The Journal of Animal & Plant Sciences, 24(4): 2014, Page: 1241-1245
ISSN: 1018-7081

# *IN VITRO* TOXICITY EVALUATION OF CULTURE FILTRATES OF *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI* ON GROWTH AND PHYSIOLOGY OF TOMATO UNDER CHROMIUM (VI) STRESS

S. Khurshid, A. Shoaib and A. Javaid

Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan.
Corresponding Author: drsaba16@gmail.com

## ABSTRACT

*In vitro* toxicological influence of culture filtrates of *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & Hans was investigated on seed germination, seedling growth and physiology of tomato (*Lycopersicum esculentum* Mill.) under chromium(VI) stress. Original culture filtrates of the fungus and three concentrations of Cr(VI) viz. 50, 75 and 100 mg $L^{-1}$ were used in laboratory bioassays either alone or in different combinations. Experiment was carried out at 25 ± 2 °C in triplicate using completely randomized design and different growth and physiological assays were recorded in 15-days old tomato seedlings. Germination, growth and biomass was declined significantly up to 40%, 85% and 70% due to original culture filtrate of the *F. oxysporum* and by 40-60%, 10-50%, 50-80% under combine stress of original culture filtrate of the fungus along with increasing concentration of Cr(VI), and by 10-20%, 0-25% and 30-60% due to increasing concentration of Cr(VI) alone. Chlorophyll contents was significantly declined due to original culture filtrate of the *F. oxysporum* f. sp. *lycopersici* alone or combined with Cr(VI), while non-significantly different due to solitary effect of Cr(VI) over control treatments. Whereas, catalase and peroxidase activities increased significantly in treatments supplemented with Cr(VI) alone or combination with fungus and decreased due to culture filtrate of fungus alone as compared to control. The present study concluded that culture filtrates of *F. oxysporum* f. sp. *lycopersici* are hazardous to tomato seedlings either alone or in combination with Cr(VI).

**Key words:** Chromium, fungal culture filtrates, *Fusasium oxysporum*, *Lycopersicum esculentum*, tomato.

## INTRODUCTION

Toxicogenic fungi mostly belong to genus *Aspergillus, Penicillium* and *Fusarium* with ability to utilize variety of substrates to produce their toxins as low molecular weight secondary metabolites during metabolic processes (Jalonder and Gachonde, 2011). The metabolites are products of some cyclic peptides, phenols, and plant growth regulators (Madhosing, 1995). *Fusarium oxysporum* f. sp. *lycopersici*, the most common soil-borne ascomycetous fungus is notorious for causing wilt in tomatoes (Park *et al.,* 2013). The fungus provoke devastating losses in agriculture and lead to food contamination by producing well-known biologically active mycotoxins like fusaric acids, fumonisins, beauvericin, enniatin, moniliformin and trichothecenes. These fungal toxins are known to cause destruction of plants by causing necrosis, chlorosis, wilting and sometimes by inhibiting seed germination (Idris *et al.,* 2003). Mycotoxins have been recognized as environmental pollutants that are present virtually in all parts of the world and their toxicology in food arising life-threatening infections in humans have been documented globally (López-Berges *et al*., 2013).

Due to extensive industrialization, chromium (Cr) contamination has become a setback in Pakistani agriculture (Rizwan *et al*., 2009). Cr(VI) is considered as the second most common contaminant in groundwater, soil and sediments (Kar *et al*., 2008). The water quality of major cities of Pakistan is deteriorating because of unchecked disposal of Cr loaded untreated tannery effluent that left residual effects in food chain (Qadir *et al.,* 2008). Discharge rate of effluent only from tanneries is approximately 1.1 million liters per day. In fact, there is no safe level of Cr and even a very dilute content can cause adverse health effects (Sen and Ghosh, 2010). Plants, the primary producers are the first ones that are being exposed to the contaminants present in the soil. This phtotoxicity causes chlorosis, tissue necrosis, reduced enzyme activity, damage to membrane and diminished photosynthesis (Srivastava and Thakur, 2006; Scoccianti *et al*., 2008).

Tomato (family Solanaceae) is the most intensively studied model species for plant-pathogenic interaction as well as for biomechanical studies (Zamir and Giuliano, 2012). It is the most ubiquitous vegetable of Pakistan with the annual production of 529.6 thousand tones, cultivated on an area of 52.3 thousand hectares (Anonymous, 2011). Cultivation of tomato has been limited by a variety of biotic (fungal, bacterial, viral, nematodes diseases) and abiotic (environmental factors) deteriorating factors (Saeed and Khan, 2011). Earlier studies were focused on the negative response of tomato to either Cr or *F. oxysporum* f. sp. *lycopersici* stress alone

(Liu *et al.,* 2008; Maia *et al*., 2011). However, the growth and physiological response under simultaneous stress of toxin of fungal pathogen and metal needs to be addressed. The present study was, therefore, accomplished to investigate the solo and simultaneous influence of culture filtrates of *F. oxysporum* and Cr(VI) on tomato seedling growth and physiology.

## MATERIALS AND METHODS

**Preparation of fungal culture filtrates:** *F. oxysporum* f. sp. *lycopersici* was isolated from infected tomato plants collected from tomato fields. The fungus was cultured on 2 % malt extract agar medium and identified on the basis of morphological characters (El-Kazaz *et al*., 2008). Fungal culture filtrate was prepared by inoculating 100 mL autoclaved malt extract broth with 2 mm *F. oxysporum* f. sp. *lycopersici* disc in 250-mL flasks, incubated for 10 days under constant shaking at 100 rpm (25 ± 3 ºC; pH 5.5). Fungal culture filtrate was collected by filtration of the fungal mat through Whatmann filter paper No.1.

**Preparation of metal solutions:** A stock solution of 1000 mg $L^{-1}$ of potassium dichromate ($K_2Cr_2O_7$) (Merk, Germany) was prepared by dissolving 2.825 g of salt in 100 mL of water and final volume of 1000 mL was made by addition of double distilled water. Further dilutions of 50, 75 and 100 mg $L^{-1}$ were prepared by adding appropriate quantity of sterilized double distilled water. A similar set of metal concentrations was also prepared in fungal culture filtrates instead of distilled water.

**Plant growth bioassays:** Seeds of tomato variety LA-2662 were procured from Vegetable Research Center, Ayub Agriculture Research Institute, Faisalabad, Pakistan. Healthy seeds were separated and surface sterilized using 0.1% sodium hypochlorite solution for 3 minutes and rinsed 4 times with sterile distilled water. Sterilized seeds were placed in sterilized Petri plates (9-cm) lined with double layer of filter papers, moistened with 3 mL of each of different concentrations of Cr(VI) solutions. In second set, Petri plates were supplied with 3 mL of culture filtrate amended with each of different doses of Cr(VI). Two control treatments were devised; one received 3 mL of sterilized distilled water and second received 3 mL of culture filtrates of *F. oxysporum* f. sp. *lycopersici.* Each treatment was replicated three times with 25 seeds each. The Petri plates were arranged in a completely randomized design and incubated at 25 ± 2 °C and 10 hours daily light period. Data regarding germination, length, and fresh and dry weight of shoot and roots were recorded 15-days after seed sowing. Germination rate, Germination index (GI) and relative germination rate (RGR) were calculated for each treatment (Li, 2008).

$$\text{Germination (\%)} = \frac{\text{No. of germinated seeds}}{\text{Total No. of seeds}} \times 100$$

$$\text{Germination Index} = \frac{\text{No. of germinated seeds}}{\text{No. of days required}}$$

$$\text{Relative germination rate} = \frac{\text{Germination \% in treatment}}{\text{Germination \% in control}}$$

**Physiological assays:** Chlorophyll extraction was performed by homogenizing 500 mg fresh leaf material with 10 mL of chilled 80% acetone. Resultant homogenate was centrifuged at 800 rpm for 15 minutes and supernatant was analyzed for chlorophyll content (Li, 2008). For antioxidant enzymes extraction, 0.5 g leaves were ground in chilled mortar using 5 mL phosphate buffer. Homogenate was centrifuged at 13000 rpm for 20 minutes at 4 ºC and supernatant was further employed for enzymes activities assays. For catalase (CAT) activity, 1.0 mL supernatant was added to reaction mixture containing 3.0 mL of 0.1 M phosphate buffer (pH 6.8) and 1 mL of $H_2O_2$ (0.01M). Ten mililitres of 2% $H_2SO_4$ was added after 1 minutes at 20 ºC. 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 (Machly and Chance, 1967). Peroxidase 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 0.01M pyrogallol. This solution was filled with 0.05 M $H_2O_2$ (5:5 in $H_2O_2$ and distilled water), incubated at 25 °C and 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 denoted the quantity of the enzyme that inhibited 50% of the auto-oxidation rate of pyrogallol at 25 °C. The enzyme activity was expressed as unit $mg^{-1}$ protein (Arnon, 1949).

All the data were analyzed through analysis of variance technique and means were compared by Duncan's Multiple Range Test (P 0.05) to separate mean differences (Steel *et al.,* 1997).

## RESULTS AND DISCUSSION

In general, all the fungal culture filtrates and Cr(VI) concentrations, either alone or in combinations, suppressed plant growth, biomass and physiology over control treatment. The highest decline in plant growth and alteration in physiology was recorded in fungal culture filtrates treatments.

Original culture filtrates of *F. oxysporum* f. sp. *lycopersici* did not exhibit significant reduction in seed germination and relative germination rate, whereas

germination index was significantly declined by 38% over control. Shoot and root lengths were significantly decreased by 50% and 85%, respectively, over control. Similarly, seedlings fresh biomass was significantly reduced by 86% and dry biomass by 70% over control. Total chlorophyll (chlorophyll a + b) content and catalase activity was decreased by 20% and 36%, respectively, while peroxidase activity was increased 2-folds over control (Table 1 and 2). Reduction in growth and alteration in physiological parameters was recorded due to the effect of culture filtrates of *F. oxysporum* may be attributed to inhibitory action of variety of mycotoxins and enzymes present in the culture filtrate (Karaman and Matavuly, 2005). Fusarium toxins in culture filtrate have been well-known for their properties related to virulence of pathogenic strain (Xu *et al*., 1993). Therefore, negative influence of mycotoxins may be well-correlated with hypersensitive response in plant tissue that results in liberation of reactive oxygen species which induce high level of lipid peroxidation mediating damage to DNA or protein in tomato tissues (El-Khallal, 2007). Therefore, it could be speculated that pathogen toxin disturb normal plant physiology by facilitating nutrients leakage from the macerated tissues (Nafie, 2003) thus consequences with overall reduction in plant growth and biomass (El-Khallal, 2007; Houssien *et al*., 2010; Maia *et al*., 2011).

When tomato seeds were exposed to different concentrations of Cr(VI) solution, germination rate, germination index and relative germination rate were significantly dropped by 10-20% with increase in metal concentration from 50 to 100 mg $L^{-1}$. Root and shoot lengths were reduced by 0-25% and both fresh and dry weight were declined by 30-60% at metal concentration of 50-100 mg $L^{-1}$ over control (Table 1). Total chlorophyll content was declined up to 6%, and there was non-significant difference among the treatments for this parameter as compared to control. Catalase as well as peroxidase activities significantly increased with increase in metal concentration as compared to control (Table 2). The toxicology of Cr(VI) might be owing to its adverse effect on seed germination and early seedling growth that could results in functional abnormality in hydrolytic enzymes, auxin synthesis and osmotic regulation (Noggle and Fritz, 1991; Barton *et al.,* 2000). Metals are also reported to depress the uptake of $O_2$ hence inhibit normal physiological process (Sharma and Sharma, 2003). The decline in chlorophyll content of chromium treated seedlings might be due to either inhibition of chlorophyll biosynthesis or correlated with the increasing Cr concentrations (Panda and Choudhury, 2005; Scoccianti *et al*., 2006; Liu *et al.,* 2008). Cr(VI) is thought to be involved in termination of enzymes responsible for chlorophyll biosynthesis by degrading  -aminolevulinic acid dehydratase (ALA) thus results in upsurge of ALA and decline in the chlorophyll content (Vajpayee *et al*., 2000). Chromium-induced oxidative stress resulted in elevated production of reactive oxygen species, producing oxidative damage and disturb normal cell functioning. Catalase and peroxidase are antioxidant defense mechanisms generated in plants against danger posed by the presence of reactive oxygen species (Meloni *et al.,* 2003). The activities of antioxidant enzymes assures their role in antioxidant defense so various employed Cr(VI) concentrations results in alteration of their enzyme activities (Sinha *et al.,* 2002).

Combined effect of culture filtrates of *F. oxysporum* f. sp. *lycopersici* and Cr(VI) exhibited a significant inhibition of 10-20% in germination and relative germination rate and 40-60% in germination index with increase in metal concentration. Root and shoot lengths were declined by 10-50% and 20-50%, respectively, over control. Likewise, fresh and dry biomasses of the seedlings were reduced by 50-80% in a mixture prepared by adding various concentration of Cr (VI) in fungal culture filtrates (Table 1). Total chlorophyll contents were decreased by 30-40%, while catalase activity was increased up to 14%. However, peroxidase activity showed non-significant response towards simultaneous influence of Cr(VI) concentrations and fungal culture filtrates (Table 2). Negative growth of plants under simultaneous action of culture filtrate of pathogen and Cr(VI) could be related

**Table 1. Effect of culture filtrates of *F. oxysporum* f. sp. *lycopersici* (FO) alone and in combination with various Cr(VI) concentrations on growth of tomato.**

| Treatments | Germination (%) | Germination index | Relative germination rate | Shoot length (cm) | Root length (cm) | Seedling fresh weight (mg) | Seedling dry weight (mg) |
|---|---|---|---|---|---|---|---|
| Control | 100±0$^{a}$ | 1.1±0a | 1±0.01$^{a}$ | 5±0.01$^{a}$ | 2 ±0.01$^{a}$ | 870±0.01$^{a}$ | 83 ±0.01$^{a}$ |
| FO culture filtrate | 100±0$^{a}$ | 0.68±0.01$^{b}$ | 1±0.02$^{a}$ | 2.5±0.02$^{cd}$ | 0.3±0.0$^{c}$ | 100 ±0.01$^{f}$ | 25 ±0.02$^{e}$ |
| Cr(VI) 50 mg $L^{-1}$ | 89±0.01$^{b}$ | 0.98±0.07$^{a}$ | 0.89±0.01$^{bc}$ | 5 ±0.02$^{a}$ | 2 ±0.01$^{a}$ | 480±0.01$^{c}$ | 49 ±0.02 $^{c}$ |
| Cr(VI) 75 mg $L^{-1}$ | 82±0.02$^{bc}$ | 0.91±0.03$^{a}$ | 0.82±0.04$^{cd}$ | 4 ±0.01$^{b}$ | 2 ±0.02$^{a}$ | 390 ±0.01$^{d}$ | 34±0.01$^{d}$ |
| Cr(VI) 100 mg $L^{-1}$ | 79 0.01$^{c}$ | 0.87±0.02$^{b}$ | 0.79±0.06$^{cd}$ | 4 ±0.01$^{b}$ | 1.5±0.03$^{ab}$ | 331±0.0$^{d}$ | 32±0.02 $^{d}$ |
| FO + Cr(VI) 50 mg $L^{-1}$ | 90 ±0$^{b}$ | 0.55±0.06$^{c}$ | 0.9±0.01$^{abc}$ | 4 ±0.01$^{b}$ | 1.4±0.01$^{ab}$ | 320 ±0.01$^{d}$ | 39 ±0.01$^{d}$ |
| FO + Cr(VI) 75 mg $L^{-1}$ | 86 ±0.02$^{b}$ | 0.51±0.02$^{c}$ | 0.86±0.03$^{c}$ | 3 ±00.02$^{c}$ | 1±0.02 $^{b}$ | 240 ±0.02$^{e}$ | 26 ±0.01$^{e}$ |
| FO + Cr(VI) 100 mg $L^{-1}$ | 79 ±0$^{c}$ | 0.48±0.04$^{d}$ | 0.79±0.04$^{cd}$ | 2.5±0.01$^{cd}$ | 1 ±0.01$^{b}$ | 210±0.01$^{e}$ | 25 ±0.02$^{e}$ |

Values with different letters in a column show significant difference (P  0.05) as determined Duncan's Multiple Range Test
± indicates standard errors of means of three replicates.

with negative outcome generated by synergistic action of both. It might be possible that Cr toxicity weaken roots and provide more absorption side to fungus to get inside roots during early germination stages and, therefore, weaken plant while oxygen depletion generated by Cr prompted that negative effect on overall growth, biomass, chlorophyll and enzyme assays.

**Table 2. Effect of culture filtrates of *F. oxysporum* f. sp. *lycopersici* (FO) and Cr(VI) on chlorophyll content, peroxidase and catalase activity of tomato.**

| Treatments | Chlorophyll content (mg g$^{-1}$) | Peroxidase activity (Unit$^{-1}$ min$^{-1}$ mg$^{-1}$ protein) | Catalase activity (Unit$^{-1}$ min$^{-1}$ mg$^{-1}$ protein) |
|---|---|---|---|
| Control | 0.89±0$^{a}$ | 0.254±0.01$^{d}$ | 5.5±0.02$^{b}$ |
| FO culture filtrate | 0.74±0.01$^{b}$ | 0.43±0.02$^{c}$ | 3.5±0.03$^{d}$ |
| Cr(VI) 50 mg L$^{-1}$ | 0.87±0.02$^{a}$ | 0.454±0.03$^{c}$ | 6.3±0.04$^{a}$ |
| Cr(VI) 75 mg L$^{-1}$ | 0.85±0.03$^{a}$ | 0.77±0.02$^{b}$ | 6.5±0.02$^{a}$ |
| Cr(VI) 100 mg L$^{-1}$ | 0.84±0.01$^{a}$ | 0.96±0.04$^{a}$ | 6.9±0.03$^{a}$ |
| FO + Cr(VI) 50 mg L$^{-1}$ | 0.62±0.04$^{c}$ | 0.35±0.02$^{c}$ | 5.5±0.02$^{b}$ |
| FO + Cr(VI) 75 mg L$^{-1}$ | 0.61±0.03$^{c}$ | 0.33±0.02$^{c}$ | 6±0.03$^{b}$ |
| FO + Cr(VI) 100 mg L$^{-1}$ | 0.57±0.02$^{d}$ | 0.31±0.01$^{d}$ | 6.5±0.02$^{a}$ |

Values with different letters in a column show significant difference (P 0.05) as determined Duncan's Multiple Range Test
± indicates standard errors of means of three replicates

**Conclusions:** The present study concluded that *F. oxysporum* f. sp. *lycopersici* culture filtrates and Cr(VI) either present alone or in combination have detrimental influence on seed germination, seedlings growth and physiology of tomatoes.

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