Morphophysiological Effects of Chromium in Sour Orange (*Citrus aurantium* L.)

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Additional index words. phytotoxicity, germination, antioxidant enzymes

Abstract. This is the first study on the performance of sour orange (*Citrus aurantium* L.) in soil contaminated with chromium (Cr). A greenhouse experiment was conducted to determine the phytotoxic effect of Cr on seed germination and seedling growth of sour orange. Cr treatments were applied as $\text{Cr}(\text{NO}_3)_3$ in five concentrations (0, 50, 100, 150, and 200 ppm). A gradual increase in Cr concentration leads to inhibition of seed germination and other growth parameters. Phytotoxicity, relative water content (RWC), seed vigor index (SVI), and the tolerance index (TI) show a significant decrease up to 100 ppm as a result of the presence of metal. Biochemical constituents, nutrient uptake, antioxidative enzymes, and lipid peroxidation under Cr stress were also investigated. The results indicate that concentrations greater than 100 ppm Cr cause an increase in plant antioxidative enzymes—superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX)—and increased lipid peroxidation. On the other hand, sour orange effectively generated an enzymatic antioxidant defense system (especially CAT) to scavenge hydrogen peroxide (H_2O_2), resulting in less H_2O_2 in shoots with greater Cr concentrations. A majority of Cr accumulated in the shoots and low translocations to shoots. However, sour orange is the best universal rootstock for citrus because of its resistance to salinity and disease. It also grows well in heavy soils. Based on the results of this study, sour orange might be a potential candidate plant for phytoremediation of contaminated water and phytostabilization of Cr-contaminated soils.

Increasing industrialization and urbanization has led to the anthropogenic contribution of heavy metals throughout the biosphere, with the largest availability in soil and ecosystems. These industrial activities are responsible for very high heavy metals concentrations in the environment, which may be 100- to 1000-fold greater than natural concentrations in Earth's outer crust (Lasat, 2002). Among the heavy metals, Cr has received highlighted attention because of its strong toxicity and relatively less-known mode of action (Gill et al., 2015). Once it enters the environment, its toxicity is determined to a large extent by its chemical form, which is also responsible for its mobility and bioavailability (Kotas and Stasicka, 2000). High concentrations of heavy metals induce oxidative stress by increasing the formation of reactive oxygen species (ROS), such as the superoxide radical (O_2^-), singlet oxygen ($^1\text{O}_2$), and H_2O_2 in plant cells. This process is responsible for peroxidative damage to fatty acids, nucleic acids, proteins, and chlorophyll (Gallego et al., 2002), thus disrupting the normal metabolism of a cell. Meanwhile, the generation of active oxygen species

(AOS), particularly H_2O_2 , has been proposed as part of the signaling cascade that leads to protection from stresses (Gallego et al., 2002). For protection from oxidative stress, plant cells contain both oxygen-radical detoxifying (antioxidant) enzymes, such as CAT, POD, and SOD; and nonenzymatic antioxidants such as ascorbate, glutathione, and α -tocopherol (Asada, 1996). Hyperaccumulator plants represent a resource for remediation of heavy-metal-polluted sites because they can tolerate, uptake, and translocate heavy metals into their biomass at levels toxic to most organisms (Zavoda et al., 2001). In addition to the knowledge of uptake, translocation, or compartmentation of heavy metals in plants, an

understanding of the tolerance mechanisms to improve plants of biotechnological interest is also important (Ali et al., 2000). A species of multiple use, the sour orange (*Citrus aurantium* L.) is also known as bitter or seville orange. It is a universal rootstock for citrus and is used widely in the Mediterranean region (Navarro et al., 1975). It is used mainly as a rootstock because of its resistance to *tristeza virus* and salinity; it grows well in heavy soils and it is tolerant to flood compared with other citrus rootstocks (Shatnawi et al., 1999). It is fairly drought resistant as a result of its deep and highly spreading root system. The nutritional quality of sour orange juice is related largely to its vitamin C content and its antioxidant capacity (Samson, 1980). This study aims to investigate the potential application of sour orange (*Citrus aurantium* L.) to determine the effect of different concentrations of Cr on seed germination, seedling growth, and antioxidant enzymes.

Materials and Methods

Plant materials and growth conditions

Sour orange seeds were acquired locally from ripened fruits, washed with water, and germinated on trays containing five Cr levels (0, 50, 100, 150, and 200 ppm) added as $\text{Cr}(\text{NO}_3)_3$. The zero level was used as the control and was germinated in distilled water. Seeds were allowed to germinate in greenhouse conditions at a temperature of 25 °C and in the dark for 2 weeks. Each treatment of Cr was represented by five replicates. Seed germination was investigated every day, and germinated seeds were counted daily until maximum germination to determine the percentage of germination. Seeds were considered germinated when the length of the radicle was more than 2 mm. The germination rate was calculated by counting the number of germinated seeds at 24-h intervals for 2 weeks. The growth parameters of root length and shoot length of seedlings were selected for the study following the standard procedure. After 1 month of germination, seedlings were transferred to plastic containers containing soil and were treated with the same Cr concentrations, as were their

Fig. 1. Effect of chromium (Cr) on seed germination and mean germination of sour orange (*Citrus aurantium* L.). Vertical bars represent $\pm$ SD. Values with the same letter are not significantly different at the 5% level.

Received for publication 11 Dec. 2018. Accepted for publication 3 Feb. 2019.

I gratefully acknowledge the University of Jordan for its financial support and facilities help during laboratory work and analysis.

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replicates. The total incubation experiment lasted for 31 d.

Growth parameters measurements

Percent RWC, percent phytotoxicity, SVI, and TI. For RWC analysis, plants were separated into roots and shoots. Wet plant biomass [fresh weight (FW)] was determined immediately. The samples were dried in a hot air oven for 48 h at 65 °C for determination of dry weight (DW). Percent RWC was calculated as $RWC = \frac{FW-DW}{FW} \times 100$ (Chen et al., 2009). The phytotoxic effect of the metal on root and shoot growth in terms of percent

phytotoxicity was calculated after 1 month of seed germination using the formula by Chou and Lin (1976):

$$\% \text{Phytotoxicity} = \frac{\text{Shoot or root length of control} - \text{Shoot or root length of treatment}}{\text{Shoot or root length of control}} \times 100.$$

SVI was calculated using the following formula (Iqbal and Rahmati, 1992): $SVI = (\text{Mean root length} + \text{Mean shoot length}) \times \% \text{Germination}$.

The tolerance of sour orange seedlings to various concentrations of Cr was determined by measuring the TI and was calculated using the following formula (Koornneef et al., 1997):

$$TI = \left(\frac{I_{me}}{I_c} \right) \times 100,$$

where I_{me} is the increase in root length in the metal ion solution and I_c is the increase in root length in the control after 15 d.

Determination of biochemical constituents. Chlorophyll pigments were extracted from fresh leaf samples using 80% (v/v) acetone and chlorophyll a and b contents were estimated spectrophotometrically at 665, 649, and 470 nm according to the method of Lichtenthaler (1987), and are expressed in milligrams per gram FW. The Biuret method (Racusen and Johnstone, 1961) was adopted for the estimation of soluble protein contents.

The reaction mixture consisted of 0.1 mL supernatant and 1.0 mL Biuret reagent. The control setup contained 0.1 mL distilled water instead of supernatant. The optical density of the reaction mixture was measured at 545 nm with a spectrophotometer (JENWAY ultraviolet-600, UK). Data Analysis Software, Version 1.2. Other biochemical constituents such as protein (Lowry et al., 1951), sugars (Nelson, 1944), and starch (Dubais et al., 1956) were determined according to their references. The analysis of total nitrogen (N) was performed using the Micro-Kjeldahl digestion procedure; crude protein content was determined by multiplying total N by a factor of 6.25. Sugar concentrations in extracts were determined using the phenol-sulfuric acid method without removing the aqueous ethanol or methanol solvent. A 5-mL sample was taken from each combined extract, mixed with 3 mL deionized water, and separated into two phases by centrifuging at 2500 rpm for 5 min. The chloroform phase was discarded and the methanol:water phase was analyzed for sugar. The residues were oven-dried at 50 °C overnight to remove the residual solvent and were stored at -20 °C for starch analysis.

Determination of antioxidative enzyme activities. Leaf tissues from sour orange seedlings were homogenized separately in a pre-chilled mortar and pestle under ice-cold conditions with 2.0 mL extraction buffer [50 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 1 mM EDTA]. The homogenate was centrifuged at 10,000 rpm for 15 min. The supernatant was used for the measurement of SOD (Beauchamp and Fridovich, 1971), CAT (Beer and Sizer, 1952), APX (Nakano and Asada, 1987), and POD (Zhang et al., 1995) antioxidative enzyme activities. The SOD activity was estimated by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). CAT activity was determined by measuring the decomposition of H_2O_2 (Beer and Sizer, 1952). APX activity was determined according to the method of Nakano and Asada (1987). POD activity (measured in milligrams per gram of tissue) was determined as revealed by Racusen and Foote (1965). The reaction mixture for POD contained 0.1 mL crude enzyme extract, 0.2 mL 1% guaiacol solution, 2.5 mL 0.1 M phosphate buffer (pH 7.2), and 0.2 mL 0.3% H_2O_2 solution, whereas the assay mixture for POD in the control contained 0.2 mL distilled water instead of guaiacol solution. The activity was estimated by measuring the absorbance of the mixture at 470 nm.

Plant and soil analysis. All the plants and soil samples were prepared using the wet digestion method (Piper, 1942). Soil samples were air-dried, crushed to pass through a 0.2-mm sieve, and stored in polythene bags for analysis (Bhide and Sundaesan, 1983). Each soil sample (10 g) was treated with 2 mL perchloric acid and 5 mL of HNO_3 concentration, and was stored in vials for further analysis. A half gram of each sample was treated with 3 mL Merk hydrofluoric acid, 1 mL Merk perchloric acid, and 7 mL 65% Suprapur Merk nitric acid. The water

Fig. 2. Relationship between relative water content (RWC) of shoots of sour orange (*Citrus aurantium* L.) and chromium (Cr) concentration in soil. Vertical bars represent $\pm$ SD. Values with the same letter are not significantly different at the 5% level.

Fig. 3. Shoot and root length of sour orange (*Citrus aurantium* L.) after direct exposure to chromium (Cr). Vertical bars represent $\pm$ SD. Values with the same letter are not significantly different at the 5% level.

Fig. 4. Phytotoxicity percentage of shoots and roots of sour orange (*Citrus aurantium* L.) after direct exposure to chromium (Cr). Vertical bars represent $\pm$ SD. Values with the same letter are not significantly different at the 5% level.

Fig. 5. Seedling vigor and tolerance index of sour orange (*Citrus aurantium* L.) after direct exposure to chromium (Cr). Vertical bars represent $\pm$ sd. Values with the same letter are not significantly different at the 5% level.

Fig. 6. Similar trends of the effect of chromium (Cr) on chlorophyll, starch, protein and sugar of sour orange (*Citrus aurantium* L.) leaf. Vertical bars represent $\pm$ sd. Values with the same letter are not significantly different at the 5% level.

used for washing and diluting was milli Q element distilled water from Millipore. The digested sample was made up to 25 mL and was analyzed for the metal Cr using atomic absorption spectroscopy (Varian AAA 220 FS). The entire analysis was conducted in clean-air room of class 10,000 (American Public Health Association, 1990). Concentrations of Cr were analyzed using inductively coupled plasma atomic emission spectrometry (Leeman Laboratories, Inc., New Hampshire) as described by Duan (2003). For other nutrient determinations, the oven-dried shoot samples were ground to pass through a 0.5-mm sieve using a laboratory mill. The ground material was mixed thoroughly and weighed. Samples (≈ 0.5 g) were ashed overnight at 55 °C in a muffle furnace, and then the ash was dis-

solved in 2 M HCl to determine potassium (K), zinc (Zn), copper (Cu), manganese (Mn), and iron (Fe) content. K concentration was analyzed using flame photometry (M410, Corning), and Zn, Cu, Mn, and Fe concentrations were determined using atomic absorption spectroscopy (Perkin Elmer 2380). Phosphorus concentration was determined using a spectrophotometer (Spectronic 20 D). Ultraviolet-visible absorption spectrophotometry (ultraviolet-VIS recording spectrophotometer 2401 PC, SHIMADZU) was used to detect the absorbency of the injected samples at a certain wavelength (470 nm) in the visible spectrum. N content was determined according to Jackson (1958).

Measurement of MDA contents. Lipid peroxidation was determined by measuring the total amount of malondialdehyde (MDA)

as described by Davenport et al. (2003). Briefly, fresh root and leaf tissues (0.2 g) were homogenized using 2 mL 5% (w/v) trichloroacetic acid (TCA) in an ice bath and centrifuged at 10,000 rpm for 10 min at 4 °C. About 2 mL supernatant was mixed with 2 mL 0.67% (w/v) thiobarbituric acid, incubated in a boiling water bath for 30 min, then cooled and centrifuged. The absorption of supernatant was carried out at 450, 532, and 600 nm.

MDA content (measured in micromolecules per gram) was calculated as

$$\text{MDA} = [6.45 \times (A_{532} - A_{600}) - (0.56 \times A_{450}) \times Vt] = W,$$

where volume of crude enzyme (Vt) = 0.0021; leaf weight (W) = 0.2 g.

Determination of H_2O_2 . H_2O_2 content was measured according to the method described by (Prasad et al., 1994) with the following modification: Two grams of root tissues were ground in 50 mM potassium phosphate buffer (pH 7.8). To the homogenate, 5% TCA was added (TCA:mixture, 1:0.7). The mixture was centrifuged at 10,000 g_n for 10 min. The supernatant was collected. About 1.6 mL of the resulting supernatant was mixed with 0.4 mL 50% TCA, 0.4 mL 10 mM ferrous ammonium sulfate, and 0.2 mL 125 mM potassium thiocyanate. The absorbance of the reaction mixture was monitored at 480 nm.

Statistical analysis

All the treatment groups were arranged in a completely randomized design, with five replicates for each Cr treatment. Means were separated by the Duncan multiple range test at $P < 0.05$. The results were analyzed by using SAS statistical software (SAS Institute Inc., Cary, NC). The least significant difference was used for comparisons between treatment means.

Results

Effect of Cr seed germination and %RWC. Because of the considerably increased Cr concentration, there was a marked decrease in growth parameters. Seed germination is the first visible indicator of plant growth and is regulated by a number of physical and physiological processes. In the current study, Cr affected seed germination differently in sour orange. The maximum seed germination was in the control (100%), whereas in the Cr-treated seeds, germination decreased significantly with an increase in metal concentration, with a minimum germination of 200 ppm (21%) (Fig. 1). Mean germination increased as the Cr level increased (Fig. 1). The greatest Cr concentration reduced shoot RWC compared with the control (Fig. 2).

Effect of Cr on root and shoot length, phytotoxicity, and SVI. A significant reduction in root growth ($P < 0.05$) was observed in sour orange seedlings growing under Cr stress as indicated by a decrease in root length in the presence of metal concentrations (Fig. 3). The proportion of growth in shoot

Fig. 7. Effect of chromium (Cr) on the activity of antioxidant enzymes, hydroxides, and lipid peroxidase in the shoots of sour orange seedlings. Vertical bars represent $\pm$ SD. Values with the same letter are not significantly different at the 5% level. CAT = catalase; SOD = superoxide dismutase; POD = peroxidase; APX = ascorbate peroxidase; MDA = malondialdehyde; H₂O₂ = hydrogen peroxide.

length decreased at greater concentrations of Cr in the medium. Some percentage of yellowing of the shoots was observed in the greater concentrations as a result of the death of some tissues in cultures. Percent phytotoxicity and the SVI are represented in Fig. 4. The phytotoxicity percentage of shoots and roots undergoing Cr treatments showed an increasing trend with increasing Cr concentrations in sour orange. The greatest percent phytotoxicity value of shoots and roots was found at a 200-ppm Cr concentration, and a strong phytotoxic effect of the metal was observed against root growth metal concentration. Percent phytotoxicity of shoots was 39.3% at 50 ppm and 90.4% at 200 ppm, whereas in roots the minimum was seen at 50 ppm (66.02%) and the maximum at 200 ppm (97.67%). The SVI and TI were less than that of the control and showed a significant decrease in seedling vigor resulting from the presence of Cr. In the experimental groups, the SVI decreased from 310.4 at 200 ppm to 1068.4 at 50 ppm Cr concentration (Fig. 5).

Effect of Cr on biochemical contents. Figure 6 shows chlorophyll, protein, sugar, and starch content. The total chlorophyll

demonstrated a decreasing trend with increasing concentration of Cr concentration. Among the different biochemical parameters, sugar content showed a decrease with increasing Cr concentration. The minimum sugar concentration was observed in 200 ppm; the maximum was seen in the control. Here also there was a significant contribution of Cr concentration toward decrease in the protein content in the leaves under different treatments and control condition. The lowest protein concentration was observed in 200 ppm. The starch content decreased as the Cr concentration increased. The highest declines were observed at 150 and 200 ppm.

Effect of Cr on antioxidant enzyme activity and level of H₂O₂ and MDA in the shoots of sour orange plants. The activities of antioxidant enzymes CAT, POD, SOD, and APX are induced by oxidative stress produced as a result of heavy-metal contamination. In the current study, seedlings of sour orange showed an initial decrease in antioxidant enzymes (Fig. 7). CAT is an important antioxidant enzyme in plants used to detoxify the effect of H₂O₂. SOD activity increased when the Cr concentration increased, with the

greatest reductions occurring at 150 ppm and 200 ppm. Chromium treatments, regardless of source, increased POD activity. Cr(NO₃)₃ at 150 and 200 ppm resulted in the greatest increase. All Cr treatments had no effect on APX. Concentrations of Cr increased H₂O₂ levels in shoots compared with the control, except for Cr at 150 and 200 ppm. Cr treatments had a significant effect on MDA, and the greatest increase was at 200 ppm.

Analysis of total minerals in plant and soil. Cr concentration increased in soil containing Cr(CO₃)₃ at 200 ppm than in the other treatments (Fig. 8). Cr(CO₃)₃ at 200 ppm resulted in the greatest increase in shoot and root followed by the remaining treatments. The greatest Cr concentration was seen in roots: shoot range, 1.15–4.08 mg·kg⁻¹; root range, 2.43–9.62 mg·kg⁻¹. The greatest amount of total Cr in soil was 16.69 mg·kg⁻¹. The increase in the concentration of Cr in the soil from 50 to 200 ppm was accompanied by alterations in shoot nutrient concentrations (Figs. 9 and 10). Increasing Cr concentrations from 150 to 200 ppm in the soil decreased N, P, K, Zn, Mn, and Fe contents in the shoots and roots of sour orange.

Discussion

Heavy-metal accumulation in soils is one of the concerns in agricultural production because of their adverse effects on crop growth and food quality (Naser et al., 2011). Heavy metals inhibit plant growth by reacting with biochemical constituents and by affecting water relations and metabolism (Gajewska and Sklodowska, 2008; Singh et al., 2007). The direct interaction of metal with cellular components can initiate a variety of metabolic responses and leads to a shift in the development of the plant (Assche and Van, 1990). Because seed germination is the first physiological process affected by Cr, the ability of seed to germinate in a medium containing Cr is indicative of its level of tolerance to this metal (Peralta et al., 2001). The decrease in seed germination by Cr is related to the inhibitory effect of metal on the activity of α and β amylase, which hydrolyze starch to sugar required by developing embryos (Zeid, 2001). Protease activity, on the other hand, increases with Cr treatment, which could also contribute to the reduction in germination of Cr-treated seeds (Zeid, 2001). Seed germination of the sour orange was reduced to 21% with 200 ppm Cr concentration. Similar inhibition of germination percentage at greater concentrations of Cr was observed in cowpea (*Vigna unguiculata*) (Lalitha et al., 1999), sugarcane (*Saccharum officinarum*) (Jain et al., 2000). A decrease in shoot length is obvious because destruction of root cells by Cr may cause a decrease in nutrient and water mobility from the roots to the shoots. The reduction in root and shoot length under Cr stress may be the result of due the inhibition of cell division and/or elongation in root cells, which results from tissue collapse and thus affects

Fig. 8. Total metal concentrations shoot (A), in root (B), and in soil (C). Cr = chromium.

absorption of water and nutrients by the roots (Diwan et al., 2010; Lu et al., 2004). The reason for high accumulation in the roots of the plants could be because Cr is immobilized in the vacuoles of the root cells, thus rendering it less toxic, which may be a natural toxicity response of the plant (Peralta et al., 2001).

From the experimental results of the current investigation, the increased total chlorophyll content at the lower level of Cr was obviously a result of better growth. The formation of chlorophyll pigment depends on an adequate supply of Fe because it is the main component of protoporphyrin, a precursor to chlorophyll synthesis. An excessive supply of Cr seems to prevent the incorporation of Fe into the protoporphyrin molecule, resulting in the reduction of chlorophyll pigment. Our findings are in agreement with the earlier findings of Bera et al. (1999).

The protein content in the leaves of sour orange decreased, which may be the result of a decrease in the N content; N is the precursor for the synthesis of amino acids, which are the building blocks of protein in the case of sour orange plants (Nag et al., 1981). A decrease in sugar content in the leaves under different treatments of Cr might be an attempt

Fig. 9. Influence of chromium (Cr) concentrations on macro mineral acquisition of sour orange (*Citrus aurantium* L.). N = nitrogen; P = phosphorus; K = potassium.

to overcome Cr stress on plants by decreasing carbohydrate synthesis (Rellén-Álvarez et al., 2006).

Enzymes are the most sensitive indexes for adaptation and response of plants to stress (Panda et al., 2003). The activities of antioxidant enzymes were investigated to determine whether Cr exposure influenced these antioxidant enzymes. The activity of CAT, SOD, APX, and POD in sour orange was generally enhanced in response to Cr exposure. In sour orange, the enzyme activity increased significantly at 200 ppm compared with the control group, followed by a decrease up to 150 ppm, and then an increase at 150 ppm, thus indicating a high production of H₂O₂ in the presence of high concentrations of Cr. A high concentration of Cr induces oxidative stress by increasing the formation of ROS such as O²⁻, ¹O₂, the hydroxyl radical, and H₂O₂ in plant cells, which are responsible for POD damage to fatty acids, nucleic

Fig. 10. Influence of chromium (Cr) concentrations on micro mineral acquisition of sour orange (*Citrus aurantium* L.). Fe = iron; Mn = manganese; Zn = zinc.

acids, protein, and chlorophyll content (Gallego et al., 2002). SOD is a major ROS scavenger, and its enzymatic action results in H₂O₂ and oxygen formation, whereas CAT and POD enzymes are involved in scavenging H₂O₂. This shows that the physiological response of the crop varieties to metal stress varies and depends on the genetic makeup of the plant that controls the tolerance mechanism of the plant. In one study, an increase in CAT activity at high Cr concentrations was observed in wheat seedlings (Dey et al., 2009) whereas another demonstrated a decrease in enzyme activity resulting from heavy-metal stress (Sazanova et al., 2012), indicating a difference in physiological response of different cultivars toward heavy-metal stress. Increasing Cr concentrations in the soil gradually decreased the N, P, K, Fe, Mn, and Zn contents of the sour orange shoot system. This is in agreement with the study by Moral et al. (1995), who found that an increase in stress in the growth medium was

followed by a marked decrease in mineral content. This inhibition of uptake may be the result of increased competition. At high Cr concentrations, when severe root was observed, reduced uptake of these elements may be the result of a breakdown of membrane function. Because of their toxic effects, heavy metals cause disruption of a number of physical actions in plants, such as transpiration, stoma movements, water intake, photosynthesis, enzyme activities, germination, and protein synthesis. These results suggest that *Citrus aurantium* is more tolerant to Cr stress because its roots take up less Cr, which results in lesser transport to the shoots. In addition, it is more effective in establishing an enzymatic antioxidant system.

Conclusion

Accumulation of Cr in plant tissues induced physiochemical changes in sour orange (*Citrus aurantium* L.) seedlings. The results of the current study show that sour orange is affected seriously by Cr at high concentrations. Data indicate that these plants may be grown directly in soils contaminated with moderate amounts of Cr. Further studies are needed to understand completely the mechanisms of Cr tolerance by *Citrus aurantium* and its potential in phytoextraction.

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