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Effects of vesicular arbuscular mycorrhiza *Glomus intraradices* on photosynthetic pigments, antioxidant enzymes, lipid peroxidation, and chromium accumulation in maize plants treated with chromium

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Abstract: Contamination of soil and ground water by chromium (Cr), due to its wide industrial use, has become a serious source of concern over the past decade. In this study a glasshouse experiment was conducted to investigate the effects of the mycorrhizal fungus *Glomus intraradices* on Cr toxicity in maize plants. Half of the plants were inoculated with the arbuscular mycorrhizal fungus (AMF). Cr was supplied in the form of potassium dichromate at 0.00, 0.10, 0.25, and 0.50 mM through irrigation water in a sand culture. At the end of the experiment, it was observed that Cr had significantly decreased the chlorophyll content in the maize leaves. The mycorrhizal plants had greater chlorophyll content than the non-mycorrhizal plants. Moreover, increasing the chromium concentration caused an increase in the malondialdehyde (MDA) content in the shoots and roots of the whole plant; however, the AM plants showed a lower MDA content than the non-AM plants. Cr caused an induction in guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) activity in the roots of the non-mycorrhizal plants but no significant induction was observed in APX activity in Cr-treated AM roots. The activity of GPX in the roots of AM plants was lower than in those of non-AM plants under chromium treatment. Measurements of chromium concentration indicated that Cr mainly accumulated in the roots of the maize plants. The Cr concentration significantly decreased in the shoots through AM symbiosis.

Key words: Antioxidant enzymes, chromium, lipid peroxidation, mycorrhizal fungus, photosynthetic pigments

Kromla muamele edilmiş mısır bitkilerindeki fotosentetik pigmentler, antioksidant enzimler, lipid peroksidasyonu ve krom birikimi üzerine vesikülar arbukülar Mikoriza *Glomus intraradices*'in etkileri

Özet: Krom (Cr) ile muamele edilmiş mısır bitkilerinin bazı fizyolojik ve biyokimyasal parametreleri üzerine mikorizal mantar *Glomus intraradices*'in etkilerini araştırmak amacıyla bir sera denemesi yapılmıştır. Bitkilerin yarısı arbuskülar mikorizal mantar (AMF) ile aşılanmıştır. Cr, 0,00, 0,10, 0,25, 0,50 mM konsantrasyonlarındaki potasyum dikromat olarak sulama suyu ile kum kültürüne verilmiştir. Denemenin sonunda, Cr'un mısır yapraklarındaki klorofil miktarını önemli ölçüde azalttığı görülmüştür. Mikorizal bitkiler, mikorizal olmayan bitkilerden daha çok klorofil içeriğine sahiptir. Malondialdehid miktarı (MDA) lipid peroksidasyon indeksi olarak ölçülmüştür. Krom konsantrasyonundaki artış bütün bitkilerin sürgün ve köklerindeki MDA içeriğinde artışa neden olmuştur. Fakat, AM bitkiler, AM olmayan bitkilerden daha az MDA içeriği göstermiştir. Ayrıca, Cr, mikorizal olmayan bitkilerin köklerinde gayakol peroksidaz (GuPX) ve askorbat peroksidaz (APX) aktivitisinde bir indüklenmeye sebep olmuştur, fakat, Cr ile muamele edilmiş arbuskülar mikorizal (AM) köklerindeki APX aktivitesinde önemli bir indükleme gözlenmemiştir. AM bitkilerin köklerindeki GuPX aktivitesi, krom muamelesi altındaki AM olmayan bitkilerden daha düşüktür. Krom konsantrasyonunun ölçümü, kromun başlıca mısır bitkilerinin köklerinde biriktiğini göstermiştir. Krom konsantrasyonu AM ile simbiyoz yaşayan sürgünlerde önemli ölçüde azalmıştır.

Anahtar sözcükler: Mikorizal fungus, krom, fotosentetik pigmentler, lipid peroksidasyonu, antioksidant enzimler

Introduction

Chromium is the seventh most abundant metal in the earth's crust (1) and is used on a large scale in many different industries, including leather processing and finishing, metal plating, paints and pigments production, tanning, catalytic manufacturing, and in chromic acid production. In nature, Cr has several oxidation states but, exists in 2 different stable states, including Cr III and Cr VI. Cr VI is highly soluble in water and is the most toxic form of chromium (2). Although Cr is necessary for glucose metabolism in humans and animals, at high concentrations it is very toxic and carcinogenic. Several researchers indicated that Cr stimulates the growth of plants at low concentrations (1 μm) (3,4). However, there is no evidence for the biological role of Cr in plants today (5). In contrast to other toxic metals such as cadmium, mercury, lead, and aluminum, plant scientists have paid little attention to Cr (6). For plants Cr is toxic in any concentration while levels differ according to the plant species (7). The symptoms of Cr toxicity have been reported by several researchers. The initial symptoms of Cr toxicity appeared as severe wilting and chlorosis in plants. Sharma et al. (2003) observed interveinal chlorosis in maize (8). Leaf chlorosis has been reported in *Nymphea alba*, tomato, potato (9,10). Moreover, Cr causes growth inhibition in plants. Cr phytotoxicity can result in the inhibition of seed germination, pigment degradation, and the induction of oxidative stress (11,12). The presence of heavy metals in toxic concentrations can result in the formation of reactive oxygen species (ROS). Consequently, oxidative stress and lipid peroxidation occur. Plants have several means of heavy metal detoxification. Heavy metals can induce antioxidant enzymes activity such as catalase (CAT), guaiacol peroxidase (GPX), superoxide dismutase (SOD), and glutathione reductase (GR). Furthermore, heavy metals cause an induction in phytochelatins and metallothioneins biosynthesis in plants. Phytochelatins are not induced under chromium toxicity (13). It seems that metallothioneins may help plants in Cr detoxification (2). Furthermore, mycorrhizal fungi are recognized as biological agents that potentially increase the tolerance of plants to heavy metal toxicity. The reduction of growth due to Cr interference with nutritional elements uptake can be improved through the application of mycorrhizal inoculation. Karagiannidis and Hadjisavva Zinoviadi (1998) showed that vesicular arbuscular mycorrhizal fungus *Glomus mosseae* enhanced yield in wheat and simultaneously decreased the chromium content in these plants (14). Davies et al. (2002) reported that AMF had a positive effect on tissue mineral concentration, growth and gas exchange in Cr treated sunflower plants (15). Since there is very little information about the effects of vesicular arbuscular mycorrhizal fungi on chromium toxicity, this study investigated the effects of *Glomus intraradices* on Cr toxicity in maize plants.

Material and methods

The seeds of maize (*Zea mays* cv. 704) were sterilized in a 10% hypochlorite solution for 20 min and were then transferred to petri dishes for germination. Three day seedlings of similar size were cultured in pots containing washed and sterilized sand. Before transplanting, half of the pots were inoculated with the vesicular mycorrhizal fungus *Glomus intraradices*. This experiment was performed under glasshouse conditions including minimum and maximum temperature at 28 °C and 34 °C respectively, a relative humidity of 60%-70%, and a 16:8 (day/night) photoperiod. Cr was supplied as potassium dichromate at 0.00, 0.10, 0.25, and 0.50 mM in irrigation water. After 25 days of sowing, .plants were supplied with a modified Hoagland's nutrient solution with half P concentration and treated with different concentrations of potassium dichromate every other day. This experiment was conducted as a completely randomized design with 3 replications. Forty-five days after transplantation, plants were harvested and divided into roots and shoots and were stored in liquid nitrogen for enzyme assay.

Chlorophyll determination

Fresh leaf samples from each treatment (0.5 g) were homogenized with an 80% (v/v) acetone in total darkness. The amount of chlorophyll was estimated spectrophotometrically at 645 and 660 nm according to the method outlined by Wellburn and Lichtenthaler (16).

Enzyme extraction and assay

Fresh leaf and root samples (0.5 g) from each treatment were homogenized in an ice cooled mortar in 0.1 M Tris-HCl (pH 7.5) containing 3 mM $MgCl_2$ and 1 mM EDTA. In order to estimate ascorbate peroxidase activity 0.2 mM ascorbate was added to the extraction buffer. The homogenates were centrifuged at 5000 ×*g* for 20 min and the supernatant was used for the enzyme assay (17).

Ascorbate peroxidase

The activity of ascorbate peroxidase (APX) was measured according to the method of Nakona and Asada (18). It was found that 3 mL of the reaction mixture contained 50 mM phosphate buffer (pH 7), 0.1 mM H_2O_2, 0.5 mM sodium ascorbate, 0.1 mM EDTA, and 100 µL enzyme extract. The enzyme activity is expressed as µmol of oxidized ascorbate min^{-1} g^{-1} FW.

Guaiacol peroxidase

The guaiacol peroxidase activity was measured according to the method of Chang and Kao (19). The reaction mixture contained 2.5 mL of 50 mM phosphate buffer (pH 7), 1 mL of 1% guaiacol (w/v), 1 mL of 1% H_2O_2. Then 0.3 mL of enzyme extract was added to the reaction mixture. The increase in absorbance due to the oxidation of guaiacol, with an extinction coefficient of 26.6 mM^{-1} cm^{-1}, was recorded at 420 nm for 1 min. Enzyme activity was expressed in terms of µmol of oxidized guaiacol min^{-1} g^{-1} FW.

Lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced using a thiobarbituric acid (TBA) reaction as suggested by Popham and Novacky (20). One gram of the fresh root and leaf samples from each treatment were homogenized with 5 mL of 1% trichloroacetic acid (TCA) and centrifuged at 10,000 ×*g* for 20 min. One milliliter of 20% TCA containing 0.5% TBA was added to 1 mL of the supernatant. The mixture was heated at 95 °C for 30 min. The absorbance was determined at 532 and 600 nm. Lipid peroxidation was expressed as malondialdehyde content in µmol g^{-1} FW.

Chromium analysis

For the estimation of Cr content, plants were harvested and rinsed with deionized water. Then they were separated into roots and shoots. Samples were dried and their ash was obtained in a forced-draught oven at 650 °C for 48 h. The Cr was measured according to the modified method of Davies et al. (21). Finally, an atomic absorption spectrophotometer (PG-990) was used to estimate the Cr content in ash samples.

Statistical analysis

This experiment was conducted using a completely randomized design. Data were analyzed statistically using a general linear model for analysis of variance followed by a Tukey test.

Results

Photosynthetic pigments

Photosynthetic pigments (chlorophyll a, chlorophyll b, and total chlorophyll) showed a reduction under chromium treatment in both AM and non-AM plants (Figures 1 and 2). Pigment content negatively correlated with chromium concentration in irrigation water. However, in mycorrhizal plants higher chlorophyll content was observed in comparison with non-AM plants.

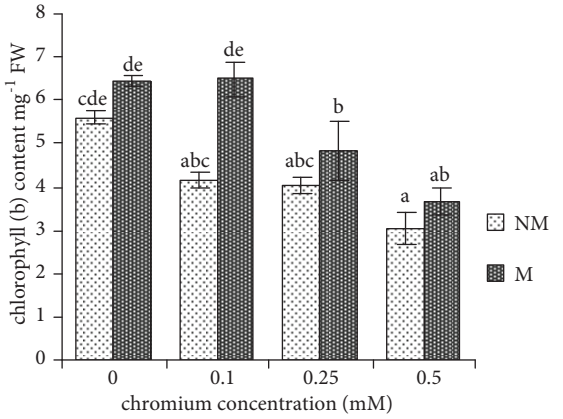

Figure 1. Chlorophyll a content in mycorrhizal and non-mycorrhizal maize leaves treated with 0.00, 0.10, 0.25, and 0.50 mM potassium dichromate. Data are means of 3 replicates ± SE. Different letters indicate significant differences in each treatment ($P \leq 0.05$) determined by ANOVA followed by a Tukey test.

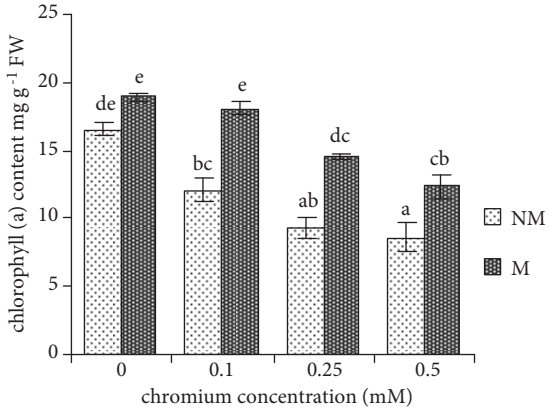

Figure 2. Chlorophyll b content in mycorrhizal and non-mycorrhizal maize leaves treated with 0.00, 0.10, 0.25, and 0.50 mM potassium dichromate. Data are means of 3 replicates ± SE. Different letters indicate significant differences in each treatment ($P \leq 0.05$) determined by ANOVA followed by a Tukey test.

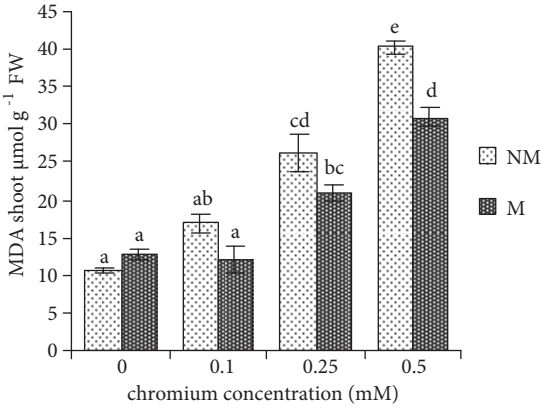

Figure 4. The amount of malondialdehyde (MDA) in mycorrhizal and non-mycorrhizal leaves treated with 0.00, 0.10, 0.25, and 0.50 mM potassium dichromate. Data are means of 3 replicates ± SE. Different letters indicate significant differences in each treatment ($P \leq 0.05$) determined by ANOVA followed by a Tukey test.

Lipid peroxidation

The amounts of MDA increased significantly by increasing the Cr supply. However, in mycorrhizal plants, the MDA content was lower in roots and shoots (Figures 3 and 4). The MDA content specially decreased in the shoots of AM plants in comparison with non-AM plants. The highest MDA content was observed at 0.50 mM Cr treatment in the shoots of non-AM plants. According to statistical analyses, the increase in root MDA content was slight and no interactive effect was observed between the Cr and mycorrhizal treatments in the roots.

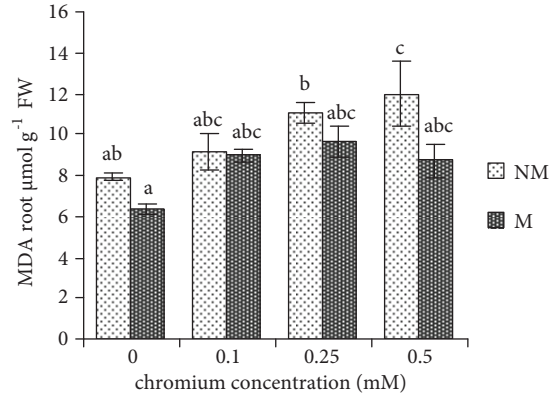

Figure 3. The amount of malondialdehyde (MDA) in mycorrhizal and non-mycorrhizal roots treated with 0.00, 0.10, 0.25, and 0.50 mM potassium dichromate. Data are means of 3 replicates ± SE. Different letters indicate significant differences in each treatment ($P \leq 0.05$) determined by ANOVA followed by a Tukey test.

Antioxidant enzymes

Cr caused an induction in antioxidant enzymes activity in the roots of AM and non-AM plants. In the roots of non-AM plants antioxidant enzymes activities, especially GPX, were induced significantly by increasing the Cr concentration; however, no significant induction in APX activity was observed in mycorrhizal roots (Figure 5). At 0.0 mM Cr, we observed a slight induction in APX activity in mycorrhizal roots as compared with non-mycorrhizal roots. Mycorrhizal roots showed lower GPX activity than non-AM ones (Figure 6).

Chromium analysis

Measurements of Cr indicated that Cr mainly accumulated in the roots of the plants. The Cr concentration in the shoots was very low. There was a 72% increase and 36.5% and 83.5% decrease in Cr concentration at 0.1, 0.25, and 0.50 mM Cr treatment, respectively, in the shoots of AM plants in comparison with non-AM plants. Interestingly, at 0.50 mM potassium dichromate, the concentration of Cr was higher in non-AM plants than in AM plants. The Cr concentration in AM roots was similar to that in non-AM roots at 0.10 and 0.25 mM Cr supply, while at 0.50 mM chromium, non-AM roots accumulated more Cr. Data are shown in the Table.

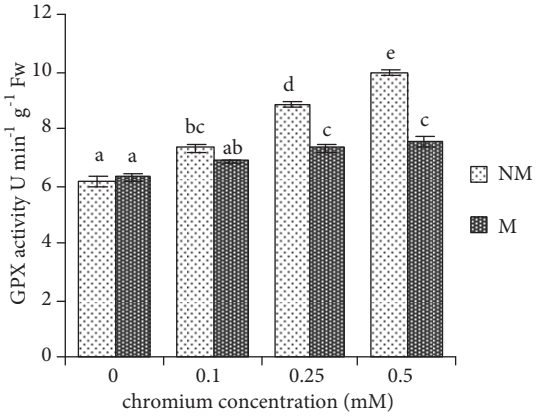

Figure 5. Ascorbate peroxidase activity in mycorrhizal and non-mycorrhizal roots treated with 0.00, 0.10, 0.25, 0.50 mM potassium dichromate. Data are means of 3 replicates ± SE. Different letters indicate significant differences in each treatment (P ≤ 0.05) as determined by ANOVA followed by a Tukey test.

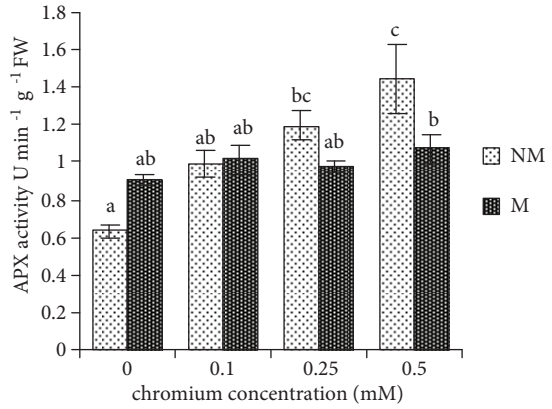

Figure 6. Guaiacol peroxidase activity in mycorrhizal and non-mycorrhizal roots treated with 0.00, 0.10, 0.25, and 0.50 mM potassium dichromate. Data are means of 3 replicates ± SE. Different letters indicate significant differences (P ≤ 0.05) as determined by ANOVA followed by a Tukey test.

Table. Effect of chromium and mycorrhizal treatment (AM) on Cr uptake in maize tissues.

Concentration (mM)	**Chromium accumulation (µg/g)**			
	Root		**Shoot**	
	Control	AM	Control	AM
0.00	ND***	ND	ND	ND
0.10	483.8 ± 16.6*[a]	489.8 ± 13.7[a]**	22.2 ± 2[a]	38.2 ± 3.4[b]
0.25	788.3 ± 35.2[b]	885.5 ± 12.5[b]	83.2 ± 5.5[c]	52.8 ± 1.4[b]
0.50	1792.0 ± 26.3[d]	1377.1 ± 14.5[c]	500.8 ± 5.8[d]	82.3 ± 2.3[c]
Significance				
Cr	211.320****		3173.704	
AM	3.168		689.407	
Cr × AM	8.350		653.195	

* Data are means of 3 replicates ± SE

** Different letters indicate significant differences determined by ANOVA followed by a Tukey test

*** not determined

Discussion

Plant stress caused by excessive concentration of heavy metals is alleviated by mycorrhizal fungi (22,23). Mycorrhizal fungi may increase the tolerance of plants to heavy metals through the following mechanisms: immobilization of heavy metals by compounds secreted by the fungus, precipitation in polyphosphate granules in the soil, adsorption in fungal cell walls, and chelating of metals inside the fungus (24).

In this study, significant reduction in pigment content of non-AM plants was observed. Bioaccumulation of Cr and its effects on photosynthetic pigments in various crops and trees have been reported previously (25,26). Cr degrades δ-aminolevulinic acid dehydratase and consequently inhibits chlorophyll biosynthesis (9). Furthermore, lipid peroxidation causes degradation of photosynthetic pigments (27).

Cr can deactivate many enzymes by replacing Mg ions at their active sites. It also reduces chlorophyll content in a similar way (10). Phosphate is a mineral nutrient with poor solubility that limits the growth of the plant. Phosphate contributes to pigment biosynthesis in its role as an energy carrier. Because P and Cr are competitive elements, higher levels of Cr reduce leaf tissue P levels (15). In this study, we observed that AM plants had higher chlorophyll content than non-AM ones. Moreover, Demir (2004) and Selvaraj (2006) indicated an increase in chlorophyll content by mycorrhization in pepper plants and *Prosopis juliflora*, respectively (28,29). AM fungi facilitate the uptake of P from the soil in the plants. Therefore, mycorrhizal fungi can enhance pigment content by increasing P uptake.

Cr has a high oxidative potential that can produce reactive oxygen species. ROS cause oxidative damages in plants. In response to oxidative stress, plants have developed defense systems to scavenge the ROS. APX and GPX can protect the cells from oxidative injury under Cr-induced oxidative stress. APX is known as a member of the ascorbic acid glutathione cycle. This enzyme eliminates poisonous H_2O_2 from plant cells (30). Furthermore, GPX participates in lignin biosynthesis and might build up a physical barrier against heavy metal poisoning; it also plays an important role as an eliminator of H_2O_2 (30). Hyperactivity of the SOD, GPX, and CAT and reduction in APX activity were observed under chromium treatment in *Ocimum tenuiflorum* by Rai et al. (30). In contrast, Shanker et al. (2004) observed an increase in APX activity in green gram plants that were treated with 50 μM Cr (31). There are few reports on antioxidative systems in mycorrhizal fungi in either pure culture or symbiosis (32). Different enzyme activity patterns of AM and non-AM plants were observed in Jackbean by Andrade et al. (33). Schüzendübel et al. (2001) reported that the activities of unspecific peroxidase increased after 14 days in non-mycorrhizal Cd-exposed roots but not in mycorrhizal ones (32). Higher GPX activity in non-AM roots of maize plants than AM roots were reported under Cd treatment (34). In this study, we observed additional activity of GPX in non-AM roots than AM ones under Cr treatment that may suggest a higher tolerance to chromium toxicity in AM plants. APX activity was not affected by mycorrhization. The slight induction of APX activity in AM roots as compared to non-AM roots at 0.0 mM Cr is probably due to the roots producing a defense response to the mycorrhizal infection.

Under Cr stress lipid peroxidation is initiated as a result of oxidative stress. Malondialdehyde (MDA) is a product of lipid peroxidation. In wheat, the process of lipid peroxidation and the amount of MDA increased with the increase in concentration and duration of Cr exposure (35). Increased MDA content has also been reported in mosses exposed to Cr (36). Chromium-induced loss of membrane permeability, coupled with increased MDA production, has also been observed in *Vallisneria spiralis* (37). The primary toxic effect of Cr seems to be membrane damage as a result of the high oxidative potential of Cr (VI). In this study, the reduction of MDA in AM plants in comparison with non-AM plants may be a result of AMF reducing oxidative stress and ROS production in heavy metal toxicity. AM fungus probably reduces oxidative stress by reduction of Cr concentration in the plant tissues and consequently ROS production and lipid peroxidation reduce.

In this study, we observed that in AM plants, especially in the shoots, the Cr concentration was lower than in non-AM plants. Mycorrhizal fungus, *Glomus intraradices* significantly decreased translocation of Cr to the shoots. Mycorrhizal fungi can immobilize metals. The fungi may immobilize metals in several ways including the secretion of special compounds and the precipitation of heavy metals in polyphosphate granules in the soil. For example, Glomalin is an insoluble glycoprotein that is released and which immobilizes heavy metals by binding them in the soil. Furthermore, the binding of heavy metals to chitin in the fungal cell walls causes a reduction in the translocation of heavy metals to the shoots of the plants. Also fungal vesicles may be involved in storing toxic metals and thereby avoiding their translocation to upper parts of the plant (24). Some studies indicated lower translocation of heavy metals to shoots by mycorrhization. Rivera-Becerril et al. (2002) and Medina et al. (2005) reported that Cd had been stabilized in the root systems of AM plants (22,38). Several studies have reported an increased retention of Zn in the roots of AM plants such as clover and maize (39-41). Moreover, Davies et al.

reported that mycorrhizal sunflower plants had similar and lower Cr tissue concentrations with Cr(III) and Cr(VI) treatments, respectively. However, AM plants had greater total plant accumulation of Cr because of their larger biomass (15). Therefore, it is suggested that AM plants with larger biomass could be used as phytoremediators of chromium stress in contaminated soils.

In conclusion, we observed that mycorrhizal symbiosis by *Glomus intraradices* could increase the tolerance of maize plants to chromium toxicity. The increase in photosynthetic pigments and reduction in lipid peroxidation indicates that mycorrhizal fungus, *G. intraradices,* can alleviate the toxic effects of chromium in maize. Furthermore, the lower activity of antioxidant enzymes in the AM roots of chromium treated maize plants as compared with non-AM plants may be due to the higher tolerance of mycorrhizal roots to Cr toxicity. Although the mechanisms that enable mycorrhizal plants to tolerate heavy metal toxicity are not well known, it seems that the mycorrhizal fungus *Glomus intraradices* decreases the translocation of Cr to shoots and thereby increases the tolerance of maize to Cr toxicity.

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