Chromium toxicity to tomato (*Lycopersicum esculentum* Mill) susceptible to Fusarium wilt pathogen

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Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), and toxicity of Cr(III) and Cr(VI) in agricultural soils adversely affect growth and physiology of tomato (*Lycopersicum esculentum* Mill). The present study was conducted *in vitro* to assess growth and physiology of tomato under single and combined stress of conidial suspension of FOL, and Cr(III) or Cr(VI). Polygonal interactions of metal–pathogen–plant were investigated in Petri plates lined with sterilized filter papers provided with conidial suspension of FOL (1×10^6) and six different concentrations (50–300 ppm) of Cr(III) and Cr(VI) under controlled laboratory conditions in completely randomized design. Maximum inhibition in growth, biomass and chlorophyll contents of tomato seedlings was due to conidial suspension of FOL. Peroxidase activity increases while catalase activity decreases significantly due to conidial suspension of FOL. The toxicity of Cr ions was influenced by their concentration in the solution and speciation. Therefore, growth, biomass and physiology of tomato seedlings were more significantly affected by ions of Cr(VI) than those of Cr(III). The drastic influence of both oxidation states of metal ions increases with increase in concentration of the metal ions. When FOL was given in combination with Cr(III) or Cr(VI), negative effect on the studied parameters of tomato seedlings was less pronounced compared to solitary influence of either FOL or Cr ions.

Keywords: Chromium, *Fusarium oxysporum*, physiological response, tomato growth and yield.

TOMATO (family Solanaceae), native to Central and South America, is one of the most popular and significant commercial horticulture crops grown throughout the world next to potato[1]. Pakistan ranks 35th among the tomato-producing countries, with annual production of 468.14 thousand tonnes cultivated on 46.23 thousand hectares. Punjab, Sindh, Khyber Pakhtun Khwa and Balochistan provinces have 14%, 10%, 35% and 45% share respectively, in tomato production[2]. More than 200 pathogens can drastically affect tomato production and yield[3]. *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd & Hans is a highly destructive phytotoxic soil-inhabiting fungus that deteriorates tomato growth and yield on a massive scale by causing Fusarium wilt[4,5]. The toxicological interest in *F. oxysporum* arises from its ability to produce a wide range of chemically diverse toxic compounds, like moniliformin, fusaric acids, beauvericin, fumonisins, enniatin and trichothecenes that impede normal plant growth[6]. Further, accumulation of *Fusarium* toxin in the end-product can be dangerous for both human and animal health[7].

Chromium (Cr) is a transitional heavy metal that exists in different oxidation states ranging from 2 to 6, but Cr(III) and Cr(VI) are the most dominant because of their immovability in natural environment[8]. Many countries, including Pakistan are facing problems of contamination of soil and water with Cr from different sources, including electroplating, tanning industries and textiles[9]. A higher level ranging from 40 to 2000 ppm of Cr has been reported in soil and water of different areas in Pakistan[10–12], and its concentration above 0.05 ppm is considered to be toxic for humans[13]. Although Cr(III) is vital for humans and animals[14], its essentiality for plants is debatable[15]. Cr(III) ions at higher concentrations are known to induce Fe-type deficiency response, thus altering plant water relation and decreasing physiological accessibility of water[15,16]. By contrast, Cr(VI) is toxic for both plants and animals. In plant species, Cr(VI) can damages roots, decrease enzyme activity, cause necrosis, chlorosis and decline plant growth[17,18].

Plants may suffer simultaneously from the wilt disease and metal toxicity under contaminated soil conditions. However, toxic effect of Cr on tomato with reference to wilt disease is poorly understood. The metal can arrest the growth and multiplication of pathogenic fungi, and also limit the growth and various metabolic activities of plant, including photosynthesis, synthesis of proteins and enzymes[19]. Based on the above arguments, the present study was undertaken to analyse the effect of different concentrations of Cr(III) or Cr(VI), either alone or combined with conidial suspension of FOL on the growth, chlorophyll content as well as catalase and peroxidase activities of tomato seedlings.

FOL was isolated from infected tomato plants collected from tomato fields. The fungus was sub-cultured on 2% malt extract agar (MEA) medium and the seven-day-old culture was utilized for preparation of conidial suspension. A heamocytomter was used for conidial count and 1×10^6 conidia were used in each treatment.

Cr(III) and Cr(VI) stock solutions were prepared by dissolving 2.82 g potassium dichromate ($K_2Cr_2O_7$) and 7.69 g chromium nitrate ($Cr(NO_3)_3 \cdot 9H_2O$; Merk, Germany) in 1000 ml of double-distilled water respectively. Further dilutions of 50, 100, 150, 200, 250 and 300 ppm of Cr(III) and Cr(VI) were prepared from the stock solutions by diluting them appropriately with double-distilled sterilized water to make a final volume of 100 ml of each concentration of metal.

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Seeds of tomato variety LA-2662 were procured from Vegetable Research Institute, Ayub Agriculture Research Institute, Faisalabad, Pakistan. Healthy, sterilized tomato seeds were placed in pre-sterilized Petri plates (9 cm) with filter papers moistened with 3 ml of each of the six different concentrations of Cr(III) and Cr(VI) solutions. In another set of Petri plates, 1.5 ml of all the above-mentioned metal concentrations along with 1.5 ml of conidial suspension (1×10^6) of FOL were added. A third set of Petri plates was supplied with 3 ml of conidial suspension (1×10^6) of FOL. Control treatment received 3 ml of distilled sterilized water. Each treatment was replicated thrice. There were 25 treatments in total. The Petri plates were kept at 22° ± 2°C in a completely randomized design. Data regarding germination, length of shoot and root, fresh and dry weight of seedlings were recorded 15 days after germination.

Chlorophyll extraction was performed by homogenizing 500 mg fresh leaf material with 10 ml of chilled 80% acetone. The resultant homogenate was centrifuged at 800 rpm for 15 min and supernatant was analysed for chlorophyll content[20]. For extracting antioxidant enzymes, leaves (0.5 g) were ground in chilled mortar using phosphate buffer (50 mM). Homogenate was centrifuged at 13,000 rpm for 20 min at 4°C and supernatant was further employed for enzyme activity assays. For catalase (CAT) activity, 1.0 ml supernatant was added to the reaction mixture containing 3.0 ml of 0.1 M phosphate buffer (pH 6.8) and H_2O_2 (l ml of 0.01 M). Ten millilitres of 2% H_2SO_4 was added after l min at 20°C. The reaction mixture was titrated against 0.005 N $KMnO_4$ to determine the quantity of H_2O_2 utilized by the enzyme. Catalase activity was expressed as number of moles of H_2O_2 utilized $min^{-1}mg^{-1}$ protein[21]. Peroxidase (PO) activity was determined by taking enzyme extract (0.5 ml) in 2 ml of 0.1 M phosphate buffer (pH 6.8) and 1 ml of 0.01 M pyrogallol. Solution was filled with 0.05 M H_2O_2 (5 : 5 in H_2O_2 and distilled water), incubated at 25°C and the reaction was stopped by adding 2.5 N H_2SO_4 (24.5 ml of H_2SO_4 + 100 ml of distilled water). Absorbance was recorded at 420 nm to determine the amount of purpurogalline formed against blank. One unit of enzyme activity is defined as the amount of the enzyme that results in 50% inhibition of the auto-oxidation rate of pyrogallol at 25°C. The enzyme activity is expressed as unit mg^{-1} protein[22].

All the data were analysed by analysis of variance followed by Tukey's HSD test using the software Statistics 8.1.

There were no disease or metal toxicity symptoms in control treatments at the 15th day of growth, and all other treatments significantly suppressed/altered plant growth, biomass and physiology. Therefore, the maximum germination (100%), length (7 cm), fresh weight (870 mg) and dry weight (83 mg) of tomato seedlings were recorded in control treatment. The highest reduction in plant growth and biomass was recorded due to conidial suspension of FOL. Whereas conidial suspension of FOL showed no drastic effect on germination rate, while this treatment significantly reduced length of shoot and root by 40% and 70%, respectively, and fresh and dry weight of seedlings by 80% and 40% respectively, over control. The highest inhibitory effect of conidia suspension of FOL on overall growth and biomass of tomato seedling indicated the extreme sensitivity of the plants. Presently, infection of FOL seems to be responsible for post-emergence mortality in tomato plants that was probably not interlinked with internal infection of seeds[23]. It has been documented that microconidia of the *F. oxysporum* germinate by root exudates of host and non-host tomato plants[24]. Even in some cases stimulation in conidial germination has been reported due to root exudates[25]. Therefore, it could be speculated that conidia of FOL germinate and invade intercellularly in the cortex and xylem colonizing the vascular tissue due to increased exudation from the roots of the tomato plant. Inside the plant, FOL probably plugs and ultimately destroys the whole xylem vessels[26] and would likely produce toxins that would cause disturbance in plant functioning and facilitate nutrients leakage from the macerated plant tissues. Therefore, nutrients leakage from the macerated tissue could probably be utilized by the pathogen with consequences of reduction in seedling growth and biomass[19].

The inhibitory effect of Cr(III) and Cr(VI) on seedling growth and biomass was more pronounced at higher concentrations, and Cr(VI) showed more inhibition on studied parameters than Cr(III). Also, Cr(III) alone, induced insignificant effect on germination rate in the concentration range 50–200 ppm, while it considerably dropped by 10% at both 250 and 300 ppm. Shoot and root length declined progressively and significantly by 10–50% and seedling weight (fresh and dry) decreased significantly by 20–70% with increase in Cr(III) concentration (50–300 ppm) over control (Figure 1 *a*–*e*). When Cr(VI) alone was supplied, there was significant decline of 10–30, 10–44, 20–98, 40–90 and 40–76% in germination rate, shoot length, root length, fresh weight and dry weight respectively, with increasing Cr(VI) concentration (50–300 ppm) compared to control (Figure 2 *a*–*e*). Cr(VI) was found more toxic than Cr(III), because the former is a strong oxidizing agent, corrosive, water-soluble, readily transported and available to the plants[27,28]. Further, Cr(III) uptake was found to be passive while that of Cr(VI) was active[29]. Increasing concentration of ions of either oxidation state of the metal was observed to be toxic possibly because of the adverse effect of Cr on auxins synthesis on tomato seedlings[30]. The reduction in germination of tomato seeds under Cr toxicity at a given concentration could be an inhibitory action of Cr on the amylase activity that consequences in interruption of sugar transport to the embryo axes[31]. Direct contact of roots with Cr ions possibly causes oxygen depletion and

prevents from absorbing water with inhibition of cell division resulting in reduced root growth and biomass. The toxic influence of Cr on root growth may alter the transport of important nutrients (e.g. Ca, K, Mg) across the plasma membrane into cytoplasm which in turn interrupts the physiological activity. Lesser nutrients and water transport to the aerial plant parts also inhibit shoot growth[32]. Overall inhibition in plant growth results in less dry biomass, similar to the problems faced by plants growing in Cr-contaminated soil; therefore, fewer yields would be expected from such plants.

FOL in combination with Cr showed significant reduction in the studied parameters compared to control. However, the reduction was less to those obtained with either pathogen or metal alone. Germination rate and shoot length were statistically insignificantly different due to Cr(III) ions combined with conidial suspension of FOL over the concentration range 50–300 and 50–100 ppm respectively. However, lengths of shoot and root declined significantly by 10–50 and 30–40% respectively, with increase in Cr(III) concentration from 100 to 300 ppm combined with conidial suspension of FOL. Whereas

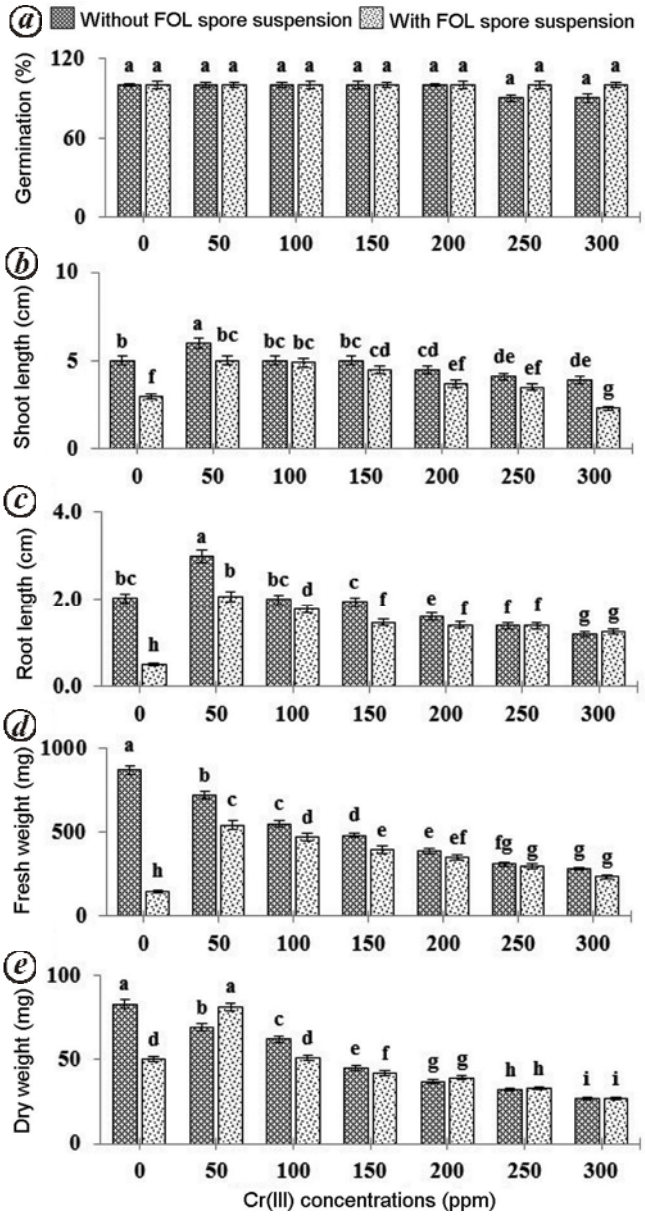

Figure 1. ***a–e***, Effect of different concentrations of Cr(III) and *Fusarium oxysporum* f. sp. *lycopersci* (FOL) on growth and biomass of *Lycopersicum esculentum*. Values with different letters on the top show significant difference ($P \leq 0.05$) as determined by Tukey's HSD test.

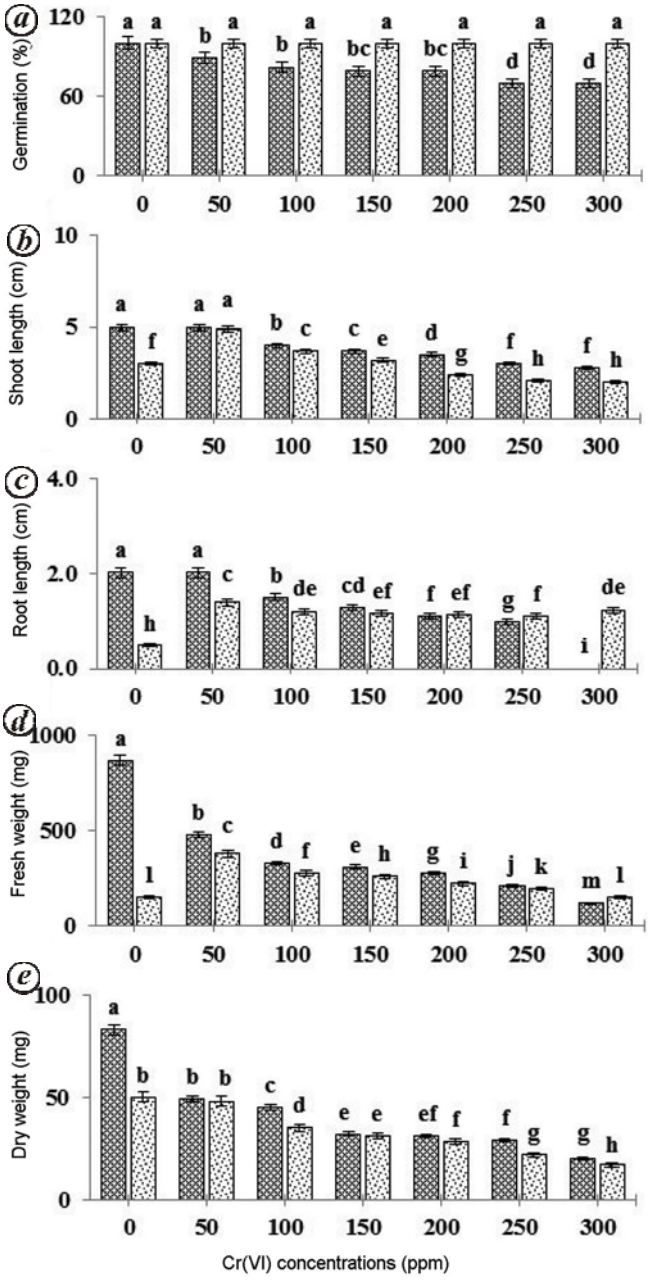

Figure 2. ***a–e***, Effect of different concentrations of Cr(VI) and *Fusariumoxysporum*f. sp. *lycopersci* (FOL) on growth and biomass of *L. esculentum*. Values with different letters on the top show significant difference ($P \leq 0.05$) as determined by Tukey's HSD test.

Table 1. Physiology of tomato under single or combined action of conidial suspension of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and Cr(III)

Treatment	Chlorophyll content (mg g^{-1})	Peroxidase activity (unit min^{-1} g^{-1})	Catalase activity (unit min^{-1} g^{-1})
Control	0.089 ± 0 a	0.25 ± 0 a	5.5 ± 0 i
SS of FOL	0.07 ± 0.01 de	0.5 ± 0.01 a	3.5 ± 0.01 l
50 ppmCr(III)	0.085 ± 0.01 a	0.42 ± 0.01 a	6.3 ± 0.01 g
100 ppm Cr(III)	0.082 ± 0.01 ab	0.45 ± 0.01 a	6.9 ± 0.01 f
150 ppm Cr(III)	0.079 ± 0.01 bc	0.51 ± 0.01 a	7.1 ± 0.01 e
200 ppm Cr(III)	0.079 ± 0.01 bc	0.64 ± 0.01 a	7.2 ± 0.01 d
250 ppm Cr(III)	0.077 ± 0.01 bc	0.72 ± 0.01 a	7.4 ± 0.01 c
300 ppm Cr(III)	0.074 ± 0.01 cd	0.82 ± 0.01 a	7.6 ± 0.01 b
FOL + Cr(III) 50 ppm	0.076 ± 0.01 c	0.27 ± 0.01 a	5.1 ± 0.01 j
FOL + Cr(III) 100 ppm	0.071 ± 0.01 de	0.31 ± 0.01 a	5.9 ± 0.01 k
FOL + Cr(III) 150 ppm	0.066 ± 0.01 e	0.34 ± 0.01 a	6.2 ± 0.01 h
FOL + Cr(III) 200 ppm	0.061 ± 0.01 f	0.39 ± 0.01 a	7.1 ± 0.01 e
FOL + Cr(III) 250 ppm	0.054 ± 0.01 g	0.41 ± 0.01 a	7.4 ± 0.01 c
FOL + Cr(III) 300 ppm	0.048 ± 0.01 h	0.46 ± 0.01 a	7.8 ± 0.01 a

Values with different letters show significant difference ($P \leq 0.05$) as determined by Tukey's HSD test.

Table 2. Physiology of tomato under single or combined action of conidial suspension of FOL and Cr(VI)

Treatment	Chlorophyll content (mg g^{-1})	Peroxidase activity (unit min^{-1} g^{-1})	Catalase activity (unit min^{-1} g^{-1})
Control	0.089 ± 0 a	0.25 ± 0 a	5.5 ± 0 a
SS of FOL	0.081 ± 0.01 b	0.39 ± 0.06 i	3.5 ± 0.01 de
50 ppmCr(VI)	0.069 ± 0.02 d	0.45 ± 0.05 g	4.5 ± 0.04 b
100 ppm Cr(VI)	0.061 ± 0.03 e	0.51 ± 0.03 e	4 ± 0.05 c
150 ppm Cr(VI)	0.059 ± 0.05 e	0.85 ± 0.02 c	3.7 ± 0.03 cd
200 ppm Cr(VI)	0.05 ± 0.06 f	0.87 ± 0.07 c	3.5 ± 0.06 de
250 ppm Cr(VI)	0.047 ± 0.07 f	0.89 ± 0.04 b	3.2 ± 0.07 e-g
300 ppm Cr(VI)	0.042 ± 0.03 g	0.96 ± 0.05 a	2.9 ± 0.01 gh
FOL + Cr(VI) 50 ppm	0.087 ± 0.02 a	0.31 ± 0.06 j	3.1 ± 0.04 fg
FOL + Cr(VI) 100 ppm	0.084 ± 0.04 b	0.33 ± 0.04 j	3.3 ± 0.05 ef
FOL + Cr(VI) 150 ppm	0.083 ± 0.05 b	0.39 ± 0.03 i	3.1 ± 0.07 fg
FOL + Cr(VI) 200 ppm	0.8 ± 0.02 b	0.42 ± 0.02h	2.9 ± 0.05 hi
FOL + Cr(VI) 250 ppm	0.075 ± 0.06 c	0.48 ± 0.01 f	2.6 ± 0.04 hi
FOL + Cr(VI) 300 ppm	0.071 ± 0.04 d	0.59 ± 0.03 d	2.4 ± 0.03 i

Values with different letters show significant difference ($P \leq 0.05$) as determined by Tukey' HSD test.

both fresh and dry weights of seedling decreased significantly by 40%–70% due to 50–300 ppm of Cr(III) + conidial suspension of FOL (Figure 1 *a*–*e*). Whereas under simultaneous stress of conidial suspension of fungus + Cr(VI), the germination percentage was insignificantly different due to metal concentration range 50–300 ppm and shoot length was also non-significantly affected at 50 ppm. However, when metal concentration was increased from 100 to 300 ppm, length of shoot was significantly reduced by 30%–60%. Root length, and fresh and dry weights decreased significantly by 30–50, 60–80 and 40–80%, respectively with increase in metal concentration from 50 to 300 ppm (Figures 1 and 2 *a*–*e*). The results reveal that when FOL is present in combination with Cr ions, low concentration of metal ions, i.e. 50 and 100 ppm, particularly of Cr(III), does not show significant inhibitory effect on root and shoot length, where biomass has drastically decreased. It has been documented that FOL is sensitive to both Cr(III) and Cr(VI), thus exhibiting high accumulation of total Cr at low concentration with reduction in its own growth[33]. Therefore, it could be assumed that at low concentration of Cr, both the fungus and the metal compete with each other, causing fragile tomato seedlings without affecting length of tomato seedling. Whereas, fragile seedlings cause reduction in seedling biomass. However, when metal concentrations is increased, Cr likely inhibits conidial germination (micro and macro conidia) and mycelial growth through blocking and conformational modification of essential function groups in the fungus[34]. It has been reported that Cr affects the mycelial growth of fungus at its initial growth stage[35]. Therefore, it could be assumed that under combined stress of FOL and higher concentration of Cr, tomato seedling suffers due to Cr

rather than the fungus. This is further evident from the absence of fungal conidial and typical disease symptoms on tomato seedling.

The highest chlorophyll content (0.089 mg g^{-1}) was recorded in control. Inoculation of conidial suspension of FOL significantly decreased chlorophyll content over control by 10%. Different concentrations of Cr significantly decreased the studied parameter to variable extent compared to control. There was significant reduction of 5–16% (0.085–0.074 mg g^{-1}) and 2%–20% (0.087–0.071 mg g^{-1}) in total chlorophyll content with increase in concentrations of Cr(III) and Cr(VI) respectively, within the range 50–300 ppm. When conidial suspension of FOL is given in combination with different concentrations of either Cr(III) or Cr(VI), the content of total chlorophyll reduced significantly by 20%–50% over control (Tables 1 and 2). Many workers have reported activation of defence related enzymes like phenylalanine ammonia lyase and peroxidase in plants due to infection by phytopathogens[36,37]. Whereas fusaric acid, fumonisins, beauvericin, enniatin and trichthecenes produced by *F. oxysporum* generally induce destruction of thylakoid membrane, increase in respiration and invertase enzyme activity leads to reduced chlorophyll content and rest of the plant physiological activities[38]. Reduction in chlorophyll content of tomato seedlings in the presence of Cr may result from high level of lipid peroxidation mediating cell damage along with inactivation of enzymes involved in chlorophyll biosynthesis pathway in plants[32]. However, chlorophyll content could also be reduced due to degradation of animo-levulinic acid dehydratase that decreases the availability of prophobilinogen required for chlorophyll biosynthesis[39].

Inoculation of the conidial suspension of FOL caused decrease in catalase activity by 40% (3.5 U min^{-1} mg^{-1} protein) over control (5.5 U min^{-1} mg^{-1} protein). Incorporation of 50–300 ppm of either Cr(III) and Cr(VI) alone, significantly increased the said parameter by 10%–20% (5.9–6.5 U min^{-1} mg^{-1} protein) and 15%–40% (6.3–7.6 U min^{-1} mg^{-1} protein), respectively compared to control. Under combined stress of conidial suspension of FOL and different concentrations of Cr(III), catalase activity was non-significantly different at 50 and 100 ppm and increased by 10%–30% in the concentration range 100–300 ppm. There was non-significant effect of 50 ppm of Cr(VI) in combination with conidial suspension of FOL, while the studied parameter increased significantly by 10–40% over concentration range 100–300 ppm compared to control. The lowest peroxidase activity was recorded for control (0.25 U min^{-1} mg^{-1} protein). Conidia suspension of FOL significantly increased this parameter up to two-fold, and different concentrations (50–300 ppm) of Cr(III) and Cr(VI) enhanced it by 2–3-fold and 3–4-fold, respectively. Under combined stress of pathogen and Cr(III), peroxidase activity increased significantly up to two-fold and pathogen along with Cr(VI) improved the studied parameters up to five-fold over control (Tables 1 and 2). Catalase and peroxidase enzymes have been documented as potent scavengers of superoxide radicals in the cell that acts by preventing the accumulation and diffusion of H_2O_2 across the cell membrane and prevents oxidative damage to the cell constituents. The reduction in activity of catalase due to FOL indicates cytotoxicity due to overproduction of reactive oxygen species[40]. Peroxidase activity is generally induced against stress and in plant–fungal interaction, it is likely to limit the spread of fungal infection in plant cell (hypersensitive response)[41]. However, in the present study it seems that activation of peroxidase is linked with increase in susceptibility of the plant to fungal infection[42]. The enhancement in peroxidase as well as catalase activity due to Cr ions either alone or combination with FOL stress might have been a direct reaction to the generation of superoxide radical. The hyperactivity of peroxidase and catalase under Cr-contaminated wastewater has been implicated as constant detoxification of H_2O_2 (ref. 32). Higher increase noticed due to Cr(VI) indicates greater generation of singlet oxygen than Cr(III) or pathogen action[16].

We conclude that maximum reduction in plant growth, biomass and physiology occurred due to conidial suspension of FOL. When FOL was present in combination with Cr(III) or Cr(VI), drastic effect on seedling growth, biomass and physiology was less pronounced compared to solitary influence of either FOL or Cr ions. The Cr ions were more toxic for tomato seedlings when present at higher concentration (100–300 ppm). Also Cr(VI) showed a more drastic effect than Cr(III).

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