



Evaluation of tolerance potential of *Aspergillus niger* and *Trichoderma viridae* against hexvalent chromium

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Abstract

In this study bioremediation potential of fungi inhabiting in heavy metal contaminated soil of Aik drain was investigated. Out of the total nine species isolated, belonging to various genera, *Aspergillus niger* and *Trichoderma viridae* were selected to evaluate their potential to tolerate Cr(VI). For this purpose, PDA medium was supplemented with 100 – 1000 ppm concentration of Cr(VI) and change in the radial growth of mycelium and tolerance index (TI) of *A. niger* and *T. viridae* was investigated and compared with control (labeled as oppm). A decreasing trend was observed in the radial growth and TI of both isolates with the increase in metal toxicity. No visible growth in the mycelia was observed at 1000 ppm (Minimum Inhibitory Concentration). A negatively significant relationship of chromium stress was observed with radial growth of mycelia and TI at $p < 0.01$. Among both isolates, a minor difference was observed in their TI but comparatively *Aspergillus niger* exhibited more ability to tolerate high concentration of chromium than *Trichoderma viridae*. It is concluded that the *Aspergillus niger* and *Trichoderma viridae* both can be considered as best candidates for bioremediation due to their capability to remove heavy metals from contaminated soils.

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Introduction

Heavy metal pollution is becoming a cause of great concern due to its far reaching impacts on human as well as environmental health. Anthropogenic mishandling and unchecked use in industrial operations (electroplating and chemical processing) have enormously contributed in contamination of spheres of earth due to the bio-accumulative and persistent nature of heavy metals, even when present in low concentration (Desai *et al.*, 2016; Liu *et al.*, 2017; Gururajan and Belur, 2018). This has led to contamination of food resources posing detrimental health issues such as digestive and lung cancer, birth defects in both human and animals and stunted growth of plants (Fernández *et al.*, 2018).

Few of the widest spread and toxic heavy metals in environment include Cr (VI), Cd (II), Pb (II), Cu (II) and Zn (II). All of these metals have received great attention because these are also required by plants in traces and once up taken in excess through plant root system, becomes hazardous due to biomagnification and bioaccumulation while moving through the food chain (Liu *et al.*, 2018). Also the introduction of heavy metal into soil, a rich habitat of microorganisms, leads to physiological and morphological changes in the microbial community structure and function. The bio-accumulative and recalcitrant nature of most of the heavy metal ions pose long term threats to mineral recycling, population size of microbial communities and soil composition (Mohammadian *et al.*, 2017). But the polluted soils also serve as a great source of metal tolerant microbes (with variable tolerance potential) which can be used to bioremediate metal contaminated sites as well (Bai and Abraham, 2003; Mohammadian *et al.*, 2017).

Increased heavy metal pollution implicates to devise cleanup strategies. Although heavy metal pollution can be treated either by physical or chemical remediation techniques but all have higher cost or are less effective compared to the eco-friendly options now available to restore contaminated sites (Liu *et al.*, 2017). The choice of eco-friendly technique depends upon the tolerance threshold of organisms to survive

and perform physiological functions at various levels of metal toxicity in their habitat (Mohammadian *et al.*, 2017). These techniques either use plants (phytoremediation) to uptake heavy metals from soil/water through their extensive root system, algae (phycoremediation), fungal (mycoremediation) or bacterial (bacterial bioremediation) systems to adsorb or absorb heavy metals from environment on to or into their bodies through various physiological pathways (Oladipo *et al.*, 2018).

Mycoremediation is considered as the most cheap, highly effective and environment friendly option to tackle the issue of heavy metal pollution (Oladipo *et al.*, 2018). In fungi, there are several mechanisms that operate to detoxify heavy metals such as precipitation of metal ions on intracellular or extracellular cell surfaces, biosorption on cell surface and transformation of toxic to less toxic forms (Mohammadian *et al.* 2017). Several genera of fungi has diverse potential to remediate heavy metal pollution arising from Hg, Pb, Cd, As, Zn and Cr oxides (Liu *et al.*, 2017; Liu *et al.*, 2018).

Chromium, a geochemical element, exists in several oxidation states in environment among which Cr (III) and Cr (VI) are more stable forms. Of these two forms, Cr (VI) (mainly the CrO_4^{2-} at neutral or alkaline pH) is considered a priority pollutant globally for its known carcinogenic and mutagenic effects on exposed population by inducing oxidative stress (Fernandez *et al.*, 2010; Fernandez *et al.*, 2018). Very low concentration of Cr (III) is important for human metabolic requirements in maintaining the level of glucose and triglycerides in cells as well as for membrane and protein stability (Fernandez *et al.*, 2018). Chromium is being heavily discharged into water bodies from industries dealing in electroplating, wood processing, mining and leather tanning (Fernandez *et al.*, 2018).

Leather industry is the third largest export earning in Pakistan and uses excessive amount of chromium salt during tanning process. In Pakistan, cities like Karachi, Lahore, Kasur, Gujranwala, Multan and

Sialkot are well renowned for their leather tanning operations and pollute environment by discharging heavy amount of chromium in adjoining water bodies and open land dumps exceeding the permissible limit of 1.0 mg/L (Abbas *et al.*, 2012; GoP, 2016). To deal with this issue, this research was designed to 1) isolate and identify the fungal strains from chromium contaminated soil around Aik drain and 2) to determine the tolerance index (TI) of two selected strains (*Aspergillus niger* and *Trichoderma viridae*) based on the Minimum Inhibitory Concentration (MIC) of chromium metal. It was hypothesized that

increase in chromium stress will reduce the tolerance potential of fungal isolates.

Materials and methods

Site characteristics

Fungal strains were isolated from contaminated soil of Aik Drain, a tributary of river Chenab in Province Punjab. The drain has east to west extent originating from Pir Punjal Range in Kashmir and traverse through Sialkot and joins drain Palkhu before falling in River Chenab (Fig. 1).



Fig. 1. Location of study area (Aik drain) in Sialkot city.

The drain receives heavy pollution load from domestic units and multiple factories including tanneries located along its sides. Most of the factories located along its extent produce sports goods, surgical, textile and leather goods (Qadir *et al.*, 2008).

These industries use heavy metals one way or the other, as raw material or part of manufacturing process, posing serious concerns for the adjoining agricultural fields and exposed human population (Abbas *et al.*, 2004).

Isolation and identification of fungal strains

For the isolation of fungal strains, soil sampling was done within a 500m radius of the discharge point of leather industry into the Aik drain near Sialkot city.

The samples were transported to Microbiology Laboratory of Sustainable Development Study Center, Government College University Lahore for analyses. Composite samples of soil were made and were investigated for their mycoflora using dilution plate technique. About 1 ml of $10^3 - 10^7$ dilutions were inoculated on sterilized PDA (Potato Dextrose Agar)

plates and incubated at 25°C for 5 days. After the colonies were grown, fungal strains were purified through streak plate method and were identified on the basis of morphological and biochemical parameters (Humber, 1997) and pure cultures were maintained on PDA slants for further investigation.

Screening of fungal strains for heavy metal tolerance potential

Out of the nine strains identified, two fungal strains i.e. *Aspergillus niger* and *Trichoderma viridae*, were selected for further study. The selected strains were first screened for their tolerance potential against heavy metal (Cr⁺⁶) under consideration. For this purpose, PDA medium was supplemented with K₂Cr₂O₇ salt (500ppm) and both strains were inoculated on PDA plates and were incubated at 27°C for five days. Meanwhile, a control was run in which the strains were incubated under same conditions except the addition of metal to PDA.

The tolerance index (TI) was determined by monitoring the growth of colonies from center of inoculation towards its radial extent in case of control and test experiment. Following formula was applied to calculate the tolerance index of screened fungal strains as a ratio of radial growth of treated colony to that of untreated colony (Akhtar *et al.*, 2013);

$$TI = D_{\text{treated colony}} / D_{\text{untreated colony}}$$

Where TI = Tolerance Index, D_t = Radial extension of treated colony (cm) and D_u = Radial extension of untreated colony (cm).

Bioremediation of chromium stress

For the evaluation of bioremediation potential of isolated fungal strains, PDA medium was supplemented with various concentrations (100 ppm to 1000 ppm) of chromium salt (K₂Cr₂O₇). The selected fungal strains i.e. *Aspergillus niger* and *Trichoderma viridae*, were inoculated in the center of petri plates in triplicates and were incubated at 27 °C for five days. Meanwhile, control (labeled as 0 ppm) was run for both strains to compare for the growth pattern. The growth of fungi was monitored from the

point of inoculation or centre of the colony towards periphery. Tolerance index was calculated following Akhtar *et al.* (2013) and minimum inhibitory concentration (MIC) was determined at which no visible growth was observed.

The percentage inhibition in growth of *A. niger* and *T. viridae* was calculated by following Kannangara *et al.* (2017).

$$I (\%) = (r_1 - r_2) / r_1 * 100$$

Where I (%) = Percentage of inhibition in mycelium growth, r₁ = Radial growth of control group and r₂ = Radial growth of treated group.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 17.0 was used to estimate standard error in the means of radial extension and tolerance index of both strains. Based on study hypothesis, bivariate correlation analysis was performed to assess individual resistance of *A. niger* and *T. viridae* for chromium stress as a measure of variation in their radial growth and tolerance index.

Results and discussion

Identification of fungal isolates

The identification of strains was done on the basis of macroscopic characteristics such as colony shape, diameter, colony morphology, appearance and texture of colony, and microscopic characteristics such as presence of reproductive structures (spores), presence of sterile mycelium, septation in hyphae, conidia shape and color of hyphae. With the help of literature (Humber, 1997), pure cultures of fungus were identified such as *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus*, *Trichoderma viridae*, *Botrytis cineria*, *Cladosporium sp.*, *Saccharomyces sp.*, *Rhizopus sp.*, and *Penicillium sp.* in the polluted soil of Aik drain (Table 1).

Results of this study are consistent to the findings of Sugasini and Rajagopal (2015) who isolated *A. niger*, *A. tamari*, *T. viridae* and *Penicillium sp.* from tannery effluent in India.

Table 1. Diversity of fungal strains isolated from Aik drain.

Fungi	No. of isolates
<i>Aspergillus niger</i>	12
<i>A. flavus</i>	03
<i>A. oryzae</i>	01
<i>Trichoderma viridae</i>	10
<i>Botrytis cineria</i>	04
<i>Cladosporium sp.</i>	02
<i>Rhizopus sp.</i>	09
<i>Penicillium sp.</i>	06
<i>Saccharomyces sp.</i>	03

Screening Test for *Aspergillus niger* and *Trichoderma viridae*

The screening test for *A. niger* and *T. viridae* against chromium at 500ppm showed visible radial growth (Fig. 2). The mean tolerance index (TI) of *A. niger* was 0.55 ± 0.04 and for *T. viridae* was 0.45 ± 0.04

which showed that selected isolates were tolerant to metal under investigation.

Based on this positive response to screening test, the strains were used for further investigation keeping in consideration the objectives of this study.

Table 2. Effect of Chromium on Tolerance Index (TI) of *Aspergillus niger* and *Trichoderma viridae*.

Concentration (ppm)	<i>A. niger</i> ($M \pm SE$)		<i>T. viridae</i> ($M \pm SE$)	
	TI	Tolerance	TI	Tolerance
0 (Control)	--	--	--	--
100	0.91 ± 0.13	High	0.78 ± 0.04	Moderate
200	0.77 ± 0.04	Moderate	0.7 ± 0.04	Moderate
300	0.72 ± 0.04	Moderate	0.62 ± 0.04	Moderate
400	0.62 ± 0.04	Moderate	0.53 ± 0.06	Low
500	0.55 ± 0.04	Low	0.45 ± 0.04	Low
600	0.47 ± 0.04	Low	0.375 ± 0.04	Very Low
700	0.4 ± 0.04	Low	0.3 ± 0.04	Very Low
800	0.30 ± 0.06	Very low	0.23 ± 0.04	Very low
900	0.17 ± 0.12	Very low	0.15 ± 0.04	Very low
1000	0.0 ± 0.0	No	0.0 ± 0.0	No

Bioremediation potential of *A. niger* and *T. viridae*

The bioremediation potential of selected strains was investigated among three replicates designed for each metal concentration. Higher metal concentration caused a reduction in radial growth and tolerance index of investigated species (Fig. 3 and Table 2). The mean radius of colony in case of control (0 ppm) was 2.10 ± 0.03 cm and 1.7 ± 0.03 cm for *A. niger* and *T. viridae* while no visible growth was observed at 1000 ppm (MIC) for both species. A decreasing trend was

clearly observed in the radial extension and TI of colonies as the metal concentration increased. At 100 ppm the radial growth in *A. niger* and *T. viridae* was 1.82 ± 0.03 cm and 1.55 ± 0.11 cm showing TI of high (0.91 ± 0.13) to moderate (0.78 ± 0.04) in both isolates respectively. Similarly, at 600ppm the radial growth of colonies was observed to be 0.95 ± 0.03 cm and 0.75 ± 0.03 cm and low (0.475 ± 0.04) and very low (0.375 ± 0.04) TI for *A. niger* and *T. viridae* respectively. The minimum growth was observed at

900 ppm with 0.35 ± 0.08 cm and 0.30 ± 0.03 cm radial growth and very low TI (0.175 ± 0.12 and 0.15 ± 0.04) for *A. niger* and *T. viridae*. The decrease in tolerance index of both isolates with increase in metal concentration can be related to built-up of toxic metabolites hindering fungal growth (Ghosh *et al.*, 2017). Keeping in consideration the trend in radial growth and tolerance index, it is evident from results that, in comparison to *T. viridae*, the *A. niger* had

higher capability to either accumulate in body or reduce Cr (IV), a toxic ionic form, to less or non-toxic form i.e. Cr (III) through complex enzymatic pathways.

The difference in the metal tolerance may be due to the incidence of one or more strategies of resistance or tolerance mechanisms displayed by fungi (Iram *et al.*, 2013).

Table 3. Relationship of metal concentration with radial extension and tolerance index of selected isolates.

Variables	<i>A. niger</i>	<i>T. viridae</i>
Radial Extension	-0.980**	-0.995**
Tolerance Index	-0.988**	-0.997**

** $p < 0.01$.

Fig. 4 shows that at lower metal concentrations, the tested fungal strains showed lower percentage inhibition. i.e. at 100 ppm (13.33% and 8.82%) but with the increasing metal concentration, rate of inhibition was increased i.e. at 500 ppm, inhibition in growth was observed at 47.61% and 47.05% and at

900 ppm, it was observed to be 83.33% and 82.35%. At 1000 ppm, no visible growth in mycelium was observed (100 %inhibition) for *A. niger* and *T. viridae* respectively. At this point, the fungal isolates had become very sensitive to the elevated metal concentration.

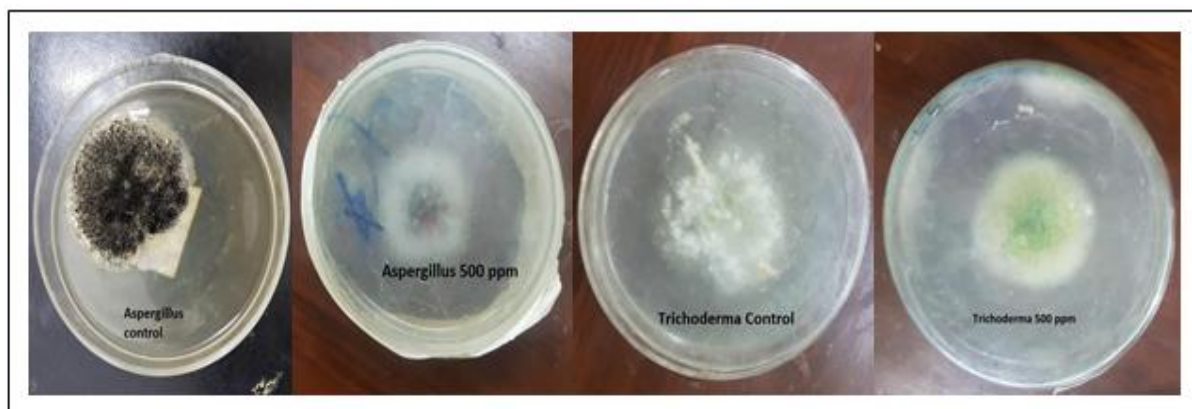


Fig. 2. Screening test for *A. niger* and *T. viridae* at 500 ppm of Cr (VI).

Findings of this study are consistent to those reported by Iram *et al.* (2012) in which tolerance potential of *Helminthosporium sp.*, *Aspergillus sp.*, *Humicolagrisea sp.*, *Scopulariopsis sp.*, *Nannizzia sp.*, *Fusarium sp.* and *Curvularia sp.*, were investigated against heavy metals and most of the isolates showed MIC at 1000 ppm as observed in present study. Similar results have been reported in another study by Rasool and Iram (2014) in which *Aspergillus*, *Pencillum* and *Fusarium* were

found tolerant to Cr, Pb, Cu and Cd metals.

Relationship between fungal growth and metal stress

Bivariate correlation analysis of independent variable of metal concentration with the dependent variables showed that metal concentration had negatively significant relationship with radial extension of colonies and tolerance index of *A. niger* and *T. viridae* at $p < 0.01$ (Table 3).

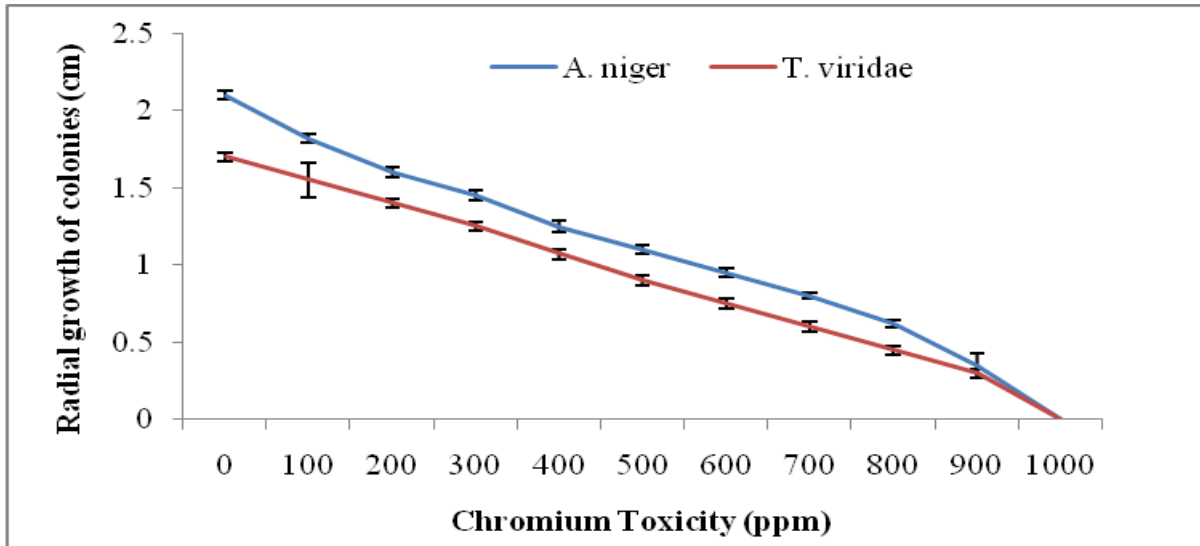


Fig. 3. Mean radial growth of colonies under chromium stress.

The analysis showed that with increase in the metal concentration both radial extension and tolerance index were decreased until minimum inhibitory concentration was achieved indicating that the strains can perform best at lower metal concentration in

order to remediate pollution. The reason for decrease in tolerance and radial growth can be attributed to the buildup of toxicity (due to formation of secondary metabolites) in culture plates with increase in metal concentration.

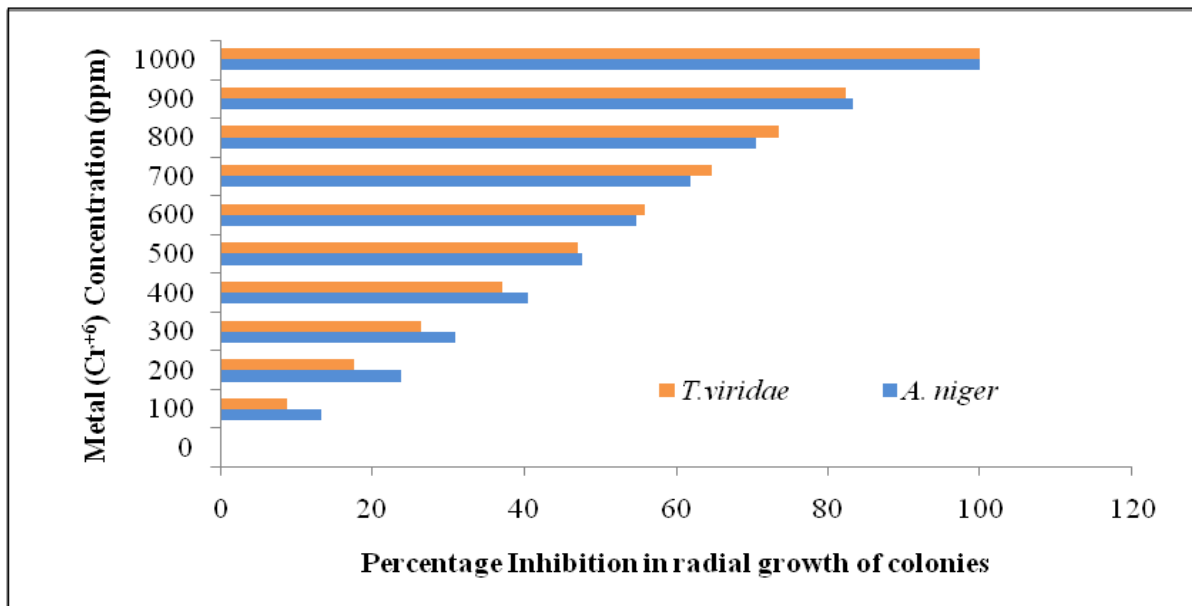


Fig. 4. Inhibition in the growth of mycelium at various metal concentrations.

In present study, growth of both strains was observed up to 900 ppm of Cr (VI) although at varying tolerance index among both strains and results are consistent to the findings of Akpor *et al.* (2015) reporting growth of *A. niger* up to 800 ppm of chromium stress. Results of this study are also consistent to the findings of Iram *et al.* (2013)

reporting *A. niger* as most tolerant strain to chromium stress among all other isolates.

Similarly Hajieghrari (2010) reported observable decrease in the linear extension of *Trichoderma sp.* under influence of various heavy metals up to a concentration of 1000ppm.

The radial extension of mycelium and tolerance index in plate culture depends on the type of compound/toxin, isolate and growth conditions. Various fungal strains exhibit differing patterns of tolerance index and growth inhibition at a particular metal concentration. Ezzouhri *et al.* (2009) isolated *Aspergillus sp.* from heavy metal contaminated site and tested it against chromium stress. After incubation for 7 days at 25°C the MIC was reported between 500 700 ppm which is contrary to the findings of this study (1000ppm). The difference in the estimates of both studies might have resulted due to differing incubation time and temperature (five days at 27°C in this study).

Bioremoval of chromium has been reported about several genera of fungi including *Trichoderma spp.*, *Aspergillus niger*, *Aspergillus oryzae* and *Fusarium oxysporum* (Jobby *et al.*, 2018). In a study, *A. niger* and *A. flavus* were studied for Cr (VI) accumulation and tolerance in which *A. flavus* accumulated 25% of Cr (VI) (Gupta *et al.* 2000). In another study, *Trichoderma inhamatum* was found to be converting Cr (VI) to Cr (III) form (Morales-Barrera *et al.*, 2008). The isolates used in this study also has potential to remove other heavy metals as well such as Cu, Zn, As, Ni and Cd so can be very efficiently used in sites contaminated by more than one type of heavy metal (Muhammadian *et al.*, 2017). Findings of this study suggest *T. viridae* and *A. niger* as successful candidates for bioremediation of metal contaminated sites. However, success of any bioremediation experiment depends on pollutant type and its concentration in media/environment, temperature, pH, availability and tolerance potential of microorganism isolated from contaminated site (Jobby *et al.*, 2018).

Conclusion

In present study, the contaminated soil of Aik drain was identified with containing nine genera of fungi out of which two of the most tolerant strains were selected. Further investigation revealed that there was a minor difference in the bio-removal of chromium by both isolates but comparatively *A. niger* was found to

be more efficient in removing chromium from culture plates than *T. viridae*. The removal efficiency of both isolates depends on their relative tolerance to test concentrations of Cr⁺⁶ supplemented in PDA plates. *A. niger* showed high tolerance to very low tolerance to Cr⁺⁶ stress while *T. viridae* showed moderate to very low tolerance as the metal concentration increased in the culture medium (100 ppm to 900 ppm). For both isolates, MIC was achieved at 1000 ppm as strains were unable to show any visible growth in their mycelium. A negatively significant relationship of metal stress with radial growth pattern and tolerance index suggests that strains are more effective to bioremediate contaminated sites at low metal concentrations but at elevated Cr (VI) stress, they become less resistant showing decrease in tolerance index and growth inhibition. The result concluded that *A. niger* and *T. viridae* both are ideal candidates for bioremediation because they were able to develop the physiological adaptation mechanism for surviving in elevated metal concentrations.

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