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## Counter Effect of *Trichoderma harzianum* Rifai. Against Cr (VI)

Sundus Akhtar<sup>1\*</sup>, Ayesha Shafqat<sup>1</sup>, Syeda Mariam Sherazi<sup>1</sup>, Sonia Aslam<sup>2</sup>, Habiba Khalil<sup>2</sup>, Rubina Shakir<sup>1</sup>, Muhammad Arslan Khan<sup>3</sup>

<sup>1</sup>Department of Botany, Minhaj University, Lahore, Pakistan.

<sup>2</sup> School of Zoology, Minhaj University, Lahore, Pakistan.

<sup>3</sup> Department of Plant Pathology, Institute of Plant Protection, MNS University of Agriculture Multan, Pakistan.

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### ABSTRACT

Industrial and sewage water ejection in river and streams on daily basis and wide use of heavy metal is contaminating our surroundings. Among all the valences, Cr (VI) is regarded as a hazardous ion, which contaminates groundwater and can be transferred through the food chain *In-vitro* study was carried out in laboratory in which impact of Cr (VI) on *Trichoderma harzianum* was studied. For those two experiments i.e., solid medium assay and liquid broth assay were conducted. In solid medium assay, the radial growth, morphological alterations (appearance of colony, changes in the morphologies of spores and hyphae) in *T. harzianum* and inhibition zone under the stress of Cr (VI) at different doses i.e., 0, 1000, 2000, 3000, 4000 and 5000 ppm were studied. The results showed that radial growth was insignificantly reduced at highest dose (5000 ppm) i.e., 3.3% as compared to control. Moreover, no inhibition zone formed, and the mycelial pattern of tested fungi was crossing the disc zones. However, at 5000 ppm of Cr (VI), about 0.2 mm of concentric zone was observed. While no phialides were observed at higher doses of Cr (VI). Furthermore, in liquid broth experiment, mycelial growth, and biochemical attributes [Total protein content (TPC), Peroxidase (POX) and Catalase (CAT) activities] were studied after 7 days of incubation. The results revealed that the fresh and dry weight of *T. harzianum* was increased up to -200% comparison to control. The level of TPC, POX AND CAT increased by -10 % to -94% with the increasing concentration of Cr (VI) i.e., 1000 to 5000 ppm. Thus, the findings showed that *T. harzianum* could be used as bioremediator against Cr (VI). Further in- situ studies need to be taken to eradicate the presence of heavy metals in the environment by using fungus for bioremediation purpose in future.

Corresponding Author: Sundus Akhtar

Email: dr.sundas@mul.edu.pk

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### INTRODUCTION

Industrial and sewage water discharge in river and streams on daily basis and extensive use of heavy metal-based fungicides, bactericides and pesticides is contaminating our environment. Various heavy metals like (Lead, Chromium, Mercury, Uranium, Selenium, Zinc, Arsenic, Cadmium, Silver, Gold, and Nickel) are entering into our food chain (Singh et al., 2011). Emission of smoke

from industries, chimneys and vehicles are also playing a role in increasing the production of heavy metal in Pakistan. Further, the wastewater from the mentioned sources mixes with the irrigation canals and finds its way to water channels. As a result, heavy metals become part of wastewater which arises from various natural, anthropogenic, and industrial activities (Mishra, 2016). If the wastewater is being used without prior treatment, the

plants are accumulating these heavy metals thus becoming the part of food chain resulting in various health problems like breast cancer in women etc. Moreover, these contaminants are constantly exceeding their threshold in the environment and affecting the balance of micro-nutrients and micro-organisms in soil also (Khan and Sajjad, 2013).

Many approaches used for the bioremediation of polluted soil and water not only include physical and chemical processes such as many bacteria and fungi engaged in their tolerance and reducing power to heavy metals. The bioremediation of the soil also constitutes a big issue in the settlement of the problem of pollution by indiscriminately harmful chemicals. The study was conducted in 2010 for testing of the possible persistence of this plant-beneficial fungus in soils polluted with mining waste and showed persistence of the *Trichoderma* sp (Shoaib *et al.*, 2019).

*Trichoderma* species belong to the hypocreale order of the Ascomycete and are incomplete filamentous fungus with telomorphes. *Trichoderma* sp. is among the most isolated soil fungus and is recognized for their biocontrol capacity to control a wide range of herbal fungi. It plays an essential function in the bioaccumulation of high amount of heavy metal present in different wastewater and soil metals (Ayad, 2018). In the present study the tolerance level of *Trichoderma harzianum* Rifai under the stress of chromium under different concentrations will be carried out.

Accumulation of Heavy metal in the soil is a biggest concern now days due to various anthropogenic and man-made activities, *Trichoderma* as useful fungi can be used to eradicate Cr (VI) heavy metal in soil. This biodegradable method can be used in future to remove heavy metal availability and mobility in the soil.

## MATERIAL AND METHODS

### Solid medium

The stock solution of 1000 mgL<sup>-1</sup> Potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) was prepared with double distilled water. Moreover, different concentrations i.e., 1000, 2000, 3000, 4000 and 5000 ppm were prepared by diluting stock solution with double distilled water (50 mL). Metal ion concentration after each experiment was examined on Z-5000 Polarized Zeeman Atomic Absorption Spectrophotometer (AAS). About 1g of agar and 1g of malt extract was added in 50 mL of distilled water and autoclaving for 45 minutes at 121°C, 15 psl pressure for

the preparation of malt extract agar medium (MEA). In order to prevent any bacterial contamination, 500 mg of Amoxal capsule was added in molten media before pouring in pre sterilized Petri plates in aseptic conditions (Akhtar and Shoaib, 2020).

### Disc diffusion method

The same procedure was carried out for the preparation of malt extract agar medium as was discussed in solid medium section. Before pouring media into Petri plates mark number of samples at the back side petri plates (such as 1, 2, 3 and so on). The media was poured into the pre-heated or pre-sterilized Petri plates under aseptic conditions and then left for solidification for 15 to 20 minutes. Furthermore, various tiny pieces of filter paper (1 mm) were soaked into different concentrations of Cr (VI) i.e., 0, 1000, 2000, 3000, 4000 and 5000 ppm and poured into the marked places in Petri plates. The inhibition zone of *T. harzianum* was noticed after four days of incubation (Shoaib *et al.*, 2019).

### Liquid broth assay

The effect of heavy metal (Cr IV) stress on the mycelial growth of *T. harzianum* (grown in liquid broth) in liquid medium was carried out by following the method of Akhtar and Shoaib (2020) with the slight modifications. The metal solution at different levels (0 to 5000 ppm) were prepared in 2% of malt extract medium and divided in three replicates containing 10 mL of each metal dose. The experiment was set in 15 mL of test tubes and further autoclaved at 121 °C, 15 psl pressure. The test tubes were left to cool down for 15 minutes followed by inoculating with the spore suspension of *T. harzianum* in aseptic condition. After that the test tubes were incubated at 30 ± 2°C for 7 days. Furthermore, after incubation fresh and dry weight of *T. harzianum* was estimated. Moreover, various biochemical alternations like protein content (Lowry *et al* 1951), catalase activity (Beers and Sizer, 1952) and peroxidase activity (Chance and Machly, 1967) in fungi were estimated after 07 days of incubation.

### Estimation of metal uptake

The metal uptake by the *T. harzianum* at different concentrations of Cr (VI) was estimated through atomic absorption Spectrometry.

### Statistical analysis

The assessment of influence of heavy metal (Cr IV) on *Trichoderma harzianum*, was evaluated by applying LSD Test and Pearson correlation. Moreover, all the statistical analyses were done by means of computer software Statistics 8.1.

## RESULTS

### Microscopic characteristics

The results showed that with the increasing concentration of Cr (VI) i.e., at 1000 to 4000 ppm the number of conidia increased as compared to 0 ppm. Moreover, the size of conidia decreased by 5.4 to 2.6  $\mu\text{m}$  at the said concentrations of Cr (VI) in comparison with negative control. However, the shape of conidia were globose to sub-globose. Moreover, the phialides were flask shaped with 4.6  $\mu\text{m}$  in size at 1000 ppm. Whereas,

not a single phialides were observed at higher doses of Cr (VI) as compared to 0 ppm.

### Radial growth

The radial growth of *T. harzianum* at different doses of Cr (VI) was observed. It was found that the radial diameter of tested fungus at 1000 to 4000 ppm of Cr (VI) was significantly ( $P \leq 0.05$ ) decreased up to 1.1% in comparison with control (9 cm). Whereas, at 5000 the %age inhibition of radial growth at 5000 ppm of Cr (VI) was 3.3 as compared to control. (Table 1).

Table 1. Radial growth of *T. harzianum* at different concentrations of Cr (VI).

Cr (VI) concentration (ppm)	Radial growth (cm)
0	9.0 <sup>a</sup>
1000	8.9 <sup>a</sup>
2000	8.9 <sup>a</sup>
3000	8.9 <sup>a</sup>
4000	8.9 <sup>a</sup>
5000	8.7 <sup>b</sup>

### Disc diffusion method

The results revealed that there was no inhibition zone formed at different levels of Cr (VI) i.e., 0, 1000, 2000, 3000 and 4000 ppm. The mycelial pattern was crossing

the disc zones that were dipped in various doses of Cr (VI). However, about 0.2 mm of the concentric zone was observed at the level of 5000 ppm of Cr (VI) in three replicates (Figure 1, Table 1).

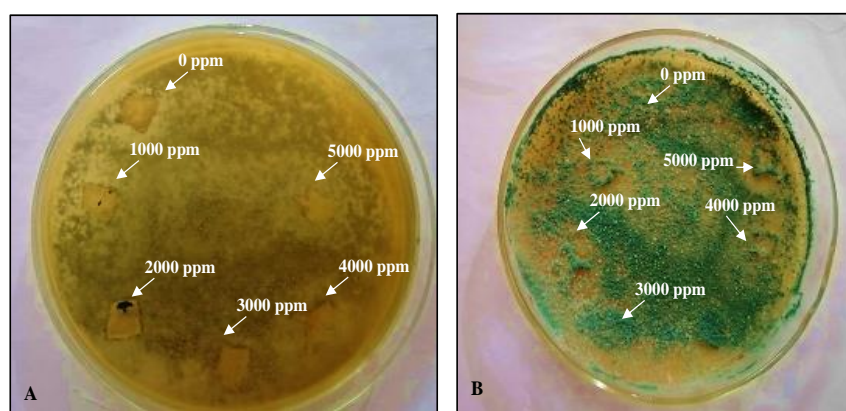


Figure 1. Disc diffusion method. A) Back side of Petri plate, B) Front side of Petri plate.

### Liquid broth assay

It was found that the fresh weight of tested fungus was significantly  $P \leq 0.05$  enhanced by -2 to -40 % as compared to control (0ppm = 1.09g) (Figure 2).

Likewise, the fresh weight of tested fungus the dry biomass of *T. harzianum* was significantly  $P \leq 0.05$  enhanced by -60 to -200% with respect to control (0ppm = 0.16 g) (Figure 3).

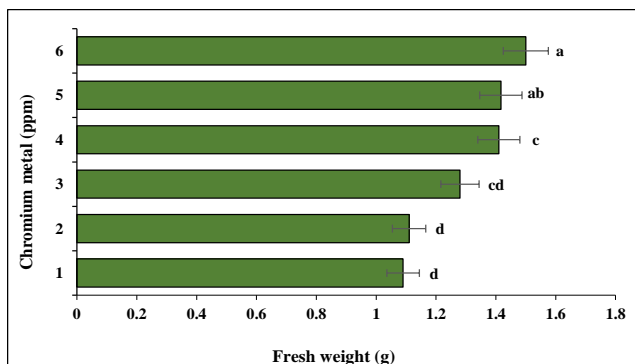


Figure 2. Fresh weight of *Trichoderma harzianum* in different concentrations of Cr (VI) after 7 days of incubation. Bars indicate standard error. Different alphabets on standard bar indicates least significant difference ( $P \leq 0.05$ ) derived from LSD test.

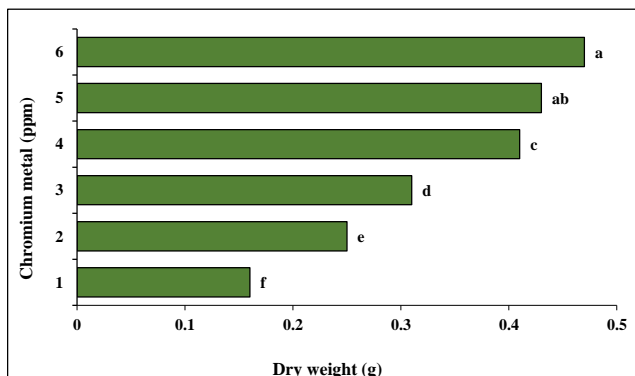


Figure 3. Dry biomass of *Trichoderma harzianum* in different concentrations of Cr (VI), after 7 days of incubation. Bars indicate standard error. Different alphabets on standard bars indicate least significant difference are  $P \leq 0.05$  derived from LSD test.

**Biochemical alterations**

**Total protein content:** It was observed that the level of total protein content of *T. harzianum* was significantly ( $P \leq 0.05$ ) more profound by -30% to -94% with the increasing concentration of Cr (VI) i.e., 1000 to 5000 ppm as compared to control (0ppm=0.24 mg g<sup>-1</sup>) (Figure 4).

**Peroxidase activity test:** The effect of Cr (VI) on the peroxidase activity of *T.harzianum* was examined. It was perceived that the level of peroxidase activity of *T. harzianum* was significantly ( $P \leq 0.05$ ) enhanced by -9.4% to -31% as compared to control (0ppm=0.74 Unit mg g<sup>-1</sup> protein<sup>-1</sup>) when the level of Cr (VI) i.e., 1000 to 5000 ppm was increased (Figure 5).

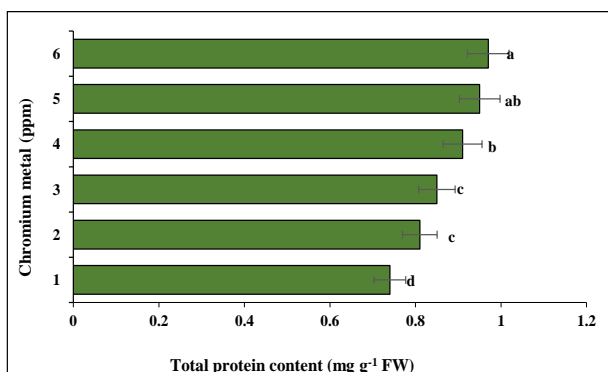


Figure 4. Total protein content of *Trichoderma harzianum* at different concentrations of Cr (VI). Bars indicate standard error. Different alphabets on standard bars indicate least significant difference are  $P \leq 0.05$  derived from LSD test.

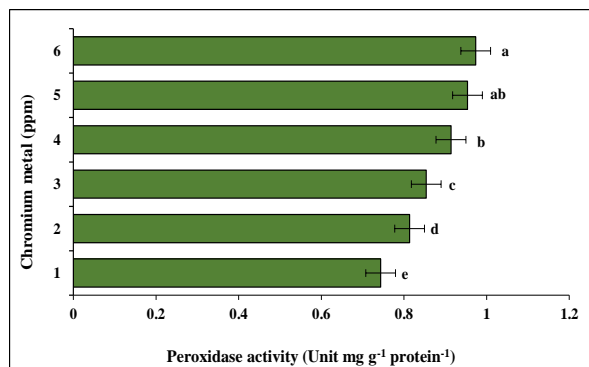


Figure 5. Peroxidase activity of *Trichoderma harzianum* at different concentrations of Cr (VI). Bars indicate standard error. Different alphabets on standard bars indicate least significant difference are  $P \leq 0.05$  derived from LSD test.

**Catalase activity:** It was observed that when the doses of Cr(VI) was increased in the growing environment of tested fungi the level of catalase activity was significantly increased by -12.8% to -42% as compared to control (0ppm = 0.54 Unit mg g<sup>-1</sup> protein<sup>-1</sup>) (Figure 6).

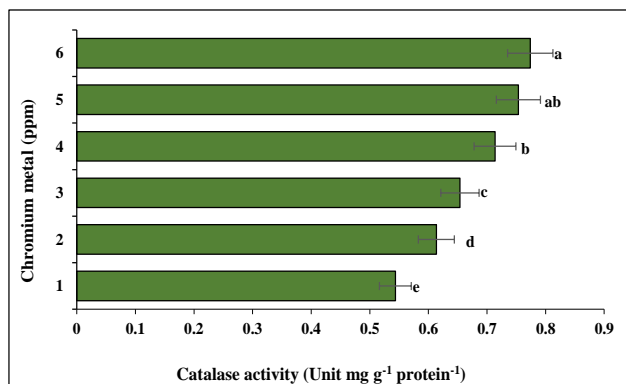


Figure 6. Catalase activity test of *Trichoderma harzianum* at different concentrations of Cr (VI). Bars indicate standard error. Different alphabets on standard bars indicate least significant difference are  $P \leq 0.05$  derived from LSD test.

**Correlation between biochemical and growth parameters**

The association between biochemical like total protein content (TPC), Catalase activity (CAT) and Peroxidase activity (POX) vs growth attributes i.e., fresh and dry weight (FW, DW). The results revealed that relationship

between FW × DW; FW × TPC, CAT, POX; TPC × CAT, POX and CAT × POX was positive, highly significant to non-significant and weakest to strong ( $P \leq 0.05$ ,  $R = 0.07$  to  $0.9$ ). Moreover, affiliation between DW × TPC, CAT, POX was negative, non-significant and weakest ( $P > 0.05$ ,  $R = 0.02$  to  $0.03$ ) (Table 2).

Table 2. Relationship between growth and biochemical attributes of *T. harzianum*.

	DW	FW	TPC	CAT
FW	0.36 <sup>ns</sup>			
TPC	-0.02 <sup>ns</sup>	0.45 <sup>ns</sup>		
CAT	-0.02 <sup>ns</sup>	0.45 <sup>ns</sup>	0.9 <sup>**</sup>	
POX	-0.38 <sup>ns</sup>	0.07 <sup>ns</sup>	0.65 <sup>*</sup>	0.65 <sup>**</sup>

DW = Dry weight; FW = Fresh weight; POX = Peroxidase activity; CAT = Catalase activity test; TPC = Total protein content.

$P < 0.05^*$	$P < 0.02^{**}$
$P \geq 0.05^{ns}$	$P \leq 0.05^*$
$P < 0.02^{**}$	$P \geq 0.05^*$

### Metal uptake

The metal uptake by the *T. harzianum* at different concentrations of Cr (VI) is depicted in the Figure 7. The

results revealed that the metal uptake by the *T. harzianum* was 75-40% with the increasing concentration of Cr (VI).

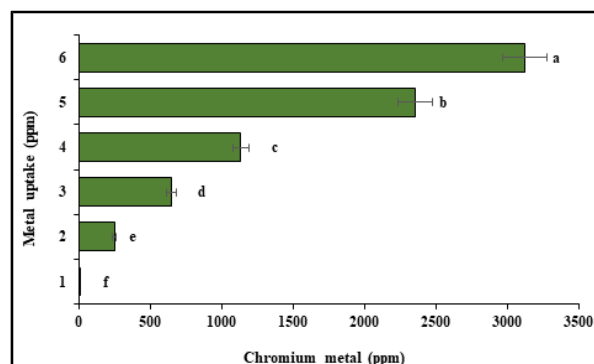


Figure 7. Metal uptake by *Trichoderma harzianum* at different concentrations of Cr (VI). Bars indicate standard error. Different alphabets on standard bars indicate least significant difference are  $P \leq 0.05$  derived from LSD test.

### DISCUSSION

In the present study macroscopic study was carried out, it was observed that the Cr (VI) did not show any direct effect on the growth of filamentous fungi. The microscopic identification at various levels of Cr (VI) alternations in the appearance of colony, changes in the morphologies of spores and hyphae was detected. Moreover, some changes took place in the mycelial morphology such as hyphae and development of hyphal coils. Further, dense clump of conidia, some disrupted spores and large sheath covering the conidia may spotted. Same findings were observed by De Padua (2021) when he treated the different strains of *Trichoderma* in the stress of nickel metal. Moreover, the results revealed that the fresh and dry weight of *T. harzianum* rather decreasing in the stress of Cr (VI) it was increased up to -200% as compared to control. Furthermore, the level of total protein content, catalase and peroxidase activity was increased by -10 % to -94% with the increasing concentration of Cr (VI) i.e., 1000 to 5000 ppm.

According to the findings of Lopez and Vazquez, (2003) almost all of the *Trichoderma* species possesses the great power of resistance and endurance against heavy metals. Such fungi have the ability to eliminate different heavy metals from dissolved solution via ion-exchange reaction. It was found that, using different salts may help in the removal of heavy metals, in response to these excellent

results obtained as an average of 90% of heavy metal content obtained within 10 minutes. The aggregation of metals of developing cells might diversifies with the age of cell. *Trichoderma* fungi possesses vast metabolic flexibility and exhibited defence against heavy metals. Lopez and Vazquez (2003) have found that *Trichoderma* own the capability to endure great level metal concentrations being the resistant cell. According to his *in vitro* analysis, *Trichoderma* specie was found as the specie which has the ability of tolerance in opposition to Cadmium (Cd), Lead (Pb), Nickel (Ni), Chromium (Cr), Zinc (Zn) and Copper (Cu). Those microorganisms which were isolated from the natural environment have great resistance to different fluctuations occur in the environment. On the basis uptake of metal mixtures of numerous ions are affected. Among other fungi, *T. harzianum* has the ability to eliminate arsenic present in agricultural soils. Arsenic is a poisonous compound that might occur in inorganic and organic forms. Various strains of *T. harzianum* may act as bio indicators of arsenic pollution. It was determined that *Trichoderma* is capable to bio volatilize arsenic and is proficient in eliminating arsenic from liquid medium (Sarkar, 2013). Moreover, it was also found in the present study that that the metal uptake by the *T. harzianum* was 75-40% with the increasing concentration of Cr (VI). It was reported by Kapoor *et al.* (1999), that dead cells and living of fungi has

the ability to eliminate the heavy metal ions from soluble solutions. Moreover, they also said that Metallothioneins and phytochelatins (a rich SH peptide) are two procedures which are vacuoles compartments and cytoplasmic protein complexation includes in intracellular metal immobilization. Fungal vacuole take part in vital roles in molecular disruption, depository of the metabolites, parameter of the cytosolic concentrations of metal ions and it also cleans toxic metal ions. Metallothioneins act as metal-binding protein which is able to control the cytosolic concentrations and secure crucial metal i.e., Cd and Zn and Cu. Biotransformation of metals occurs with the aid of fungi via chemical reactions, i.e. oxidation, dealkylation reduction, and methylation. Such reactions may induce volatilization of metal and lessen the toxicity metal. Metals may also transfer through cytoplasmic vesicles and vacuoles to further other parplant symbionts fungi mycelium.

Karcprzak and Malina (2005), stated that filamentous fungi possess the ability to yield chemical complexes within the extracellular enzymes and metal ions, in addition to the ability to secure the metals ions to their cell walls. Fungi have wide range of stored metals i.e. Cd, Cu, Pb, Ni, Cr and Zn. The arrangement is due to their cell walls that are attained from lipids, polysaccharides and protein which were gathered in significant quantities of metals ions, so, that they could provide a variety of binding sites for metals ions. In the biosorption process, the components of fungal cell walls play a significant part. Nonetheless, it is not only the removal and elimination procedure, but as well as to preserve the atmosphere and biodiversity, also permitting the reclamation of the metals. *Trichoderma* fungi is the distinct group, for example, they can adjust and develop in the fluctuating circumstances of temperature and nutrient accessibility and pH (Kapoor *et al*, 1999).

## CONCLUSION

*Trichoderma harzianum* fungi can be used for the purpose of bioremediation. Since, it retains the ability to tolerate various abiotic stresses i.e., lower pH condition and high concentration of metal. Such fungi possess the capacity and ability to bind heavy metals to their cell walls which enhance the intracellular storage of contaminants. So far, response of *Trichoderma harzianum* like microorganisms against toxic heavy metals plays a crucial role in the retrieval of contaminated areas and making the polluted free environment for us. However, future studies need to

be taken for darwing the possible pthway of chromium biosorption by the fungus and the influence of other physicochemical parameters in phytoremediation process.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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