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A SURVEY ON THE EFFECTS OF CR^{3+} STRESS ON PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF LETTUCE (*LACTUCA SATIVA* L.)

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Abstract

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This study evaluated the effects of concentrations of the heavy metal Cr^{3+} (0.5, 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15, and 20 mg L^{-1}) on the rate of photosynthesis pigments, the activity of antioxidant enzymes, the amount of non-enzymatic antioxidants, soluble and insoluble sugars in leaves and roots, the concentration rate of sodium and potassium, and the chromium accumulation rate in roots and shoots of lettuce. The results showed that a treatment of more than 1 mgL^{-1} chrome reduced the concentrations of total chlorophyll and chlorophyll a and b in the leaves of *Lactuca sativa* L. It was found that increasing the concentration of trivalent chromium significantly increased proline in lettuce leaves at concentrations higher than 3 mg L^{-1}. Increasing the chromium concentration also increased the amount of soluble sugars in leaves and roots. Increasing the chromium concentration caused the amount of soluble sugars in leaves and roots to decrease, and the decrease was higher in the leaf than in the root. Investigating the effects of Cr^{3+} on the activity of antioxidant enzymes guaiacol peroxidase (GPOX) and polyphenol oxidase (PPO) of lettuce leaves and roots determined that stress increased PPO activity in leaves more than in roots. The level of GPOX activity was higher in roots than in leaves. The results also showed that Cr accumulated more in the roots than in the shoots, and also the amount of essential minerals, ingredients such as potassium and sodium, decreased as the Cr concentration was increased.

Key words: heavy metal, Cr^{3+} stress, chlorophyll content, proline

Introduction

Chromium is considered an essential micronutrient for humans that can boost insulin function and play a key role in the normal metabolism of carbohydrates and lipids (Vincent, 2001). Clinical trials indicate that the use of dietary supplements containing chromium can reduce blood glucose levels in diabetic patients (Lukaski et al., 2007). According to research reports, some chromium supplements cause oxygen radicals and have harmful effects on DNA. As a result, the beneficial health effects of the dietary supplement are sharply reduced (Yang et al., 2006). One of the best methods to combat chromium deficiency in the human diet is to use edible plants that accumulate Cr in their tissues (Zayed et al., 1998). On the other hand, heavy metal stress is one of the major environmental stresses that effects plant metabolism. Toxicity levels are increasing in soil and water because of agricultural and industrial activities (Scoccianti et al., 2006). Due to widespread industrial use, Cr is considered a major pollutant in soil and water. It is released into the environment mostly through industries such as leather tanning, textiles, and electroplating industries (Shanker et al., 2005).

Chromium is the seventh most abundant element in the Earth (Katz and Salem, 1994) and has different oxidation states from +2 to +6. Stable forms of chromium are Cr^{2+} and Cr^{6+} (Shanker et al., 2005). Absorption of chromium (VI) is carried out actively, and in plant roots is reduced to the trivalent chromium by reductase enzyme. Trivalent chromium that formed in roots and stems of several plants such as celery which were treated with $CrO4^{2-}$ were identified. Chromium accumulation in plants causes toxicity that result in reduced root growth, biomass, and chlorosis as well as photosynthesis disorder and ultimately cell death (Scoccianti et al., 2006). Heavy metal stress leads to the production of reactive oxygen species (ROS) and thus damage to plant cells (Gajewska et al., 2006). ROS are highly reactive and damage lipids, proteins,

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and nucleic acid (Foyer, 1993). Plants have certain defense mechanisms that either prevent the formation of ROS or clear free radicals (Devi and Prasad, 1998). Antioxidants produced in cells defend biochemical activity and create a resistance in stress conditions (Asada, 1992). Measurements of proline under chromium stress could be as a useful parameter for measuring a plant's tolerance to heavy metal. Apparently proline is the only amino acid that accumulates in high levels in the leaves of many plants under stress in amounts proportional to stress intensity. One study determined that increasing the concentration of Cr caused a gradual increase in proline levels in all four genotypes of soybean, and more proline was observed in resistant genotypes (Sankar et al., 2009). The amount of proline in a plant depends on the strategies the plant uses to adapt to chromium toxicity, because proline plays multiple roles in creating resistance under stress conditions.

Vegetables are a main component of the human diet. In addition, they absorb essential elements that might absorb hazardous elements. The accumulation of these elements in vegetables is a direct threat to human health (Ejazul et al., 2007). Lettuce is one high consumption vegetable of households. In addition to food consumption, lettuce also has medicinal purposes (Yu-lin et al., 2004). The purpose of the present study was to survey the effects of different concentrations of Cr^{3+}, particularly under stress concentrations, on certain physiological factors, such as growth rate, photosynthetic pigment, activity of antioxidant enzymes, non-enzymatic antioxidants, soluble and insoluble sugars of the roots and leaves, the concentrations of mineral elements sodium and potassium and Cr accumulation in the roots and shoots of the *Lactuca sativa* L.

Materials and Methods

This research was conducted in a greenhouse at the Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran. The seeds of local varieties of lettuce (*Lactuca sativa* L.) were disinfected with 5% sodium hypochlorite for 5 minutes and washed with water. Then they were grown in pots containing soil.

Two weeks after planting the lettuce seeds in soil, plant seedlings received the Cr^{3+} treatment in the form of nitrate [$Cr(NO_3)_3.9H_2O$] with a molecular weight of 400 g in different concentrations (0.5, 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15 and 20 mg L^{-1}). Control plants were watered with distilled water; after 7 weeks the plants were harvested.

Estimation of Chlorophyll content

Chlorophyll (a, b, and total) were determined from leaf material (200 mg FW) ground in a pre-chilled mortar in acetone (80% v/v). After complete extraction, the mixture was filtered and the volume was adjusted to 10 mL with cold acetone. The absorbance of the extract was measured at 664, 647, and 470 nm using a spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan) and the pigment concentrations were calculated according to Lichtenthaler (1987).

Proline estimation

Free proline content was determined according to Bates et al. (1973). Leaf samples (200 mg) were homogenized in aqueous sulfosalicylic acid (3% w/v, 12 mL). The filtered homogenate (2 mL) was reacted with equal volume each of acid ninhydrin and acetic acid at 100°C for 1 h and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene and mixed vigorously with a stirrer. The chromophore containing toluene was aspirated from the aqueous phase and warmed to room temperature. The absorbance was recorded at 520 nm using toluene as a blank. Proline concentration (mgg^{-1} FW) was determined from a standard curve using L-proline.

Preparation of Enzyme Extract

One gram of leaf tissue was homogenized in 3 ml of prechilled phosphate buffer, (pH 7.2) in chilled pestle and mortar. The homogenates were centrifuged at 15 000 rpm for 15 min at 4˚C and supernatant collected and used for enzyme activities of PPO and GPOX.

Determination of Guaiacolperoxidase (GPOX) activity

Guaiacol peroxidase was assayed by mixing 50 µl of Guaiacol, 30 µl of H_2O_2 and 3 ml of potassium phosphate buffer and enzyme extract. Blank was prepared by adding all the reagents except enzyme extract. The absorbance was recorded at 436 nm.

Determination of polyphenol oxidase (PPO) activity

Polyphenol oxidase (PPO) activity was determined according to the method of Reymond et al. (1993). The reaction mixture containing 2.5 cm^3 of 200 mM sodium phosphate buffer (pH 6.8), 0.2 cm^3 of 20 mM pyrogallol and 0.05 cm^3 enzyme extract. The temperature of the reaction mixture was 40°C. The absorbance was recorded at 430 nm.

Carbohydrate Determination

Root and shoot samples were analyzed for carbohydrate by following a slightly modified procedure from that outlined by Chatterton et al. (1987). Samples were ground into a fine powder and a 100–200 mg portion was placed in a sealed vial and used for the determination of soluble sugars and starch, as reported by Wilson and Al-Hamdani (1997).

Chromium Content

Individual aliquots (0.1–0.7 g) of dried root and shoot tissues from each treatment were refluxed for 15 min in 10 mL 6 N HNO_3. To the samples, 5 mL concentrated HNO_3 was added and the reflux was continued until approximately 5 mL of solution was left. After the solution had cooled, 2 mL of water and 5 mL of 30% hydrogen peroxide were added. The samples were heated slowly and hydrogen peroxide was added to the solution, 1 mL at a time, until effervescence ceased. The samples were allowed to cool and HCl was added in a ratio of 1:2 (vol/vol) following the procedure outlined by Cabrera-Vique et al. (1997). After all the plant tissue was digested, the solution was then brought up to 75 mL with distilled H_2O. The metal content extracted by the acid was determined with a Buck Model 210 VGP atomic absorption spectrophotometer at 357.9 nm.

Sodium and potassium content

Dried plant tissues (0.1 g) were digested in a concentrated nitric/perchloric acid (2:1, v/v) mixture. Sodium and potassium concentrations were determined directly by atomic emission spectrometry.

Statistical Analysis

The experiment was organized as a completely random design with four replicates for each treatment. The data were analyzed as a randomized complete block design. Duncan was used in mean separations for the treatments that showed significant F values at 5% level of probability of the ANOVA.

Results

The effects of Cr^{3+} on the amount of chlorophyll a, chlorophyll b, and total chlorophyll

ANOVA results for chlorophyll a, b, and total chlorophyll showed that treatment significantly decreased the chlorophyll content in the leaves. This reduction was observed in treatments of more than 0.5 mg L^{-1} and in the 1 mg L^{-1} treatment, the reduction was more significant than the control.

In treatments of 12.5, 15, 20 and 25 mg L^{-1} minimal reductions were observed in chlorophyll a, b and total chlorophyll and no significant difference was noted (Figure 1). These results also showed that chlorophyll a was reduced more than chlorophyll b. The lowest and highest decreases were observed in the 0.5 mg L^{-1} and 25 mg L^{-1} treatments, respectively. Compared to the control, these reductions were significant (Figure 2).

The effect of Cr^{3+} on the amount of proline in lettuce leaves

With increasing Cr^{3+} concentrations, the amount of proline increased. In the 7.5 and 10 mg L^{-1} treatments the maximum amount of proline was observed compared to the control, and it was significant. In treatments with more than 10 mgL^{-1} Cr^{3+} proline levels were reduced again. The lowest level of proline was observed in 25 mg L^{-1} treatments that were significantly different from the control. The amount of proline in 0.5, 1, 2, 3, 4, 12.5, 15, 20 and 25 mg L^{-1} treatments was not significantly different compared to the control (Figure 3).

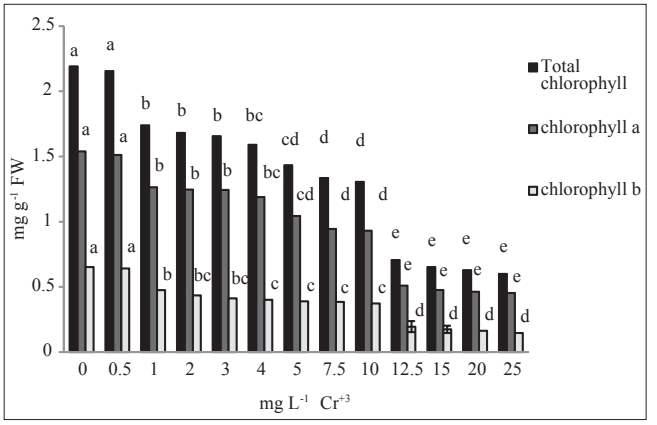

Fig. 1. Effects of different Cr concentrations on chlorophyll a, b and total chlorophyll content in lettuce leaves

Note: Means by the same letter in each column are not significantly different based on the Duncan test ($P < 0.05$)

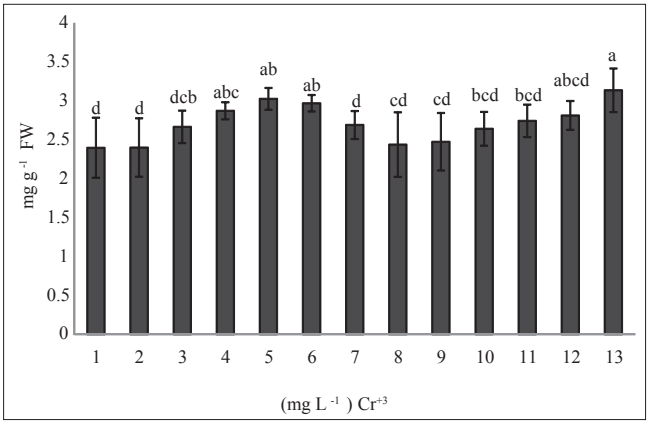

Fig. 2. Effects of different Cr concentrations on ratio of chlorophyll a to b in lettuce leaves

Note: Means by the same letter in each column are not significantly different based on the Duncan test ($P < 0.05$)

The effect of Cr $^{3+}$ on soluble sugars in leaves and roots of lettuce

Comparison showed that increasing the chromium concentration in lettuce roots and leaves increased the amount of soluble sugar. The largest increase in soluble sugars in leaves and roots was seen in the 7.5 mg L^{-1} treatment, which was a significant difference compared to the control. The concentration of soluble sugars in the leaves decreased, and the largest decrease was observed at 25 mg L^{-1} concentrations, which was not significant compared with the control. In concentrations higher than 7.5 mg L^{-1} Cr^{3+} the amount of soluble sugars decreased and this decrement was less than the controls (Figure 4).

The effect of Cr^{3+} on the amount of insoluble sugars (starch) in leaves and roots of lettuce

According to the mean values of different Cr^{3+} concentrations in grown plants, the control plants showed the highest starch content in roots and leaves, the lowest starch concentration in leaves being 10 mg L^{-1} Cr^{3+} and in roots 7.5 mg L^{-1} Cr $^{3+}$, which is significantly different than the control group. Starch levels in leaves of treatments higher than10 mg L^{-1} Cr^{3+} and in roots of treatments higher than 7.5 mg L^{-1} were not significantly different (Figure 5).

The effect of Cr^{3+} on enzyme activity of polyphenol oxidase (PPO) in leaves and roots of lettuce

The results showed that by increasing the Cr^{3+} concentration, the enzyme activity in leaves and roots increased significantly compared with the control. The highest amount of enzyme activity was observed in the leaves of the 20 mg L^{-1} treatment but in concentrations higher than 20 mg L^{-1} enzymes' activity fell again; however, this reduction was less than the control. In roots, the most polyphenol oxidase enzyme' activity was observed in the 12.5 mg L^{-1} treatment which, compared with the control, was significant. In concentrations higher than 12.5 mg L^{-1}, polyphenol oxidase enzyme activity was reduced again, but this reduction was less than that of the control. Compared with the activity of this enzyme in the leaves and roots of lettuce plants under Cr^{3+} treatment, it became clear that the activity of this enzyme is higher in leaves than in roots (Figure 6).

The effect of Cr^{3+}on Guaiacol peroxidase (GPOX) enzyme activity in leaves and roots of lettuce

The results showed that increasing the Cr^{3+} concentration increased the enzyme activity in leaves and roots significantly compared with the control. The highest amount of enzyme activity was seen in the leaves of the 10 mg L^{-1} treatment. In concentrations higher than 10 mg L^{-1} enzymes' activity fell again; this reduction was more than the control and had a significant distance with the control. In roots the highest level of Guaiacol peroxidase enzyme activity was observed in the 7.5 mg L^{-1} treatment which, compared to the control, was significant. In concentrations higher than 7.5 mg L^{-1} activity was reduced again, but it didn't have a significant distance with the control. Compared with the activity of this enzyme in leaves and roots of lettuce plants under Cr^{3+} treatments, it became clear that that the activity of this enzyme is higher in roots than in leaves (Figure 7).

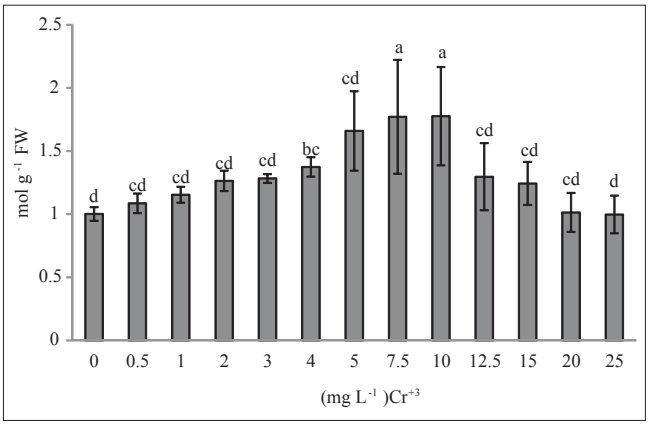

Fig. 3. Effects of different Cr concentrations on proline content in lettuce leave

Note: Means by the same letter in each column are not significantly different based on the Duncan test ($P < 0.05$)

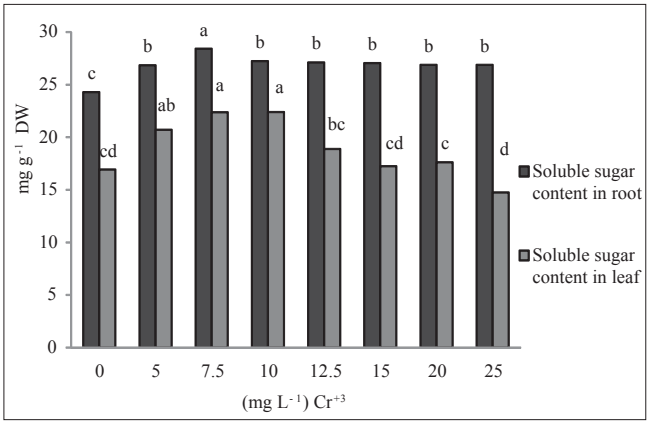

Fig. 4. Effects of different Cr concentrations on soluble sugar content in lettuce root and leaf

Note: Means by the same letter in each column are not significantly different based on the Duncan test ($P < 0.05$)

The effect of Cr $^{3+}$ on the amount of sodium in leaves and roots of lettuce

Results of variance analysis showed that the impact of Cr^{3+} on the amount of sodium in leaves and roots was significant at $p < 0.05$. The mean values of sodium in different Cr^{3+} concentrations decreased such that in plants treated with 0.5 mg L^{-1}.

Despite the gradual reduction in the amount of sodium in the leaves of plants in the environment with increasing concentrations of Cr $^{3+}$, significant differences compared with control plants at 4 mg L^{-1} concentrations were observed. Plants treated with 0.5, 1, 2 and 3 mg L^{-1} did not have significantly different sodium contents. Sodium levels decreased in roots treated with Cr^{3+} with increasing chromium concentrations; controls and plants treated with 0.5 and 1 mg L^{-1} trivalent chromium had the highest amounts of sodium, and 25 mg L^{-1} treated plants had the least amount of sodium, which had no significant difference with the plants in the 12.5, 15 and 20 mg L^{-1} treatments. The mean sodium content in leaves and roots of treated plants showed that sodium was reduced more in roots than in leaves (Figure 8).

Effect Cr^{3+} on the amount of potassium in leaves and roots of lettuce

Results of variance analysis showed that the impact of trivalent chromium on the amount of potassium in leaves and roots was significant at $p < 0.05$. Based on the mean values in

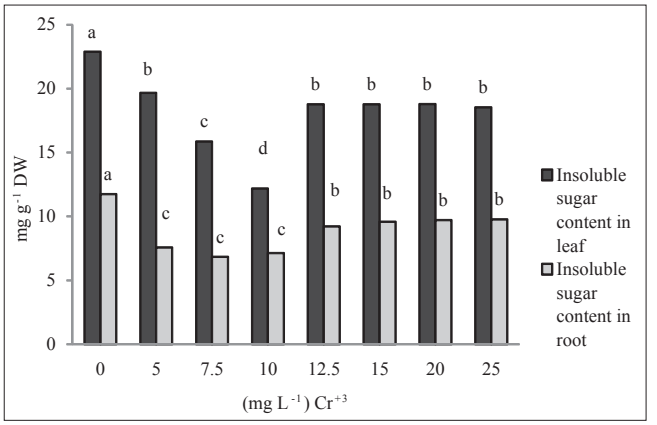

Fig. 5. Effects of different Cr concentrations on insoluble sugar content in lettuce root and leaf

Note: Means by the same letter in each column are not significantly different based on the Duncan test (P < 0.05)

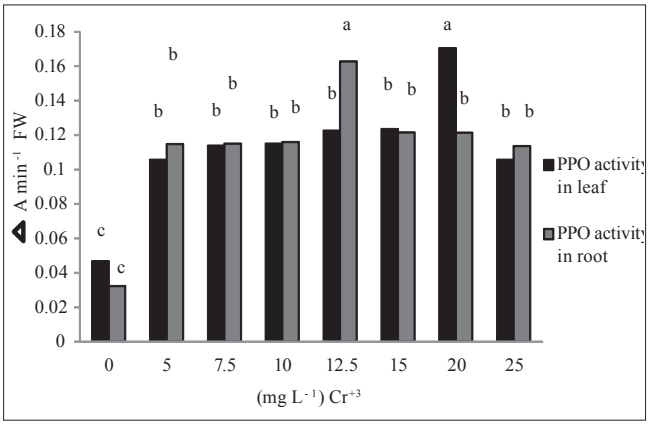

Fig. 6. Effects of different Cr concentrations on PPO activity in lettuce root and leaf

Note: Means by the same letter in each column are not significantly different based on the Duncan test (P < 0.05)

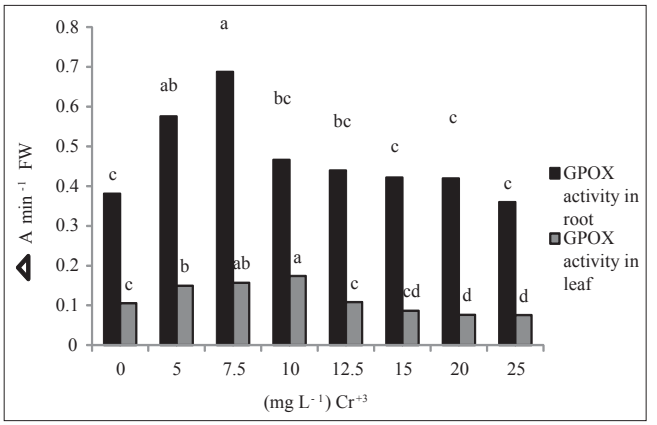

Fig. 7. Effects of different Cr concentrations on GPOX activity in lettuce root and leaf

Note: Means by the same letter in each column are not significantly different based on the Duncan test (P < 0.05)

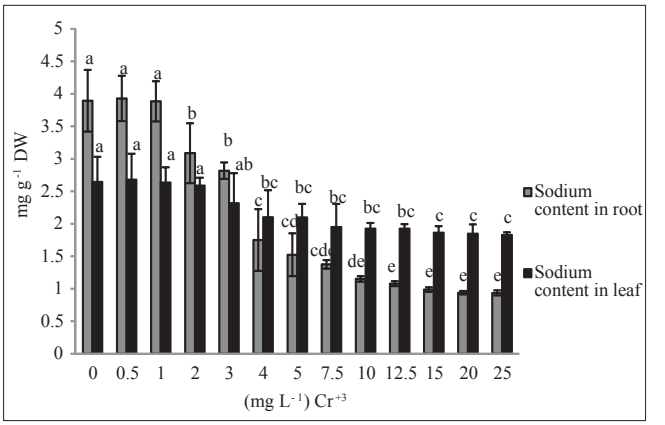

Fig. 8. Effects of different Cr concentrations on sodium content in lettuce root and leaf

Note: Means by the same letter in each column are not significantly different based on the Duncan test (P < 0.05)

grown plants in different concentrations of Cr^{3+}, plants treated with 0.5 mg L^{-1} had the highest amount of potassium in the leaves, and the levels were significant compared with the control plants. The lowest potassium concentrations in leaves were observed in 25 mg L^{-1} concentrations despite a gradual decline in the rate of decrease in the concentration of 4 mg L^{-1}. Potassium concentrations in the leaves were significant compared to the control plants (Figure 9).

A gradual reduction in the amount of potassium was observed in the roots of lettuce plants. The reduction in the 3 mg L^{-1} treatment was significant compared with the control. The lowest potassium value was observed in the 25 mg L^{-1} treatment; Compared with the 12.5, 15 and 20 mg L^{-1} treatments, there were no significant differences (Figure 9).

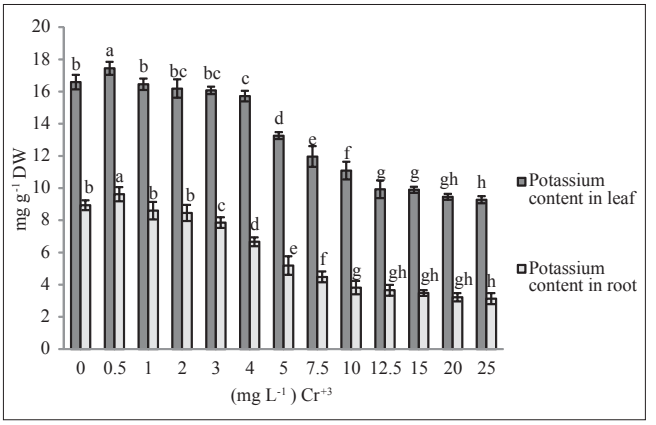

Fig. 9. Effects of different Cr concentrations on potassium content in lettuce root and leaf

Note: Means by the same letter in each column are not significantly different based on the Duncan test(P<0.05)

Accumulation Rate of Cr $^{3+}$ in shoots

The results showed that increasing the concentration of Cr^{3+} increases its concentration in lettuce shoots. The 25 mg L^{-1} treatment with an average of 0.074 mg g^{-1} of dry tissue had the highest amount of Cr, and the 0.5 mg L^{-1} treatment with a mean of 0.0029 mg had the lowest amount of Cr. Thus the rate of accumulation of trivalent Cr in the highest treatment 24.891 was equal to its amount in the lowest treatment (Figure 10).

Accumulation Rate of Cr $^{3+}$ in the roots

ANOVA analysis of data related to the amount of trivalent chromium levels in the roots of treated plants showed significant increases under different treatments of the element chromium. Plants treated with 25 mg L^{-1} and having a mean

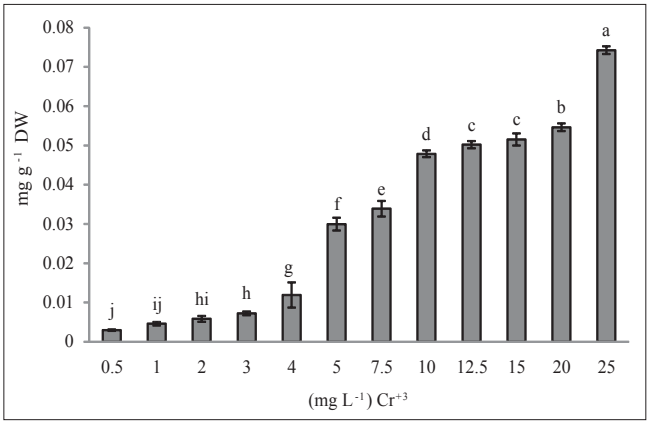

Fig. 10.Chromium accumulation in shoot of lettuce as influenced by different Cr concentrations

Note: Means by the same letter in each column are not significantly different based on the Duncan test (P<0.05)

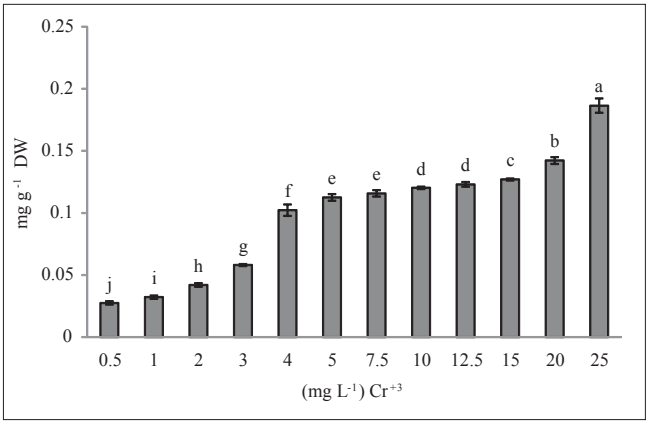

Fig. 11. Chromium accumulation in root of lettuce as influenced by different Cr concentrations

Note: Means by the same letter in each column are not significantly different based on the Duncan test (P<0.05)

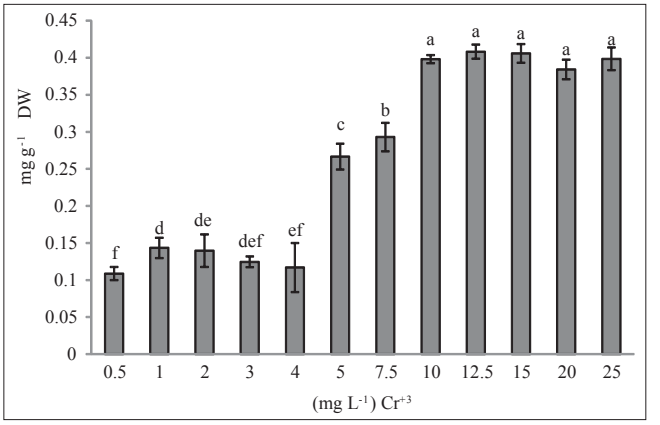

Fig. 12. Effects of different Cr concentrations on ratio of chromium content in shoot to root in lettuce plants

Note: Means by the same letter in each column are not significantly different based on the Duncan test (P<0.05)

of 0.186 mgg^{-1} of dry weight had the highest rate of 0.5 mg L^{-1}, and plants treated with 0.5 mg L^{-1} 0.5 mg $L^{-1}$and having a mean of 0.0275 mgg^{-1} of dry weight had the lowest rate of Cr^{3+} in the roots. Thus, the accumulation of trivalent chromium in roots in the highest level of treatment 6.781 was equal to that of the lowest treatment (Figure 11).

Results indicate that the chromium concentration in the roots is several times more than its concentration in the shoots. The highest concentration of Cr (25 mg L^{-1}) is 2.5 times higher than that in shoot, while the lower treatments of Cr (0.5, 1, 2, 3 and 4 mg L^{-1}) had 10 times more than its amount in shoots. Thus, increasing Cr^{3+} trivalent at the root was remarkable.

Ratio of chromium content in shoot to root in lettuce plants

In treatments higher than 10 mg L^{-1} the ratio chromium content in shoot-to-root was increased more than in treatments below 10 mg L^{-1}. In treatments of 1, 2, 3 mg L^{-1} no significant difference were observed (Figure 12).

Toxicity symptoms of Cr3+ in lettuce

Visible signs of toxicity were observed only in treated plants with high concentrations of Cr. As time goes by, the signs of chlorosis in the 5 mg L^{-1} treatment and the signs of necrosis in treatments of more than 7.5 mg L^{-1} were observed in lettuce leaves. The leaves gradually changed to a terracotta color and small necrotic spots were visible, Stems were very fragile and thin. Root growth at this concentration had also fallen sharply, so the roots were shorter and brittle.

Discussion

The results of the study showed that treatments of more than 1 mg L^{-1} Cr^{3+} decreased the concentrations of total chlorophyll and chlorophyll a and b in *Lactuca sativa* L. plants. A reduction in the amount of chlorophyll based on chromium treated plants such as lettuce (Nazz and Pandey, 2010), seedlings of celery (Scocciant et al., 2006), green beans, and cauliflower has been previously reported (Shanker et al., 2005). In another study, reductions in all photosynthetic pigments, especially chlorophyll a, in lettuce, cucumber, and bean plants were observed (Vassilev et al., 2007). Reductions in the amounts of chlorophyll a and b and the amount of total chlorophyll in maize (Zou et al., 2009), parsley (Zaker et al., 2006), green beans (Bera et al., 1999), cauliflower (Chatterjee and Chatterjee, 2000), edible plants (Sharma and Sharma, 1996), *Salvinia minima* (Nichols et al., 2000), bean (Hussain et al., 2006), kudzu (Connell and Al-Hamdani, 2001), *Eichohornia crassipes* (Mishra et al., 2009), and soybean (Sankar Ganesh et al., 2009) have been reported. Changes in chlorophyll levels can cause decreased absorption of Fe and decrease the chlorophyll biosynthetic enzymes involved in chlorophyll biosynthesis and the replacement of Mg^{2+} in the molecular structure of chlorophyll by some heavy metals under treatment or reduced antennae size of the complex due to the chromium ion (Dhir et al., 2009).

The reduction of Fe and Cr in the presence of magnesium in bean, soybean, wheat, and sugar beet plants has been reported (Shanker et al., 2005). Reduced chlorophyll content of plants treated with Cr^{3+} could be related to the decreased activity of ALAD (delta-amino levulinic acid dehydratase) and protochlorophyll reductase (Vassilev and Yordanov, 1997). The ALAD is a metalo-enzyme that involved in the biosynthesis of chlorophyll, and it is thought that Cr substitution with magnesium in the active site of the enzyme decreases ALAD activity. Reduced activity of this enzyme leads to the decreased use of ALA and so reduces the amount of porphobilinogen (PBG) which is essential for chlorophyll biosynthesis (Vajpayee et al., 2000). Deactivation of enzymes involved in the biosynthesis of chlorophyll can help reduce total chlorophyll in plants under treatment and stress of Cr (Shanker et al., 2005). Reductions in the amount of chlorophyll and thus photosynthesis results in reduced photosynthetic production with which to grow organs, and thus reduces growth (Hussain et al., 2006). On the other hand, Cr with decreased absorption of N and Mg, which are essential components in the structure of chlorophyll, cause a reduction in chlorophyll concentration. Membrane permeability changes and the ultrastructure of chloroplast due to lipid peroxidation, which is induced in response to heavy metals such as chromium, could also be involved in reducing pigments (Horcsik et al., 2006).

The present research showed that the ratio of chlorophyll a to chlorophyll b at lower treatment is not affected by stress treatment, indicating that chlorophyll a and b have equal sensitivity toward stress on the studied plant; these findings are consistent with other researchers who have studied parsley (Zaker et al., 2006), kudzu (Connell and Al-Hamdani, 2001), and beans (Hussain et al., 2006). In this study, the ratio of chlorophyll a to chlorophyll b in a 25 mg L^{-1} treatment showed the highest decrease; this represents a further reduction of chlorophyll a to chlorophyll b under high stress of Cr. These results correspond with the findings of other researchers in maize plants (Zou et al., 2009) and celery seedlings (Scocciant et al., 2006). This reduction can cause damage faster (Appenroth et al., 2003).

In the present study it was found that increasing the concentration of trivalent Cr increased the amount of proline in lettuce leaves. Increments in concentration of more than 3 mg L^{-1} were significant, but in concentrations higher than 10 mg L^{-1}, it again fell. Compared with the control, this result is not

significant. These findings correspond with other researchers' studies. For instance, increasing proline in Cr stress was observed in all four soybean genotypes and resistant genotypes showed more proline (Sankar et al., 2009). It depends on the strategies that plants employ to adopt Cr toxicity because the proline plays multiple roles in creating resistance to stress conditions. An increase in proline concentration in lettuce plants treated with heavy metal stress represents an increase of proline in stress. In higher concentrations, its amount was reduced (Teklic et al., 2008). One study indicated that the effect of trivalent Cr on plant *Datura innoxia* increased proline (Vernay et al., 2008). Karimi and Nojavan (2008) showed an increase in proline levels in lentil seedlings that were treated with a heavy metal. With increasing concentrations of treatment, that amount was reduced further.

Heavy metal stress in *Bacopamonniera* and *Pluchea lanceolata* plants increased proline content. By increasing the concentration of metal in the environment, proline content was reduced (Kumar et al., 2004). Increases in Cr resulting in an incensement of free amino acids and proline in green algae *Chlorella* the amount of which fell at higher concentrations is probably due to cell degradation (Horcsik et al., 2006). The most obvious metabolic manifestation of abiotic stresses in higher plants is the rapid accumulation of proline. An external supply of proline can cause osmotic resistance in plants (Kumar et al., 2004). Heavy metal stress in plants increased proline synthesis. Proline as an essential amino acid is important for the plant to adjust to the osmotic pressure of heavy metal-stressed cells (Anbazhagan et al., 1998). A decrease in metabolic activity may lead to the accumulation of NADH, and in the synthesis of a molecule of proline from glutamic acid, 2 molecules of NADH are needed thus proline synthesis is a strategy to reduce acidity and accumulation of NADH (Pardha, 1991). Proline can also act as an antioxidant and by inhibiting lipid peroxidation reducing risk of free radicals and maintaining membrane integrity (Mehta and Gaur, 1999). Proline makes a complex with the metal to prevent damage to cell membranes (Wu, 1998). It may also reduce the effect of ions on enzymes and cause the stability of enzymes (Wallace, 1987). It is a source of nitrogen and carbon for growth and participation in protein synthesis during stress (Sanker et al., 2009).

In higher concentrations of chromium, proline levels are reduced which indicates that plants have other mechanisms against stress (Karimi and Nojavan, 2008). The rapid increase of proline which occurs when stress conditions are reduced coincides with the start of water potential in leaves (Gzik, 1996). Anything that reduces water potential increases the accumulation of proline, which has many biological effects (Kuznetosv and Shevyakova, 1999). Proline accumulations in plants under heavy metal stress have high relevance to the mechanism of plant resistance against osmotic changes (Sanker et al., 2009). The results of the current study showed that with increasing chromium concentration, the amount of soluble sugars is higher in the roots than in the leaves. In roots in concentrations higher than 7.5 mg L^{-1} and in leaves in concentrations higher than 10 mg L^{-1}, it is reduced. Also with increases in the concentration of Cr, the amount of soluble sugars in roots and leaves is reduced, more so in leaves than in roots.

The increase in soluble sugars and reduction in insoluble sugars in the roots and leaves of the Kudzu plant were significant at 4 and 8 mg L^{-1}, respectively. Increases in carbohydrate reserves in the *Azolla caroliniana* in the presence of chromium and *Salvinia minima* in the presence of aluminum have been reported (Connell and Al-Hamdani, 2001). The highest increases were seen in the amount of sugar in lettuce plants treated with 50% diluted wastewater containing Cr. When plants were treated with undiluted wastewater containing Cr, the amount of sugar was reduced again and was consistent with signs of toxicity in plants (Nazz and Pandey, 2010). High concentrations of chromium in rice plants caused a decrease in glucose (Singh et al., 2005). Karimi and Nojavan (2008) reported increments in the levels of soluble carbohydrates in lentil seedlings treated with cadmium. Water potential and turgor pressure decrement by Cr is due to reduced stomata conductance and root surface, and ultimately decreased of water absorption (Shanker et al., 2005). In spinach, a reduction in osmotic potential causes the chloroplast to wrinkle and the stroma to acidify, ultimately resulting in reduced photosynthesis.

The highest effect of osmotic stress on photosynthetic activity is effect on 1, 6-bis phosphatase activity caused by the acidification of the stroma (Berkowitz and Gibbs, 1983). Stress induction causes a reduction in cell expansion. This reduces the conversion of soluble sugars into the structural polysaccharides, so they accumulate in the plant. Increase the activity of enzymes degrading non-soluble sugars, such as invertase and sucrose synthase that cause a reduction in sugars on the one hand and increases their production on the other (Verma and Dubey, 2001). Cr with impaired absorption of minerals and potassium reduction in plants (Shanker et al., 2005) causes a reduction in synthesis enzyme of the starch enzyme and will prevent conversion of glucose to starch; that itself will increase sugar in the plant (Marshener, 1995). The heavy metal content reduces the amount of water transported to the leaves and the peroxidation of cell membranes, thus causing changes in the ultrastructure of cell organelles and the activities of key enzymes in a carbohydrate metabolic way. This is followed by the accumulation of metal in the cells, and the content of reduced sugars in the plant increase; that is the plant's adaptive mechanism to maintain osmotic

potential in stress situations. In addition to the role of sugars in regulating osmotic pressure, it is assumed that the plant's carbohydrate reserves are maintained at an optimal level in order to maintain basic metabolism of cells under stressful environmental conditions (Verma and Dubey, 2001).

Reduction of plant growth caused by increased chromium reduces respiration rate and this increment is resulting from mitochondrial damage. Thus, the plant, by reducing photosynthesis and increased signs of aging, will depend on root carbohydrate reserves in order to survive (Connell and Al-Hamdani, 2001). The activity of the antioxidant enzymes, Guaiacol peroxidase (GPOX) and polyphenol oxidase (PPO) in leaves and roots of lettuce plants was found to have increased. There was more PPO activity in leaves than in roots. The highest activity levels in the leaves was observed in the 20 mg L^{-1} treatment and in the roots in the 12.5 mg L^{-1} treatment, which is significant compared to the control. GPOX activity in leaves was higher than the maximum root activity and the most activity was seen in the 10 mg L^{-1} treatment in leaves and in roots in the 7.5 mg L^{-1} treatment, which is significant compared to the control. The activity of both enzymes decreased again at higher concentrations. This review is consistent with the results of other researchers. Gozdez et al. (1997) found that lower concentrations of heavy metals increased the activity of antioxidant enzymes, whereas higher concentrations reduced it. High peroxidase activity in *Leucaena leucocephala* callus was observed on treatment 15 µM of chromium (Rot et al., 1999).

The peroxidase activity in lettuce plants under stress conditions caused by the Cu^{2+} treatment significantly increased, and in concentrations of more than 25 mg Kg^{-1} DW, it was reduced by an amount that had a positive correlation between the concentration of free proline and GPOX of leaves under stress. This increase presents enzymatic and non-enzymatic antioxidant responses in lettuce (Teklic et al., 2008). In one study it became clear that heavy metals significantly increased GPOX activity in the roots of all plant species, such as lettuce, cucumbers, and beans. The maximum response was observed in cucumber plants (Vassilev et al., 2007). Sen et al. (1994) reported an increase in peroxidase activity at concentrations of 10 µg of chromium. Increased peroxidase activity in lettuce irrigated with treated wastewater containing chromium has been reported with higher decreases (Naaz and Pandey, 2010).

These results are because of the high level of damage to the immune system caused by high chromium concentrations in lettuce plants. An increase in antioxidant enzymes in sugarcane, soybean, and sunflower have been reported (Fronazier et al., 2002). The accumulation of hydrogen peroxide in sugarbeet leaf at high concentrations of Cr and its reduction at low concentration corresponds with the increased antioxidant enzyme activity. In higher chromium concentrations, the activity of this enzyme is not synchronized with ROS production (Rai et al., 2006). In this condition, photosynthesis is reduced, increased energy is stored in the chlorophylls, and then it is transformed to single oxygen, and it spoils. The reduction of CO_2 assimilation decreases to need NADPH and ATP, which leads to the accumulation of NADPH and $NADP^+$deficiency. The excess excitation energy is not absorbed by the $NADP^+$. It is absorbed by O_2 and produces superoxide radicals that cause oxidative damage, including lipid peroxidation and antioxidant systems is resulting in interference (Calatayud and Barrenos, 2004). When tensions are high, ROS production and oxidative damage occur. ROS are extremely reactive and quickly disturb normal cell metabolism (Gajewska et al., 2006).

It has been reported that modifying a plant's antioxidant defense system increases oxidative stress tolerance (Dazyet al., 2008). This change is probably due to the synthesis of new isoenzymes or the increased activity of enzymes that metabolize ROS are already included. The PPO enzyme could be used as a terminal oxidase in the respiratory chain and oxidative phosphorylation in the mitochondrial membrane to cause the energy transfer to the water molecules and thereby increase oxygen uptake and activate the oxidative pentose phosphate cycle (Singh et al., 1999).

The results of this study showed that increasing Cr decreased sodium and potassium concentrations in roots and leaves of lettuce plants. The decrease in sodium in the leaves and roots of the 4 mg L^{-1} and 2 mg L^{-1} treatments was significant. The loss of potassium in the leaves and roots of the 4 mg L^{-1} and 3 mg L^{-1} treatments was significant. The 0.5 mg L^{-1} treatment had a stimulatory effect on potassium levels in roots and leaves. These results were observed in parsley (Zaker et al., 2006), soybean and sugar beet (Zayed and Terry, 2003). Morale et al. (1995) reported that high concentrations of chromium strongly affect uptake and concentrations of essential nutrients such as Ca, P, N Fe, Mg, Na, and K in tomato plants. Thus reducing nutrient absorption is not the only cause of reduced growth; impaired respiration and photosynthesis also reduce growth.

Therefore, it is very likely that the reduction in plant growth is due to a decrease in potassium absorbed in lettuce in the treatment with Cr. Several studies have reported the direct involvement of this element through its effect on osmotic potential, water absorption, and turgor involvement in protein synthesis (Marschner, 1995). Sodium such as potassium involve in osmotic adjustment, water absorption and turgor (Marschner, 1995). In general, heavy metals, including chromium ions, have a high affinity for sulfhydryl and carboxyl groups that depend on the chemical and physical properties of the cation. Membrane ATP-ases are electrogenic pumps

and involve in cell membrane selective permeability of cell membrane. One reason that the decreased absorption of most nutrients in plants under stress due to chromium can inhibit the activity of the plasma membrane is the disruption of cell membranes and the production of free radicals. Reduced activity of proton pump outflow is reduced, resulting in greater reductions in the transmission and absorption of nutrients through the roots (Shanker et al., 2005). High concentrations of chromium interact with the mitochondrial electron transport system occurs. The negative impact on photosynthesis, ATP synthesis, and related processes such as the absorption of nutrients and growth will be affected. Therefore when the synthesis of ATP is interfered with, absorption is also impaired (Shanker et al., 2005).

The growth stimulatory effect of chromium concentrations in 0.5 mg L^{-1} chromium can be attributed to an increase in the potassium concentration. Based on the results obtained with increasing of concentrations of Cr, its accumulation in roots and leaves were increased, and in leaves were higher than in the roots. Several studies have shown that chromium accumulated more in the roots than the shoots of some plants such as broccoli, kale, peas, beans, and lettuce (Zayed et al., 1998) and *Eichhornia crassipes* (Mishra et al., 2009). Chromium is a toxic element and is therefore unnecessary for plants, and plant has not specific mechanisms for Cr uptake and its absorption is done by carriers of essential metals. Both forms of chromium are absorbed by plants, and trivalent chromium is absorbed by a passive mechanism into vacuoles or cell walls of plants and is kept immobile (Shanker et al., 2005). Absorption of chromium (VI) actively perform and reduced to the trivalent chromium by reductase enzyme in root , so that the form of trivalent chromium was identified in root and stem of some plants like celery of which was treated with CrO_4^{2-} (Scoccianti et al., 2006).

Cr is transported through the xylem to the shoot. Chromium accumulation in plants causes toxicity and results in reduced root growth, biomass, and chlorosis-impaired photosynthesis and, ultimately, cell death (Scoccianti et al., 2006.) The greater accumulation of chromium in roots could be caused by store of chrome in vacuoles of root cells as well as binding to cell wall carbohydrates (Shanker et al., 2005). In plants, especially crops, chromium at low concentration (0.05-1 mg L^{-1}) increase growth but plants need is not considered. A concentration of 1 mg L^{-1} induced changes in the metabolic processes of plants. Stimulating effects of heavy metals in low concentrations can be due to the hyperpolarization of root cell membranes and source of energy for cation absorption, resulting in cell swelling. Because some enzymes in low concentrations, such as cellular protease, increase the activity of this enzyme, it may also result in increased growth (Shanker et al., 2005).

The stimulatory effects of low concentrations of 0.1 and 0.01 µg g^{-1} chromium on the growth of *Eichhornia crassipes* (Mishra et al., 2009) were reported and corresponded with our findings which indicate a stimulatory effect of trivalent chromium concentrations in 0.5 mg L^{-1}.

Toxicity symptoms such as reduced leaf surface and burning marginal and tips of leaves of spinach plants treated with both types of chromium was observed (Singh, 2005). A decrease in plant water potential caused water stress symptoms such as bending and the formation of brown spots on the leaves (Berkowitz and Gibbs, 1983). Also chlorosis induced by heavy metals is generally related to low content of plant Fe which affect on iron mobility and absorption (Shanker et al., 2005). The results of this study were consistent with previous reports. Plants that are better able to cope with toxic metals and survive contamination with heavy metals and semi-metals are a good choice for the purposes of phytoremediation.

In the present study, in the lettuce shoots of the 25 mg L^{-1} treatment with a mean of 0.07425 mg per g dry tissue and a mean of 0.18560 mg per g dry weight, the maximum amount of Cr can be observed. Cr concentration in roots was several times more than in the shoots. The amount of root chromium in the highest concentration (25 mg L^{-1}) was 2.5 times higher than that in the shoot, while in the lower treatment 0.5, 1, 2, 3 and 4 mg L^{-1} the amount of chromium was 10 times more than in the shoots. Thus trivalent Cr concentration is considerable in roots.

Broccoli and cabbage are sulfur-friendly plants. These species also have the ability to absorb and accumulate heavy metals more than several other species. Kumar et al. (2004) reported that *Brassica* species, such as Indian mustard, showed a high ability to absorb heavy metals such as lead, chromium, cadmium, nickel, zinc, and copper thorough the roots, and these metals concentrate in their tissue. Zeid et al. (1998) also showed that, regardless of the type of chromium, these plants stored more Cr in roots, and the Cr concentration in roots is a hundred times more than in the shoot.

In leafy vegetables that don't high levels of iron in their leaves such as lettuce and cabbage, chromium movement to the shoot is less, while the iron-friendly leafy vegetables such as spinach, onion, celery, and onion transfer chromium to the shoot (Zayed et al., 1998).

In another study, it was determined that Cr accumulation was higher in the roots of lettuce plants than in the stems (Naaz and Pandey, 2010), which indicates the accumulation of heavy metals in roots and its movement to the higher parts. Poor movement of chromium to stems can be due to the maintenance of chromium in vacuoles of stem cells for detoxification; however, we know that chromium is a toxic element and unnecessary for plants. Thus there is no specific mechanism for its absorption and transmission (Shanker et al., 2005).

Zayed and Terry (2003) reported that species of *Brassicaceae* family such as broccoli, kale, and cabbage could absorb more chromium than other species without having to show signs of toxicity. Very low mobility of Cr from root to shoot is a major obstacle to the use of trees for phytoremediation. Pulford et al. (2001) confirmed that among trees in temperate regions chromium is poorly absorbed to shoot and is maintained more in roots. This finding means that the prospect of using trees as phytoremediation in contaminated places with Cr is low (Shanker et al., 2005).

Conclusions

We can conclude that chromium accumulation is less in lettuce plants than in the *Brassica* species. Because of the visible signs of toxicity and growth retardation due to the high chromium accumulation in treated lettuce plants, this plant can be used as a biomarker of chromium contamination; because of this, it is useful in research and plant treatment methods.

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