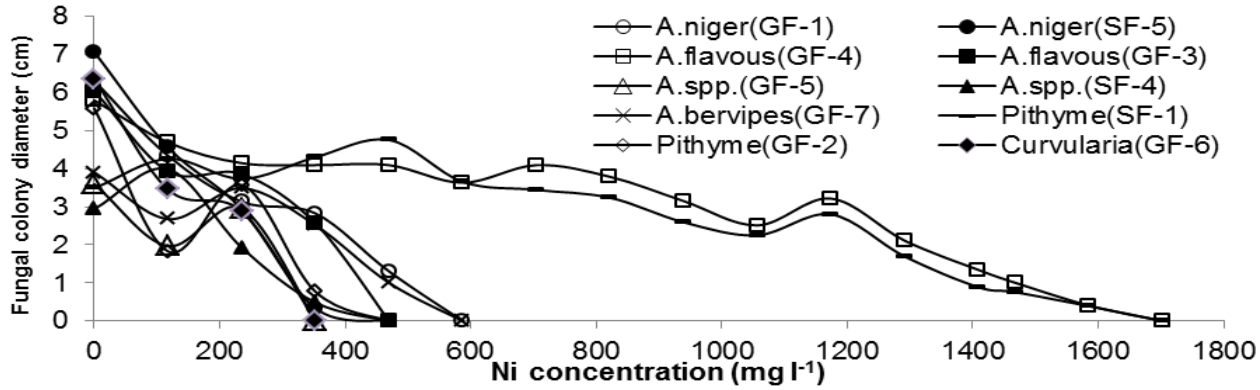

Figure 4: Effect of Ni concentrations on growth of fungal strains isolated from contaminated soils, up to 7 days at 28 °C in PDA medium

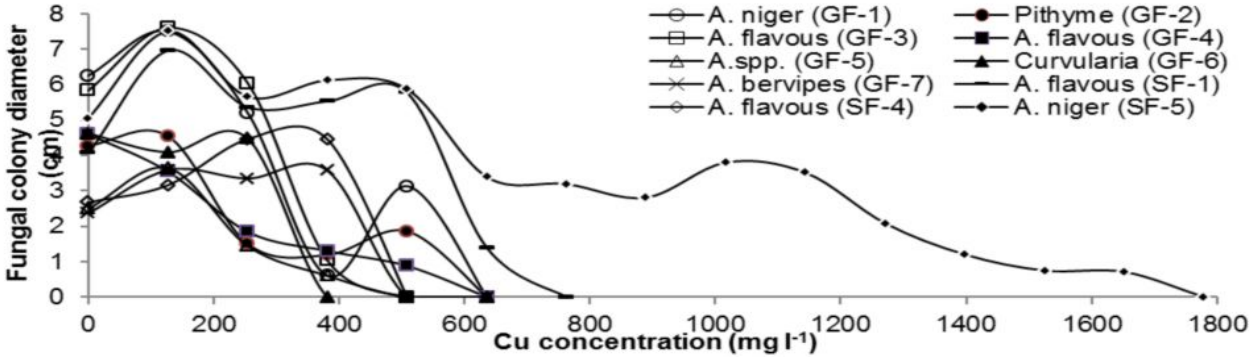

Figure 5: Effect of Cu concentrations on growth of fungal strains isolated from contaminated soils, up to 7 days at 28 °C in PDA medium

As Figure 5 depicts, *Aspergillus* genera showed highest level of Cd tolerance (9218 mg L^{-1}) while *Curvularia sp.* (GF-6) exhibited the second highest level of tolerance (5732 Cd mgL^{-1}). The order of the rest for Cd tolerance was *Aspergillus niger* (GF-1) > *Pythyme* (GF-2) > *Aspergillus sp.* (GF-5) > *Aspergillus flavus* (GF-3) > *Aspergillus flavus* (SF-4) > *Aspergillus brevipes* (GF-7) > *Pythyme* (SF-1) > *Aspergillus flavus*

isolated from contaminated environments with heavy metals have adapted to such environments.

Nickel proved relatively toxic ion among the tested metal ions (Figure 6) and 1600 mg L^{-1} Ni concentration was found maximum to be the tolerable level exhibited by *Aspergillus flavus* (GF-3) species. While *Curvularia sp.* (GF-6) showed the least resistance, i.e. 293 mg $L^{-1}$and

remaining fungal strains showed moderate to low Ni tolerance ranging from 250 to 400 mg L^{-1}. Heavy metals affect microorganisms in natural environment by reducing numbers and diversity of microbes and selecting a metal resistant population. It is also commonly assumed that metal exposure leads to the establishment of a resistant and tolerant microbial population (Gadd, 1993).

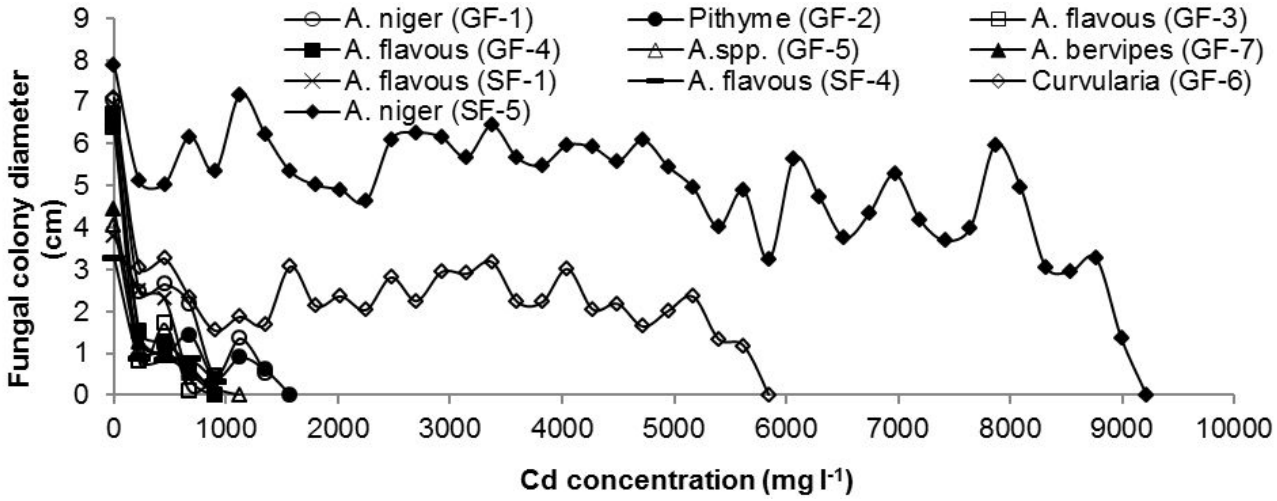

Figure 6: Effect of Cd concentrations on growth of fungal strains isolated from contaminated soils, up to 7 days at 28 °C in PDA medium

The Ni tolerance of different species was in order of *Aspergillus>Pithyum>Curvularia.* while for Cu and Cd, the order was *Aspergillus>Curvularia>Pithyum.* The metal tolerance among filamentous fungi was observed in order of Cd >Cu >Ni. These results indicated that various fungal isolates responded differently to different concentrations of copper, cadmium and nickel. There were morphological and physiological differences between fungal genera, species and strains, and therefore, their response was not same to the concentrations of the heavy metal ions (Al-Garni *et al.,* 2009). The metal tolerant fungi can successfully be used for bioremediation as it is an efficient strategy due to its low cost high efficiency and eco-friendly nature. Recent advances have been made in understanding metal-microbe interaction and their application for metal accumulation/detoxification (Rajendran *et al.,* 2002).

Conclusion

It is concluded from our research that fungal populations isolated from heavy metal-contaminated sites of Gujranwala and Sialkot have the ability to resist higher concentrations of copper, cadmium and nickel. 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 all the metals tested, which may make them promising candidates for further investigations regarding their ability to remove metals form contaminated environments. These results are very promising as a starting point for a potential application of these microorganisms in bioremediation. The isolated fungi can be used in agricultural soils, which can be polluted with heavy metals as a consequence of the incorporation of amendments such as sewage sludge or industrial/municipal solid/liquid wastes.

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