

# Bioaccumulation of chromium by *Fusarium oxysporum*

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**ABSTRACT:** We investigated the impact of both hexavalent and trivalent chromium on mycelial growth and metal accumulation in the wilt pathogen of tomato (*Fusarium oxysporum* f. sp. *lycopersici*). Experiments were conducted in liquid growth medium amended with 19 concentrations of either Cr(III) or Cr(VI). Metal-amended medium was inoculated with the pathogen and incubated for 15 days at  $25 \pm 3^\circ\text{C}$ , 50 rpm, and at pH 5. Generally, the fungal dry biomass was significantly inhibited by 14–64% and 30–95% with increase in concentrations (5–350 ppm) of Cr(III) and Cr(VI), respectively. The fungus was unable to grow at metal concentrations higher than 400 ppm. The metal accumulation by the fungus decreased with an increase in metal concentration. The fungal biomass exhibited the greater accumulation capacity (0.01–2.6 mg/g) for Cr(VI) than that of Cr(III) (0.01–1.86 mg/g). The bioaccumulation factor of the fungus for both oxidation states of Cr ranged between 0.7 and 0.9.

**KEYWORDS:** heavy metal, wilt pathogen, *Solanum lycopersicum*, tomato

## INTRODUCTION

Heavy metal pollution of water and soil is well-known dilemma of the current century. Among toxic heavy metals, chromium (Cr) is an important industrial metal utilized widely in metallurgy, refractory, plating and tannery industries, wood preservation, and pigment manufacturing<sup>1,2</sup>. Although it exists in many oxidation states, Cr(III) and Cr(VI) are the most stable forms in the environment<sup>3</sup>. Cr(III) mainly exists as hydroxides, oxides, or sulphates, and a small amount is vital for men, animals, and microorganisms. However, its requirement for plants is debatable<sup>4,5</sup>. The divalent oxyanions of Cr(VI) are 100–1000 times more toxic, more soluble in water, and more motile and strong oxidizing agent than Cr(III)<sup>6,7</sup>; hence it has been declared lethal for all living organisms<sup>8</sup>. Thus it is imperative to consider the significance of Cr for soil biota as one gram of soil may comprise 0.005–3 mg of Cr<sup>9</sup>.

In soil, reaction of microorganism varies and heavy metal contamination can shift microbial populations<sup>10,11</sup>. Fungi are more resistant to metal toxicity due to physiological adaptation, while exist as dominant community in metalliferous soil. Nevertheless, under the influence of adverse environmental condition, fungi can survive due to their innate ability to take up the pollutants as nutrients through absorption or accumulation<sup>12</sup>. In adsorption, heavy metals are deposited in the mosaic structure of the

fungal wall that provides excellent metal binding properties<sup>13</sup>. Metals, when accumulated in the cytoplasm of the fungal cells, damage the majority of enzymes and proteins by two principal mechanisms. In an extracellular mechanism, metals enter in cell and bind to ligands (e.g., citrate, oxalate) or cell wall components (chitin, glucan)<sup>14</sup>. The mechanism involves intracellular detoxification through association of metal to intracellular compounds such as metallothionein and glutathione (GSH, a small sulphur containing molecule)<sup>15</sup>.

It has been documented that various heavy metals in the medium bestowed differential responses to various fungal species and isolates of the same genus<sup>16</sup>. So far, soil is a reservoir of both saprophytic and pathogenic mycoflora. Majority of the reports are available on interaction of heavy metals with saprophytic fungi<sup>17</sup>. However, the interaction of heavy metals (particularly Cr) with plant pathogenic fungi is not well known<sup>18</sup>.

*F. oxysporum* is a highly destructive soil-borne ascomycetous fungus that infects all the agricultural and horticultural crops globally in warmer areas<sup>19</sup>. In comparison to other soil-borne fungal pathogens, the wilt pathogens have a more specialized host range (classified as forma specialis) and are adapted to colonize the vascular system of their hosts. Strains that cause wilt disease in tomatoes are classified as f. sp. *lycopersici* (FOL). Seedlings infected by FOL show yellowing of lower leaves,

often only on one side of the plant succeeded by reduced growth and eventually death of the entire plant<sup>20</sup>.

It is hypothesized that when metal laden water is used to irrigate the agricultural fields, these heavy metals interact with phytopathogens present in the respective area. Those microbes may exhibit over or under activity in terms of pathogenicity and finally integrate into the food chain. The toxicology of *F. oxysporum* has been established due to its ability to produce a wide range toxins in plants such as moniliformin, fusaric acids, fumonisins, and trichothecenes<sup>21</sup>. These toxins may become part of the food chain through accumulating in the end product<sup>22</sup>. *F. oxysporum* f. sp. *lycopersici* has the ability to degrade humic substances with subsequent increase in metal solubilization and bioaccessibility to tomato plant<sup>23</sup>. Increase in disease severity caused by *F. oxysporum* f. sp. *radicis lycopersici* increases with an increase in lead uptake by the tomato plant<sup>24</sup>. Thus under the combined stress of the heavy metal and fungal pathogen, the plant could suffer more due to the synergistic action of both stresses. So the presently planned study aimed to investigate the impact of Cr(III) and Cr(VI) on growth, metal accumulation, metal uptake capacity, efficiency, and bioaccumulation factor (BF) in wilt pathogen of tomato, i.e., *F. oxysporum* f. sp. *lycopersici* under laboratory conditions. It is assumed that *F. oxysporum* f. sp. *lycopersici* being a robust pathogen can tolerate high concentration of Cr and later can promote metal uptake by the plant.

## MATERIALS AND METHODS

*F. oxysporum* f. sp. *lycopersici* was aseptically isolated from roots of tomato plants suffering from wilt disease. The fungus was subcultured and maintained on 2% malt extract agar medium and identified on the basis of micrometry and morphological characters<sup>21</sup>.

Stock solutions of Cr(III) and Cr(VI) were prepared by dissolving each 7.69 g chromium nitrate,  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ , and 2.82 g potassium dichromate,  $\text{K}_2\text{Cr}_2\text{O}_7$  (Merk, Germany) in 1000 ml distilled water. Further dilutions of 5, 10, 20, ..., 100, 150, 200, ..., 500 ppm were prepared by adding the appropriate quantity of sterilized distilled water.

Experiments were conducted in 250 ml flask filled with 2% malt extract broth (100 ml) amended with 19 different concentrations of each of Cr(III) and Cr(VI) ranging from 0, 5, 10, 20, ..., 100, 150, 200, ..., 500 ppm. Flasks were inoculated with 2 mm disc of *F. oxysporum* f. sp. *lycopersici* and

incubated at  $25 \pm 3^\circ\text{C}$ , 50 rpm, and for 15 days. Treatments with the fungal inoculum but without addition of Cr(III) or Cr(VI) solutions were the control. Each treatment was done in triplicate and the experiment was performed using completely randomized design. After the incubation period, the biomass of fungus was separated from the culture broth by filtration and subjected to successive washings with double distilled deionized water to remove the culture broth. Dry weights of the fungus were recorded on pre-weighted filter paper.

Remaining Cr in fungal biomass as well as in culture broth was analysed by atomic adsorption spectrophotometry. The sample was digested using 3:1 mixture of concentrated  $\text{HNO}_3$  (69–70%) under reflux at a temperature of  $240^\circ\text{C}$  for 2 h 30 min. The digests were filtered through Whatman No. 1 filter paper and the filtrate was diluted up to 30 ml and subjected to analysis of Cr on Z-5000 Polarized Zeeman atomic adsorption spectrophotometer. The amount of chromium accumulated by fungal biomass and the efficiency of biomass were calculated as  $q = (C_i - C_f)V/m$  and  $E = (C_i - C_f)/C_i$ , respectively, where  $C_i$  and  $C_f$  are the initial and final concentrations of the metallic ion,  $m$  is the fungal mass in the reaction mixture, and  $V$  is the volume of reaction mixture.

The bioaccumulation factor, BF, was calculated as  $\text{BF} = C_m/C_s$ , where  $C_m$  is the metal concentration in fungus and  $C_s$  is the metal concentration added in the growth medium.

All the data were analysed through ANOVA technique and the means were compared using Tukey's test ( $p \leq 0.05$ ) to separate mean differences.

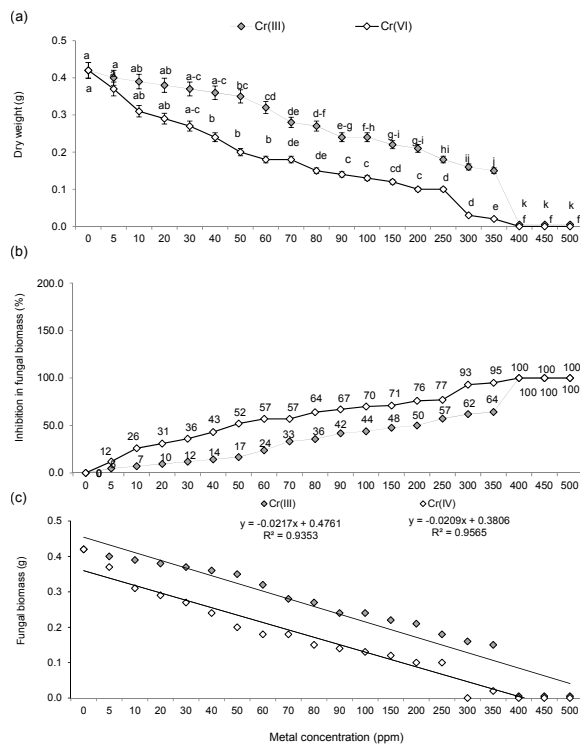
## RESULTS

ANOVA for the data recorded on the effect of nineteen concentrations (5–500 ppm) each of Cr(III) and Cr(VI) on biomass production of *F. oxysporum* f. sp. *lycopersici* is pooled in Table 1. According to ANOVA, the effect of metal oxidation states (MOS)

**Table 1** Effect of Cr(III) and Cr(VI) on the biomass of *F. oxysporum* f. sp. *lycopersici*.

| Sources of variation        | df  | SS   | MS    | F value |
|-----------------------------|-----|------|-------|---------|
| Metal oxidation state (MOS) | 1   | 0.23 | 0.23  | 798*    |
| Metal ions conc. (MC)       | 19  | 1.85 | 0.097 | 335*    |
| MOS × MC                    | 19  | 0.07 | 0.004 | 14*     |
| Error                       | 80  | 0.02 | 0.002 | 0       |
| Total                       | 119 | 2.17 |       |         |

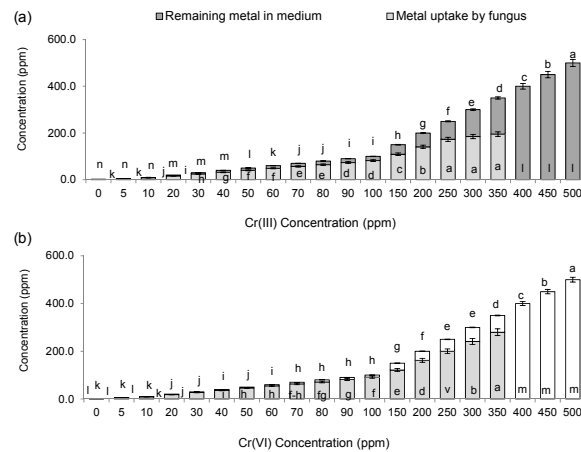
\* significant at  $p \leq 0.001$ .



**Fig. 1** Growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* due to different concentrations of Cr(III) and Cr(VI): (a) dry biomass; (b) percentage decrease in biomass over control; (c) regression analysis for the relationship between different concentrations of metal ions and fungal biomass. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ( $p \leq 0.05$ ) as determined by Tukey's HSD test.

as well as their different concentrations (MC) was highly significant ( $p \leq 0.001$ ). Similarly, interactive effect of the MOS  $\times$  MC was also found to be highly significant ( $p \leq 0.001$ ).

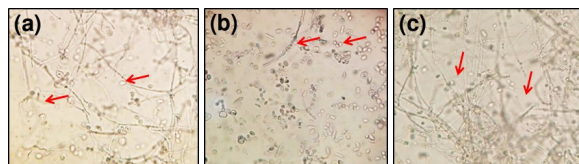
Growth assays against various concentrations of Cr(III) and Cr(VI) were calculated in terms of the percentage of growth inhibition, metal accumulation, metal uptake capacity, efficiency, BF, and regression analysis by the biomass of *F. oxysporum* f. sp. *lycopersici* (Figs. 1–4). Generally, biomass of the fungus exhibited more tendency to accumulate Cr(VI) than Cr(III). The fungus hold more capacity, efficiency, and BF against increasing concentration of Cr(VI) than that of Cr(III). Growth and, therefore, biomass were significantly decreased with increased concentrations of either Cr(III) or Cr(VI) up to 350 ppm, and beyond 400–500 ppm the fungus was unable to grow.



**Fig. 2** Metal accumulation by biomass of *F. oxysporum* f. sp. *lycopersici* at different concentrations of Cr(III) and Cr(VI). (a) Cr(III) accumulation by fungal biomass; (b) Cr(VI) accumulation by fungal biomass. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ( $p \leq 0.05$ ) as determined by Tukey's HSD test.

Under Cr(III) stress, biomass of *F. oxysporum* f. sp. *lycopersici* was insignificantly inhibited by 14% (0.35 g) at concentration range of 5–40 ppm and significantly decreased by 20–60% (0.3–0.15 g) with increase in metal concentration from 50–350 ppm over control (0.42 g) (Fig. 1a and Fig. 1b). At higher concentrations (400, 450, and 500 ppm) of Cr(III), however, the fungus was unable to grow, resulting in 100% growth inhibition. When the growth medium was supplemented with 5–20 ppm of Cr(VI), the fungal biomass was insignificantly declined by 30% over the control. However, biomass of the fungus was significantly reduced by 70% and 95% over the control with further increase in Cr(VI) concentration in the range of 30–100 ppm and 150–350 ppm, respectively, while no growth was observed beyond 350 ppm (Fig. 1a and Fig. 1b). Regression analysis ( $R^2 \leq 0.93$ ) showed a linear relationship between growth of the fungus and increase in concentrations of each of Cr(III) and Cr(VI) within the range of 10–500 ppm (Fig. 1c).

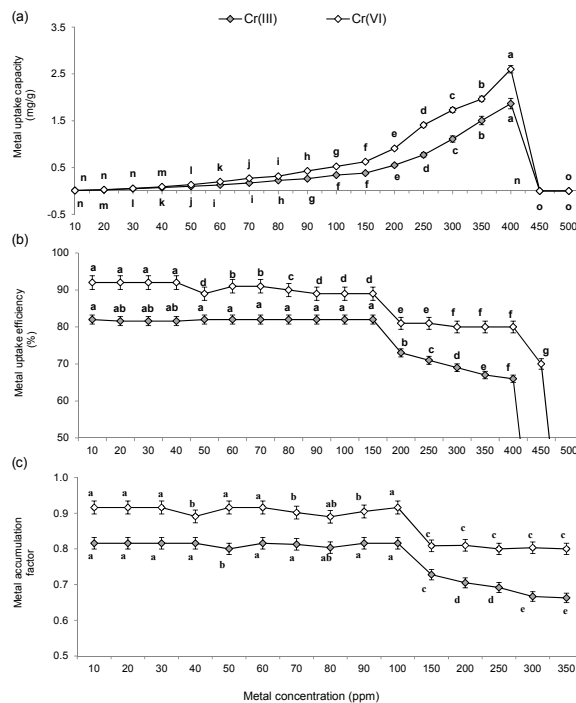
Remaining metal concentration (ppm) detected in the growth medium and in the fungal biomass is presented in Fig. 2a and Fig. 2b. The metal accumulation by the fungal biomass was decreased and the remaining amount in growth medium was increased with an increase in metal concentration. There was no precipitation of metal in the growth medium. The fungus accumulated 70–80% of Cr(III) at con-



**Fig. 3** Microscopic growth characteristics of *F. oxysporum* f. sp. *lycopersici* at 40× due to effect of metal-medium amended with 300 ppm of Cr(III) and Cr(VI) solutions: (a) control (macroconidia with hyaline hyphae and normal septation); (b) Cr(III) (shrinking of microconidia along with compartmentalization of hypha); (c) Cr(VI) (few micro conidia with shrunken hyphae and extensive hyphal branching).

centration range of 10–150 ppm and the rest of metal was detected in the growth medium. At 200 and 250 ppm, the fungus accumulated 70%, and at 300 and 350 ppm, the fungus accumulated about 66% Cr(III) (Fig. 2a). At 300 ppm of Cr(III), *F. oxysporum* f. sp. *lycopersici* showed reduction in size of microconidia along with compartmentalization of hypha with few macroconidia (Fig. 3a and Fig. 3b) as compared to the control (Fig. 3a). The fungus held greater tendency to accumulate Cr(VI) than Cr(III). Thus metal accumulation was found within a range of 80–90% due to Cr(VI) concentration regimes of 5–350 ppm (Fig. 2b). At 300 ppm of Cr(VI), orange colour of medium changed to slight yellow shade. Microscopic examination showed that the number and the size of microconidia were declined with shrunken and increased hyphal branching, while no macroconidia were observed as compared to the control (Fig. 3a and Fig. 3c). However, with further increase in either Cr(III) or Cr(VI) concentration above 350 ppm, 100% of the metal was detected in the growth medium.

Results derived from the metal accumulation data were used to calculate fungal metal uptake capacity and efficiency (Fig. 4a and Fig. 4b). Fungal biomass exhibited the greater accumulation capacity (0.01–2.6 mg/g) for Cr(VI) than that of Cr(III) (0.01–1.86 mg/g). Likewise, metal uptake efficiency was greater (80–90%) due to the effect of various concentrations of Cr(VI) than that of Cr(III) (70–80%). The fungus exhibited good BF at lower concentrations of metal as compared to higher doses, therefore it could be classified as moderately tolerant to Cr stress. BF of the fungus was 0.90 due to Cr(VI) concentrations of up to 100 ppm, and 0.81 up to concentrations of 350 ppm. On the other hand, BF of the fungus was 0.8 up to 100 ppm and



**Fig. 4** Influence of Cr(III) and Cr(VI) on (a) metal uptake capacity, (b) uptake efficiency, and (c) BF of *F. oxysporum* f. sp. *lycopersici*. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ( $p \leq 0.05$ ) as determined by Tukey's HSD test.

0.7 up to 350 ppm of the Cr(III) (Fig. 4c).

### DISCUSSION

It was found that *F. oxysporum* f. sp. *lycopersici* is sensitive to both Cr(III) and Cr(VI), thus exhibited high accumulation of total Cr at low concentration with reduction in growth<sup>25</sup>. Reduction in growth of *F. oxysporum* f. sp. *lycopersici* with increase in Cr concentration within the range of 5–350 ppm and then absence of growth at metal concentration > 350 ppm could be due to the toxic effect of the metal<sup>26</sup>. Low concentrations of Cr(III) or Cr(VI) were stimulatory for the growth of *F. oxysporum* f. sp. *lycopersici* and vice versa with increase in the metal concentration<sup>27</sup>. The reduction in growth of fungus and change in microscopic features of the fungus at higher metal concentrations could be attributed to internalization of metals in the cytosol, where it probably disrupts cell integrity and cell functions (e.g. mitochondrial electron transport chain, glycolysis, and oxidative phosphorylation) and cause growth retardation<sup>28</sup>. Besides, loops

and connective filament development together with an increase of hyphal branching indicate an increasing concentration of the metal in fungus<sup>29</sup>. Moreover, metal displacing essential metal with the contaminant metal and mutation of biomolecule consequences in failure of fungus to perform normal activities. Whereas, no growth of fungus at the highest range of metal concentrations possibly occurs owing to non-sporulation of *F. oxysporum* f. sp. *lycopersici* mycelium under extreme toxicity level or prolongation of lag phase.

The greater toxicity of Cr(VI) compared with Cr(III) may result from its more mobile ability; usually, Cr(VI) can get into the cells of the fungus more easily and cause oxidative damage during its reduction<sup>30</sup>. It could be due to the fact that microorganisms require Cr(III) in minute amounts. So this trivalent form probably incorporates into the fungus whereas no machinery is available for hydrolysis of Cr(VI), hence incorporation of hexavalent Cr directs all the resources towards initialization of defence response in fungi and lesser energy is left for growth. Further Cr(VI) toxicity was reported to link with its specific antagonism to sulphate uptake, whereas Cr(III) toxicity resulted from antagonism with iron transport<sup>31</sup>.

It was noticed that fungus exhibited 90% efficiency to uptake Cr(VI) at concentration range of 5–100 ppm, while efficiency declined by 12% at the range of 150–350 ppm. The fungus was able to uptake 80% of the Cr(III) at 5–100 ppm, and approximately 70% within the range of 100–350 ppm. However, at the remaining concentrations of 350, 400, and 500 ppm of both Cr(III) or Cr(VI), 100% of the metal was detected in the growth medium with no growth of the fungus. Effluent loaded with noxious organic compounds was found hinder the fungus growth and enhance heavy metal uptake<sup>32</sup>. In another study<sup>26</sup>, 90% of the Cr(VI) was found to be accumulated by *F. oxysporum* at 100–200 ppm, 80% for 300–400 ppm, and up to 65% for 500–1000 ppm. Likewise, 100% removal of Cr(VI) by different soil fungi including *Fusarium* sp. was recorded<sup>33</sup>. Greater metal accumulation at low metal concentration might be due to the presence of essential energy metabolism and energy coupled transport system similar to metallothionein function<sup>34</sup>. However, at low metal concentration fungus could detoxify metal by complexing it with phosphate analogues, carboxyl groups or histidine analogues<sup>35</sup>. There is also a possibility of increased melanin production in the growth medium amended with Cr that may in turn increase the metal binding

ability of the fungus. Besides, reduction in metal accumulation capacity of the fungus with increase in either Cr(III) or (VI) concentrations might be owing to saturation of the fungus metal-binding sites<sup>36</sup>. At low metal concentrations (5–100 ppm), there could be more binding sites for complexation of chromium with fungal biomass. However, at higher metal concentrations (> 100), toxicity of metal was likely to reduce fungal biomass, increase competition for less binding sites and therefore resulted in low metal uptake efficiency by the fungus<sup>37</sup>.

Growth medium supplemented with Cr(VI) caused change in colour of medium from orange-yellow to its lighter shade with the advent of incubation time. The maximum discoloration was observed at the 15th day of incubation. No alteration in colour was observed in case of Cr(III)-amended medium. Besides, no change in colour of the growth medium was noticed at concentrations above 300–350 ppm where the fungus was unable to grow. The fungus likely to utilize carbon source in the growth medium as one of the efficient electron donors to reduce Cr(VI) and was accompanied by a change in colour of the medium<sup>25</sup>.

The BF represents the pollutant concentration in fungus comparing with the environment concentration<sup>38</sup>. A value of BF greater than 1 is considered to be an efficient tool in the polluted soil bioremediation. Presently, BF of the fungus (BF: 0.7–0.9) was comparatively better at low concentrations (> 300 ppm), therefore the fungus could be ranked as moderately tolerant to Cr stress.

## CONCLUSIONS

*F. oxysporum* f. sp. *lycopersici* is moderately tolerant to both Cr(III) and Cr(VI). Increase in metal concentration within the range of 5–350 ppm resulted in gradual reduction in fungal growth and the fungus was unable to grow at 400–500 ppm. The pathogenic fungus accumulated more metal at lower than at higher concentrations. Furthermore, the fungus exhibited more tendency to accumulate Cr(VI) than Cr(III).

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