

Inoculation with mycorrhizal fungi modifies proline metabolism and increases chromium tolerance in pepper plants (*Capsicum annuum* L.)

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ABSTRACT

In general, heavy metals interfere with several physiological processes and reduce plant growth. Plants naturally establish symbiotic associations with soil microorganisms, such as mycorrhizal fungi. The aim of this research was to determine if inoculation with mycorrhizal fungi increases tolerance to Cr, evidenced by growth and biochemical parameters and the effect on roots membranes in *Capsicum annuum*. Plants were either non-inoculated or inoculated with *Glomus mosseae* or *Glomus intraradices*, and grown in the presence of different concentration of Cr ($K_2Cr_2O_4$) in soil. Pepper plants grown without Cr behaved as mycotrophic species. At the highest concentration (200 μ M $K_2Cr_2O_4$), Cr reduced root colonization by *G. mosseae* or *G. intraradices* (to 23 and 20% respectively). Moderate and high concentrations of Cr reduced all growth parameters. The interaction of inoculation and Cr increased leaf chlorophyll and proline content while reduced the leaf protein and root proline content. Carotenoid content was not affected by treatments. High Cr concentrations increased significantly electrolytes leakage in roots, either non-inoculated or inoculated plants. At the highest Cr concentration, inoculated plants had double the biomass of non-inoculated plants. Cr content in roots of inoculated plants was significantly higher than in non-inoculated plants. Chromium accumulation was low in leaves and showed no differences between treatments. Mycorrhization increased pepper plant tolerance to Cr in the soil, modifying proline metabolism to assure a more efficient response.

Key words: Electrolytes leakage, *Glomus intraradices*, *Glomus mosseae*, heavy metals.

INTRODUCTION

Heavy metals (HM) are one of the main sources of environmental pollution (Il'yasova and Schwartz, 2005) and are responsible for several environmental problems, associated with industrial and agricultural activities: decrease of microbial activity, soil fertility and crop yield (Yang et al., 2005). Interest in chromium originates from widespread use in various industries, such as metallurgical and chemical. Due to industrial process, large quantities of Cr compounds are discharged into the environment, resulting in significant

adverse biological and ecological effects (Kabata-Pendias and Pendias, 2001).

Heavy metals interfere with several physiological processes reducing the plant growth, photosynthesis and consequently the biomass (Jamal et al., 2006). Decrease in total chlorophyll, chlorophyll a and b and carotenoids have been well documented for Cr stressed plants (Panda and Choudhury, 2005). In the soil, Cr exists in two different oxidation states: trivalent (Cr^{3+}) and hexavalent (Cr^{6+}). Both Cr^{3+} and Cr^{6+} differ in terms of mobility, solubility and toxicity. Hexavalent

Cr^{6+} is more toxic and mobile than Cr^{3+} , it forms chromate and dichromate, are highly soluble in water and there is no evidence of the potential role in plant metabolism (Panda and Patra, 1997). Chromium phytotoxicity can result in inhibition of seed germination, pigments degradation and induced oxidative stress in plants (Panda and Patra, 1997, 2000). Beside, these effects, Cr can alter membrane ultrastructure in plants (Choudhury and Panda, 2005). Toxic ions penetrate cells by the same mechanisms used by essential minerals (Patra et al., 2004). The pathways for Cr^{6+} transport underlie active mechanisms involving carriers of essential anions, such as sulphate, Fe and P (Cervantes et al., 2001; Barbosa et al., 2007) and moves mainly by plant xylem. Golovatyj et al. (1999) have demonstrated that Cr accumulation was great in roots and lower in vegetative and reproductive organs.

Arbuscular mycorrhizal (AM) fungi establish symbiotic associations with many plant species. This evolution strategy has been known for more than a century but just in recent decades it has become a useful tool in horticulture, agriculture and forestry, where there are evidences of its efficiency in the competitive and sustainable development of the production systems (Pawlowska and Charvat 2004, Pawlowska, 2005). Mycorrhizal fungi enhance plant tolerance to biotic and abiotic stresses (Auge, 2001; Beltrano et al., 2003). The influence of mycorrhizal fungi on plant nutrition is important for the absorption of elements with low mobility, such as P and heavy metals (Clark and Zeto, 2000). Participation of mycorrhizal fungi in heavy metals metabolism has been proposed as a mechanism to increase plant tolerance, in some cases heavy metals can be absorbed by fungi hyphae and transported to the plant (phytoextraction), in other cases fungi contribute to heavy metals immobilization in the soil (phytostabilization) (Gaur and Adholeya, 2004; Khan, 2005).

Mycorrhizal roots may act as a barrier against metal transport from roots to the aerial part of the plant. This effect is attributed to metal adsorption on the hyphal walls, since chitin has an important metal-binding capacity (Joner et al., 2000). Another possible tolerance mechanism is the dilution of heavy metals concentrations by growth increase of mycorrhizal plants. Liu et al. (2005) determined that biomass of tomato mycorrhizal plants increased by 30% compared to non-mycorrhizal with increasing concentration of arsenic in the soil.

The hypothesis of our work was that mycorrhiza fungi have a protective action against heavy metals stress, increasing

pepper plant (*Capsicum annuum* L.) tolerance to Chromium. The aim of this investigation was to test this hypothesis by determining the effect of different Cr concentrations on pepper plant growth parameters (dry weigh, leaf area and partition of assimilated), biochemical parameters (Cr content in roots and leaves, chlorophyll, carotenoids, proline), and roots and leaf cell membranes stability in non-inoculated and inoculated plants with the mycorrhizal fungi *Glomus mosseae* or *Glomus intraradices*.

MATERIALS AND METHODS

Growth conditions: Seeds of pepper (*C. annuum* L. 'California Wonder 300') were sown in plastic pots previously filled with substrate composed of a mixture of soil (Argiudol vertic, pH 5.5, 12 mg/Kg⁻¹ total P, 3.5% organic matter, 2% total C and 0.24% total N) perlite and vermiculite (2:1:1) tinalized at 100°C for 60 minutes, during 3 consecutive days. *G. intraradices* Schenck & Smith isolate GA1 and *G. mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe (Banco de Glomeromycota In Vitro BGI. Buenos Aires, Argentina) were bulked-up through culture with *Trifolium repens* L. for four months in a semi-controlled grown chamber.

The inoculum (10% of the substrate weight), a mix of soil, spores (50 spores per g⁻¹ inoculum) mycelium and root fragments colonized by *G. mosseae* (Mmos) or *G. intraradices* (Mintra), was added to the substrate at sowing time. The same amount of sterilized inoculum plus 10 ml mycorrhizal fungal-free filtrate from the inoculum suspension was added to non-inoculated pots in order to provide the same soil conditions.

When root colonization with *G. intraradices* was approximately 50% and with *G. mosseae*, 40%, inoculated and non-inoculated plants, pepper young plants were transplanted to 500 ml pots containing the same soil. Before transplanting different doses of Cr in the $\text{K}_2\text{Cr}_2\text{O}_4$ form was added to the substrate to rich concentrations of 0 μM $\text{K}_2\text{Cr}_2\text{O}_4$ (Cr0); 10 μM $\text{K}_2\text{Cr}_2\text{O}_4$ (Cr1); 100 μM $\text{K}_2\text{Cr}_2\text{O}_4$ (Cr2) and 200 μM $\text{K}_2\text{Cr}_2\text{O}_4$ (Cr3). The experiments were conducted at La Plata (34° SL, 54' WL) (Argentina). Pepper plants were grown in a greenhouse between October to December, under natural conditions.

The treatments were: (a) control (NI), the plants received no mycorrhizal inoculation with *G. mosseae* or *G. intraradices*: NICr0, without $\text{K}_2\text{Cr}_2\text{O}_4$; NICr1, 10 μM $\text{K}_2\text{Cr}_2\text{O}_4$; NICr2, 100 μM $\text{K}_2\text{Cr}_2\text{O}_4$; and NICr3, 200 μM $\text{K}_2\text{Cr}_2\text{O}_4$; and (b) inoculated plants (M): MmosCr0 (*G. mosseae*) or MintraCr0 (*G. intraradices*), without $\text{K}_2\text{Cr}_2\text{O}_4$; MmosCr1 or MintraCr1, 10

$\mu\text{M K}_2\text{Cr}_2\text{O}_4$; MmosCr2 or MintraCr2, $100 \mu\text{M K}_2\text{Cr}_2\text{O}_4$; and MmosCr3 or MintraCr3, $200 \mu\text{M K}_2\text{Cr}_2\text{O}_4$.

Variables measured: Ten plants per treatment were harvested at the end of the experimental period (51 days after transplanting (DAT)).

Estimation of AM colonization: Fungal colonization was assessed according to Trouvelot et al. (1986) and expressed as rate of mycorrhization (Myc%) and relative arbuscules and vesicles abundance (A% and V% respectively). Roots were cleared with 10% KOH (p/v) and stained with trypan blue in lacto-phenol (Phillips and Hayman, 1970). The viability of hyphae was determined by measuring succinate dehydrogenase activity (SDH) (Schaffer and Peterson, 1993). Three replicates of 10 randomly chosen root fragments were mounted on slides and examined microscopically. Myc% was calculated as the proportion of infected roots over total root fragments and A% was calculated as the arbuscular abundance per colonized roots. V% was calculated as the abundance of vesicles per colonized roots.

Mycorrhizal dependency (MD) was calculated according to the following formula:

$$\text{MD} = \frac{\text{DW inoculated plants} - \text{DW of non inoculated plants}}{\text{DW of inoculated plants}} \times 100$$

DW: dry weight

Growth parameter: Plant height, leaves number, DW of leaves, stems and roots were obtained by drying the material in oven at 80°C until constant weight and leaf area (LA) per plant (Li 3000 leaf area meter, LICOR, Lincoln, NE, USA) was measured.

Seed germination test: Replicates of 25 seeds of *C. annuum* L. were sown on two layers of filter paper in 11-cm Petri dishes. About 5 ml of deionized water or Cr solution was added to each Petri dish as $\text{K}_2\text{Cr}_2\text{O}_4$. The concentrations used were: $10 \mu\text{M}$, $100 \mu\text{M}$ and $200 \mu\text{M}$, and a control without metal. Petri dishes were incubated in a grown chamber at 25°C and 12 h photoperiod. The seeds were considered germinated when radicle emerged 1mm. Percentage of germination was determined daily and after 15 days, the length of the root and the aerial part was measured. Each treatment was replicated four times.

Carotenoids, chlorophyll and leaf proteins content: Carotenoids and chlorophyll contents were determined in one leaf disc (1 cm diameter) per plant, and protein content was estimated in five leaf discs (1 cm diameter) per plant. The

concentration of carotenoids and the content of chlorophyll were measured according Wellburn (1994), and proteins concentration, according to Bradford method (1976) using bovine albumin as standard. All absorption spectra were recorded in a Shimadzu UV-160 spectrophotometer (Kyoto, Japan). Results were expressed as $\mu\text{g carotenoids cm}^{-2}$, $\mu\text{g chlorophyll cm}^{-2}$ or $\mu\text{g protein cm}^{-2}$.

Proline content in roots and leaves: Proline content was determined from 1 g leaf or root fresh weight (FW), according to Bates et al. (1973). Extraction was made with an aqueous solution of 3% sulfosalicylic acid and the extract obtained reacted with ninhydrin acid and glacial acetic acid. Proline concentration was measured in a spectrophotometer Shimadzu UV-160 (Kyoto, Japan) at 520 nm absorbance. Proline content was calculated per unit of FW according to:

$$\mu\text{mols proline g}^{-1} \text{FW} = \frac{[(\mu\text{g proline / ml} \times \text{ml toluene}) / 115.5 \mu\text{g} / \mu\text{mols}] / (\text{g FW} / 5)}$$

Electrolyte leakage: This technique is based on the increase of cellular membrane permeability and concomitantly greater electrolyte diffusion out of cells when tissue is injured by a stress situation. The electrolyte leakage was measured as described by Lutts et al (1996) with a few modifications. After harvest, the uppermost fully expanded leaves of 10 plants per treatment were immediately cut into discs of 1 cm diameter. Leaf disc were washed briefly three times in deionized water to remove solutes released during cutting of the discs. Five discs of each leaf were placed in a vial filled with 10 ml deionized water and maintained at 25°C for 4 h subsequently the electrical conductivity of the bathing solution was determined (ELi: initial). After the first measuring the vials were heated in boiling water for 60 min and the final electrical conductivity was obtained after equilibration at 25°C (ELf: final). Electrolyte leakage was determined by measuring the electrical conductivity of the vial solution, using a conductimeter and data were expressed as $\mu\text{S cm}^{-1}$. Relative electrical conductivity (EL) was calculated as follows:

$$\text{EL (\%)} = (\text{ELi/ELf}) \times 100$$

Determination of chromium in roots and leave: Chromium content was determined in 500 mg of root and leaf samples per treatment, according to method 3111B (APHA-AWWA-WPCF, 1998). The samples were analyzed by atomic absorption spectrometry, direct air-acetylene flame with digestion pretreatment with nitric acid.

Statistical analysis: The experiment was a 3×4 factorial, in a completely randomized design with three mycorrhizal levels (NI, Mmos, Mintra) and four levels of chromium (Cr0, Cr1, Cr2, Cr3). Data were analyzed by ANOVA, and comparisons among means were made using LSD ($P < 0.05$). For the statistical analysis all inoculation percentage values were arcsine transformed to improve homogeneity. The number of replicates was: for growth data ($n=10$), and for mycorrhizal observations ($n=3$ replicate of 30 roots fragments).

RESULTS

Mycorrhization: None of the non-inoculated plants was colonized by *G. mosseae* or *G. intraradices* and

there were very few colonization by native fungi. Without Cr, the level of plants colonization with *G. mosseae* was 44% and with *G. intraradices* was 58%. The percentage of arbuscules and vesicles was higher in plants inoculated with *G. intraradices* than in plants colonized with *G. mosseae*.

Chromium reduced inoculation with *G. mosseae* by 4%, 13% and 23% in Cr1, Cr2 and Cr3, respectively, compared to Cr0, while in plants inoculated with *G. intraradices*, reduction was 5%, 15% and 20% in Cr1, Cr2 and Cr3, respectively. Viability of hyphae, expressed by SDH activity, was higher in plants inoculated with *G. intraradices* than in inoculated with *G. mosseae*. The highest concentration of Cr reduced viability in 46% in MintraCr3 and 71% in MmosCr3 (Table 1).

Table 1. Effects of chromium concentrations on mycorrhizal colonization, abundance of arbuscules and vesicles, viable hyphae, and mycorrhizal dependence in *Capsicum annuum* L. Plants non inoculated or inoculated with *Glomus mosseae* or *Glomus intraradices* *.

| Treatments | Mycorrhizal colonization (%) | Arbuscule abundance (%) | Vesicle abundance (%) | Viable hyphae (%) | Mycorrhizal dependence (%) |
|------------|------------------------------|-------------------------|-----------------------|-------------------|----------------------------|
| MmosCr0 | 44.5 b | 30.1 a | 10 b | 30.6d | 18 |
| MmosCr1 | 41.1 b | 38.3 b | 10 b | 22.5 c | 11 |
| MmosCr2 | 38.2 ab | 35.7 ab | 11 b | 14.3 b | 54 |
| MmosCr3 | 34.4 a | 29.6 a | 4 a | 9.1 a | 33 |
| MintraCr0 | 58.2 c | 44.5 c | 45 b | 56.5 c | 33 |
| MintraCr1 | 55.3 bc | 41.7 c | 40 ab | 50.4 bc | 39 |
| MintraCr2 | 50.5 ab | 29.2 b | 38 a | 46.6 b | 64 |
| MintraCr3 | 45.6 a | 12.3 a | 36 a | 30.5 a | 57 |

* Means value followed by the same letter within each column and each inoculum are not significantly different ($P > 0.05$).

Germination test: Percentage of germination and the length of the aerial part did not show significant differences with increasing Cr concentrations. Root length was significantly reduced with increasing Cr concentrations (Table 2).

Growth parameter: Moderate or high Cr concentrations reduced all plant growth parameters (roots, stems and leaves DW, and LA). Chromium reduced the height and leaf number in all treatments. In NI, all Cr treatments reduced these parameters compared to inoculated plants.

Table 2. Effects of chromium concentrations on germination, aerial and root length in *Capsicum annuum* L*.

| Treatments | Germination (%) | Aerial length (mm) | Root length (mm) |
|------------|-----------------|--------------------|------------------|
| Cr0 | 90.5 a | 21.21 a | 43.95 d |
| Cr1 | 82.3 a | 19.85 a | 35.61 c |
| Cr2 | 85.4 a | 20.61 a | 19.85 b |
| Cr3 | 80.2 a | 20.05 a | 8.51 a |

*Means value followed by the same letter within each column are not significantly different ($P > 0.05$).

Height reduction was 16%, 14% and 22% in NI Cr3, MmosCr3 and MintraCr3, respectively, compared to Cr0 treatments (Figure 1A). Chromium concentration reduced the leaf number by 13%, 18% and 15% in NI Cr3, MmosCr3 and MintraCr3, respectively, compared to the control without chromium (Figure 1B). ANOVA showed a significant interaction (myco x chromium) for height though not for leaf number.

Chromium reduced LA in all treatments; the higher values were obtained in plants inoculated with *G. intraradices* in Cr0. Leaf area of plants inoculated with *G. intraradices* showed significantly higher compared to *G. mosseae* in all Cr treatments (Figure 1C). Myco x chromium interaction was not observed.

The total dry weight (TDW) of plants inoculated with *G. intraradices* was higher than the inoculated with *G. mosseae* in all Cr treatments. NI plants showed the lowest values and decreased significantly with Cr treatments (Figure 1D). For TDW significant myco x chromium interaction was observed.

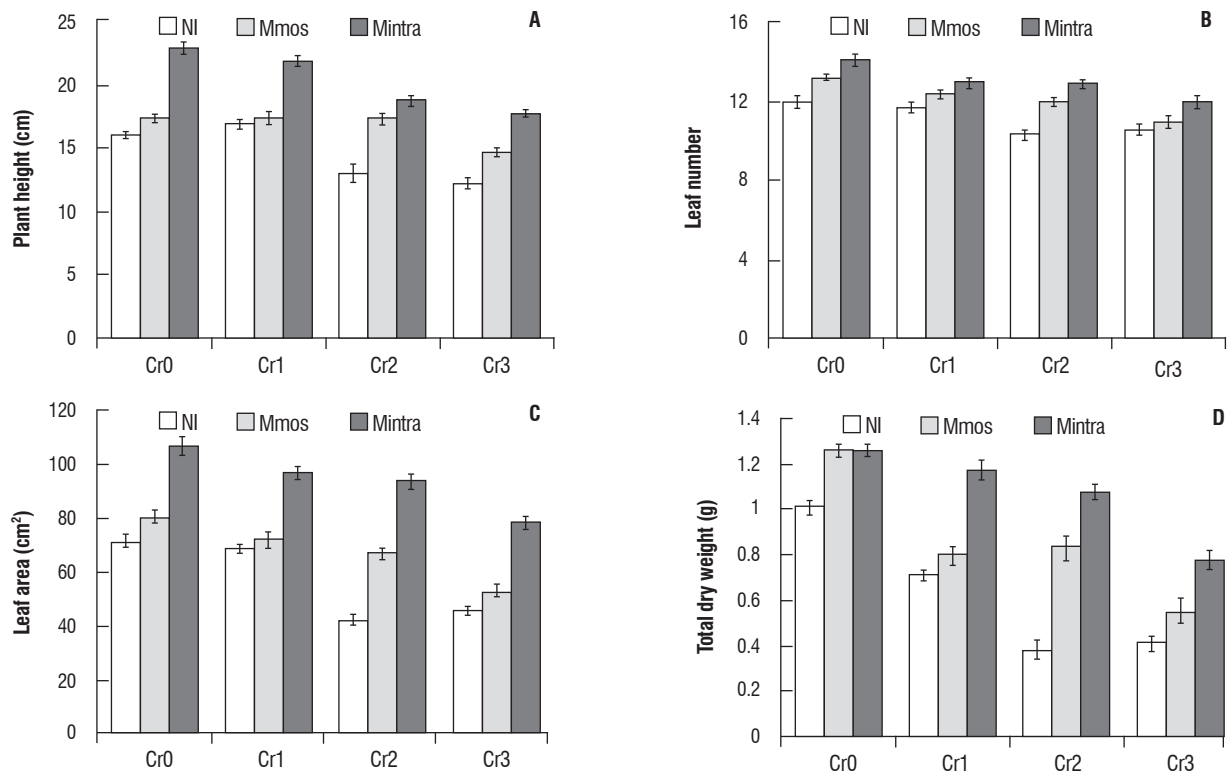


Figure 1. Effects of chromium concentrations on plant height (A), leaf number (B), leaf area (C) and total dry weight (D) of *Capsicum annuum* L. plants non inoculated or inoculated with *Glomus mosseae* or *Glomus intraradices*. NI, non inoculated; Mmos, inoculated with *Glomus mosseae*; Mintra, with *Glomus intraradices*. Cr0, without chromium; Cr1, with 10 μ M de $K_2Cr_2O_4$; Cr2, with 100 μ M de $K_2Cr_2O_4$; Cr3, with 200 μ M de $K_2Cr_2O_4$. Data are mean value \pm SE (n= 10)

Biomass partition was significantly modified in all treatments (Figure 2).

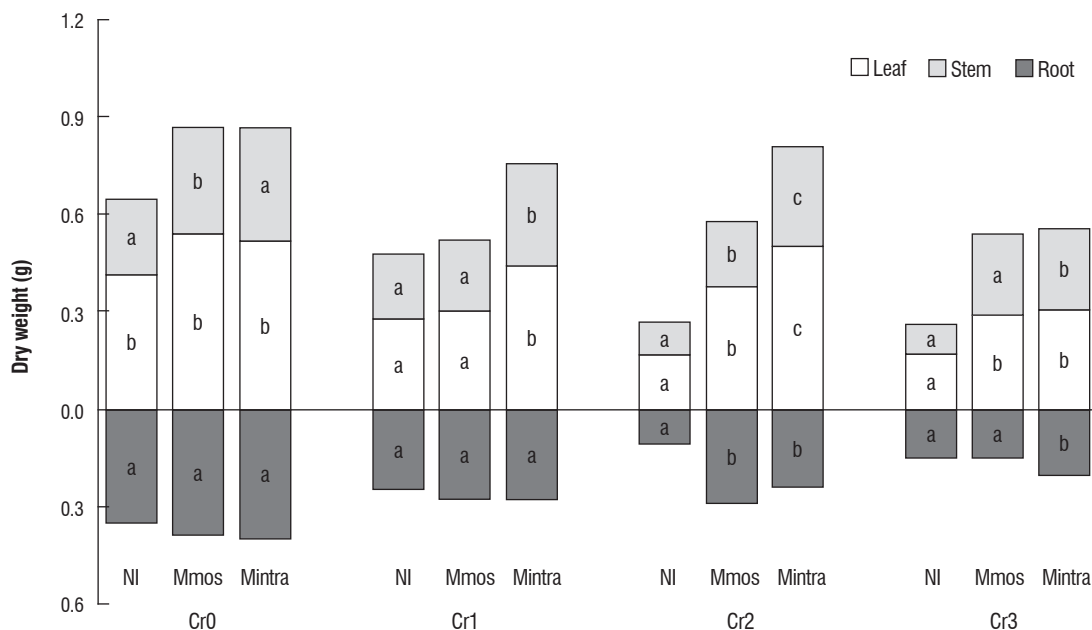


Figure 2. Effects of chromium concentrations on leaf, stem and roots DW in *Capsicum annuum* L. plants non inoculated or inoculated with *Glomus mosseae* or *Glomus intraradices*. NI, non inoculated; Mmos, inoculated with *Glomus mosseae*; Mintra, with *Glomus intraradices*. Cr0, without Cr; Cr1, with 10 μ M de $K_2Cr_2O_4$; Cr2, with 100 μ M de $K_2Cr_2O_4$; Cr3, with 200 μ M de $K_2Cr_2O_4$. The same letters between treatments are not statistically significant at $P > 0.05$.

Aerial/TDW and aerial/roots DW ratios were not affected by mycorrhization although the ratios were affected by Cr concentrations. The leaf/TDW ratio was affected only by mycorrhization. Stem/TDW ratio was affected by mycorrhization and Cr concentration. The specific leaf area was not modified by mycorrhization although this was modified by Cr concentrations.

Mycorrhizal dependency without Cr was 18% and 33% for plants inoculated with *G. mosseae* and *G. intraradices*, respectively and 33% and 57% for inoculated with *G. mosseae* and *G. intraradices*, respectively at the higher Cr concentration (Table 1).

Proteins, chlorophyll and carotenoid contents of leaves: There were significant differences in foliar protein content at the different Cr concentrations. NI plants showed higher protein contents than inoculated ones. Plants inoculated with *G. intraradices* had significantly higher protein contents than those inoculated with *G. mosseae*. Protein content

was significantly affected by mycorrhization and chromium and the myco x chromium interaction was significant (Table 3). Without chromium, chlorophyll concentration was not affected by inoculation with *G. mosseae* or *G. intraradices*. At the higher chromium concentrations, the leaves of Mmos and Mintra plants retained greater chlorophyll levels than NI. Myco x chromium interaction was significant. Carotenoids content was not affected by the treatments (Table 3).

Proline content in leaves and roots: In NI plants, the proline content of leaves diminished 32% with an increase of chromium concentration (Cr0 versus Cr3). In Mmos and Mintra plants, leaves proline content increased by 29% and 49%, respectively. In NICr3 plants, proline content of roots increased by 63% while inoculation with *G. mosseae* and *G. intraradices* induced a significant decreased in root proline concentrations (45% and 40%, respectively). Myco x chromium interaction was significant in leaves and roots (Table 3).

Table 3. Effects of chromium concentrations on leaf protein, chlorophyll, carotenoides and leaf and root proline contents in *Capsicum annuum* L. plants non inoculated or inoculated with *Glomus mosseae* or *Glomus intraradices*^a.

| Treatments | Leaf protein ($\mu\text{g cm}^{-2}$) | Chlorophyll ($\mu\text{g cm}^{-2}$) | Carotenoids ($\mu\text{g cm}^{-2}$) | Leaf proline ($\mu\text{mols.g}^{-1}$ FW) | Root proline ($\mu\text{mols.g}^{-1}$ FW) |
|---------------------|---|--|--|---|---|
| NICr0 | 5.19 f | 30.48 de | 6.31 bcd | 0.50 c | 0.11 cd |
| NICr1 | 5.07 f | 28.11 cd | 6.41 bcd | 0.39 b | 0.10 bc |
| NICr2 | 4.35 bcd | 23.69 ab | 5.95 abc | 0.33 a | 0.13 e |
| NICr3 | 4.41 cd | 21.05 a | 5.42 a | 0.34 a | 0.18 g |
| MmosCr0 | 4.64 de | 29.53 cde | 5.97 abc | 0.31 a | 0.22 h |
| MmosCr1 | 4.08 ab | 26.66 bc | 6.22 bcd | 0.30 a | 0.17 g |
| MmosCr2 | 4.61 de | 30.25 de | 7.37 e | 0.40 b | 0.14 e |
| MmosCr3 | 3.87 a | 30.69 de | 6.38 bcd | 0.40 b | 0.12 d |
| MintraCr0 | 5.73 g | 31.13 de | 6.16 abcd | 0.39 b | 0.15 f |
| MintraCr1 | 4.76 e | 30.07 cde | 6.52 cd | 0.42 b | 0.10 bc |
| MintraCr2 | 4.25 bc | 32.22 e | 6.78 de | 0.47 c | 0.09 ab |
| MintraCr3 | 3.88 a | 29.24 cde | 5.70 ab | 0.58 d | 0.08 a |
| Significance | | | | | |
| AM | (**) | (**) | ns | (**) | (**) |
| Cr | (**) | ns | ns | (**) | (**) |
| AM X Cr | (**) | (**) | ns | (**) | (**) |

^aMeans value followed by the same letter within each column are not significantly different ($P > 0.05$). ns non significant. * Significant at 0.05 level. ** Significant at 0.01 level.

Determination of Electrolyte leakage: Leakage of root solutes increased due to high chromium concentrations but not due to mycorrhization (Figure 3B). Myco x chromium

interaction was not significant. Treatments did not affect electrolyte leakage in leaves (Figure 3A).

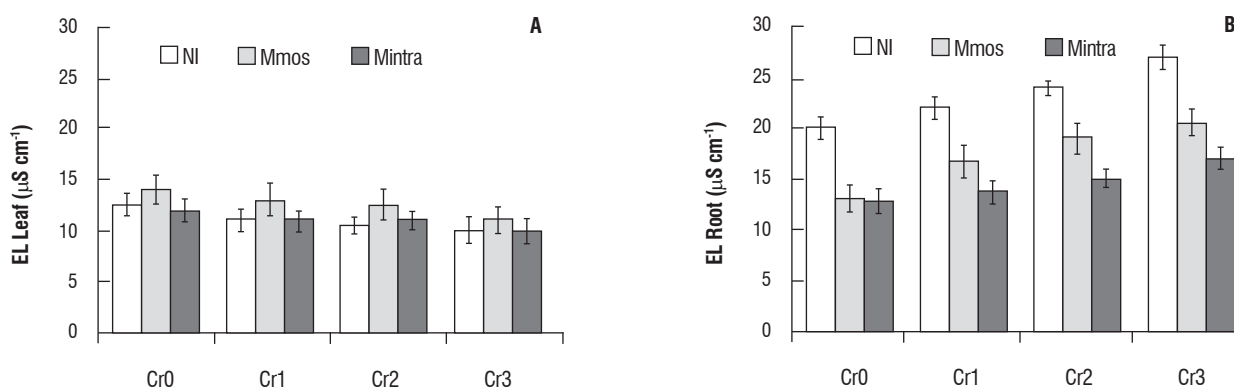


Figure 3. Effects of chromium concentrations on electrolyte leakage (EL) in leaf (A) and root (B) of *Capsicum annuum* L. plants, non inoculated or inoculated with *Glomus mosseae* or *Glomus intraradices*. NI, non-inoculated; Mmos, inoculated with *Glomus mosseae*; Mintra, with *Glomus intraradices*. Cr0, without chromium; Cr1, with 10 µM de $K_2Cr_2O_4$; Cr2, with 100 µM de $K_2Cr_2O_4$; Cr3, with 200 µM de $K_2Cr_2O_4$. Data are mean value \pm SE (n= 10).

Chromium determination in leaves and roots: At Cr0 and Cr1 no differences were observed in Chromium content in both aerial parts and roots of non-inoculated, Mmos and Mintra plants. At the highest concentration, Cr content of NI, Mmos and Mintra roots was significantly higher than aerial parts. Higher chromium content was at higher concentrations of chromium (Cr2 and Cr3) in Mmos and Mintra roots than in non-inoculated roots. No differences were observed between *G. mosseae* and *G. intraradices* (Table 4).

Table 4. Chromium content on aerial and root fraction in *Capsicum annuum* L. plants non inoculated or inoculated with *Glomus mosseae* or *Glomus intraradices*^d.

| Treatments | Fraction (mg Cr/Kg DW) | |
|------------|------------------------|---------|
| | Aerial | Root |
| NICr0 | < 2.5 a | < 2.5 a |
| NICr1 | < 2.5 a | < 2.5 a |
| NICr2 | 2.8 a | 27.4 b |
| NICr3 | 8.5 b | 59.1 c |
| MmosCr0 | < 2.5 a | < 2.5 a |
| MmosCr1 | < 2.5 a | < 2.5 a |
| MmosCr2 | < 2.5 a | 142.5 d |
| MmosCr3 | 8.3 b | 319.1 e |
| MintraCr0 | < 2.5 a | < 2.5 a |
| MintraCr1 | < 2.5 a | < 2.5 a |
| MintraCr2 | < 2.5 a | 125.5 d |
| MintraCr3 | 6.9 b | 314.5 e |

^d Means value followed by the same letter within each column are not significantly different (P>0.05).

DISCUSSION

Plant tolerance and/or resistance to heavy metals stress can be associated with one or more mechanisms, such as: (i) metal retention in roots preventing its translocation to the aerial part (Patra et al., 2004); (ii) metal immobilization in the cell wall (Cosio et al., 2005); (iii) homeostatic cellular mechanisms to regulate the concentration of metal ions inside the cell (Benavides et al., 2005); (iv) increase of tolerance to mineral deficiency or decrease of nutritional requirements; (v) increase in absorption of certain macronutrients or development of the capacity to absorb and use minerals in the presence of heavy metals (Meda et al., 2007). As a result of these tolerance and/or resistance mechanisms (alone or in combination) some plants can grow in environments contaminated with heavy metals where other species could not survive. Since seed germination is the first physiological process affected by Cr, the ability of a seed to germinate in a medium containing Cr would be indicative of its level of tolerance to this metal (Peralta et al., 2001). In our study, Cr did not affect seed germination of pepper or the growth of the aerial parts of the seedlings, though in agreement with findings by Nayari et al. (1997) and Panda et al. (2002) our results showed that Cr toxicity significantly affected root growth. Meanwhile, Rout et al. (2000) observed that germination of *Echinochloa colona* was reduced to 25% with 200 µM Cr. Higher levels (500 ppm) of hexavalent Cr in soil reduced germination up to 48% in *Phaseolus vulgaris* (Parr and Taylor, 1982).

Peralta et al. (2001) found that 40 ppm Cr⁶⁺ reduced by 23% the ability of seeds of lucerne (*Medicago sativa* cv. Malone) to germinate in contaminated soil.

Mycorrhizas can alleviate Cr toxicity and support greater plant growth in Cr rich soils (Davies Jr et al., 2001). Although little information is available on the influence of inoculation with mycorrhizal fungi as improvers of plant tolerance and phytoaccumulation of Cr (Davies Jr. et al., 2001), some authors have demonstrated that spores and pre-symbiotic hyphae of mycorrhizal fungi are sensitive to heavy metals and under certain conditions, metals inhibit spore germination and hyphal growth (Shalaby, 2003). This study demonstrate that inoculated pepper plants can tolerate the presence of Cr in the soil, and shows that in the treatments without chromium, mycorrhization was high, confirming that pepper is a mycotrophic species (Ronco et al., 2008). The increase in chromium concentration affected colonization and arbuscular and vesicular formation, in both *Glomus* species. Our data show that plants inoculated with *G. intraradices* showed the highest percentage of mycorrhization, although it was reduced with the increase of Cr in the soil. The sensitivity to Cr toxicity was revealed by reduction in arbuscular formation, followed by reduction in vesicular formation; hyphal formation was less affected. Also, high Cr levels affected hyphal viability, expressed by SDH activity, and it was higher in *G. intraradices* than in *G. mosseae*. Similar results were obtained by Pawlowska and Charvat (2004) who demonstrated that *G. intraradices* was more tolerant in the presence of other HM. Similar effects of different HM had been reported by Rivera-Becerril et al. (2002) who observed that without Cd colonization of *Pisum sativum* was 45%, meanwhile in presence of HM was by 28%. Shalaby (2003) proposed that resistance to HM was likely due to phenotype plasticity rather to by genetic changes, since tolerance was lost after one generation in the absence of heavy-metals.

Chromium has not been recognized as an essential element for plant, however Shanker et al. (2005) reported that low concentrations of chromium can stimulate the plant growth, this fact was not observed in our study. In the absence of Cr, our results showed that the higher growth was determined in plants inoculated with *G. intraradices*. The deleterious effect produced by the presence of Cr was lower in inoculated plants compared to non inoculated ones, and in those inoculated with *G. intraradices* compared to those

inoculated with *G. mosseae*. These results are in agreement with Jamal et al. (2006), who demonstrated that Cr produced a significant reduction in the growth of *Prosopis juliflora* and with Bishnoi et al. (1993) who determined that the deleterious effect of Cr was more pronounced on the growth of roots than on the stems, this could be due to Cr accumulation in the roots, as was observed in our results. Cr reduced the height, leaves number, leaf area and dry weight in all treatments. The arbuscular mycorrhizae contribute in supporting partially Cr toxicity as demonstrated by higher growth of inoculated plants in the presence of Cr compared to the non-inoculated ones, as observed by Bagyaraj et al. (1988) in several crops and by Davies Jr. et al. (2001) in sunflower. Consequently, mycorrhizal dependency, that is the relationship between biomass of mycorrhizal plants compared to non-mycorrhizal, reaching higher values than 50% at the highest concentrations of Cr, regardless of the inoculum used, similar results are presented by Davies et al. (2002) in sunflower plants in similar Cr concentrations. The plant height and leaves number were the parameters less affected. Leaves, stem and root DW were the most affected by Cr and by inoculation in agreement with Anderson et al. (1972) who observed 11%, 22% and 41% reduction in oats plant cultivated in 2, 10 and 25 ppm of Cr in soil, respectively. The biomass reduction in pepper plants could be attributed to a competition mechanism between Cr and P, demonstrated by Davies et al. (2002) and previously reported by several authors. The roots of non-inoculated plants were the most affected organ by low and high concentrations of Cr. Our data showed that the effect of mycorrhization and the presence of Cr on the DW of leaves, stem and root and the interaction is highly significant (<0.001). Decrease root growth due to heavy metals is well-documented (Tang et al., 2001). The roots may act as a barrier against Cr uptake by plants, and this effect is increased by the presence of mycorrhizal fungi.

Our data show that the effect of Cr on the root growth and on the integrity of cell membranes, assessed by the electrolytes leakage, was significantly higher in NI compared to inoculated plants and in the roots compared to the leaves. Cr affects cell membranes, though the mechanism is not known, electrolytes leakage by root tissues is significantly increased by Cr. Electrolytes leakage by leaf tissues was not affected nor by chromium nor by mycorrhization. Davies et al. (2002) proposed that the first effects on the root membranes could be attributed to the high potential of reduction of Cr⁶⁺

which is retained in the vacuoles and cell walls of the root, while Cr reaching the leaves can be mainly at the Cr^{3+} , although accumulation mechanisms of Cr are not well known, our results seem to confirm those presented by this author, since Cr content determined in the leaves did not promote significant damage in the membranes.

As found by James (2002), higher Cr content in roots than in leaves is observed, while the lower concentration is found in the stems. In this study, Cr content in leaves was low, in agreement with results by James (2002); and Sharma and Sharma (1993) for wheat and by Tripathi et al. (1999) for *Albizia lebbbeck*; these last authors proposed that leaf growth might serve as suitable bio-indicators of heavy metal pollution and used in the selection of resistant species. According to Shanker et al. (2004) the reason of the high accumulation in roots could be caused because Cr is mobilized to the vacuoles of the root cells, thus producing lower toxicity to the aerial part. It is possible that when pass the endodermis via symplast Cr^{6+} is reduce to Cr^{3+} , which is retained in the root cortex cells and reduce Cr^{6+} concentration. Although higher plants do not contain enzymes reducing Cr^{6+} , they have been widely reported in bacteria and fungi (Cervantes et al., 2001), and AM are likely to participate in this reduction. In addition to the reduction in growth and its effect on cell membrane stability, Cr^{6+} promotes inhibition of photosynthetic pigment synthesis (Vajpayee et al., 2000) and induce oxidative damages in biomolecules such as lipids and proteins (Vajpayee et al., 2001).

Our results show that carotenoids content was not modified by the presence of Cr, in discordance with Rai et al. (1992) who determined that Cr can induce degradation of carotenoids in plants, while chlorophyll content and proteins declined with increasing Cr concentration. Meanwhile, the moderating effect of AM reported in our study, as a possible expression of the protective action against stress by HM, was greater at the highest concentrations of Cr, avoiding a high degradation of both chlorophyll and proteins, in agreement with Abdul Razak (1985) who determined a decrease in the photosynthetic activity and in the chlorophyll synthesis by the accumulation of HM, and by Schützendübel and Polle (2002), that determined a significant reduction in proteins content and in enzyme activity by the interference produced of metal ions. The way in which mycorrhizal fungi modify these effects depends on several factors such as growth conditions, fungal species and metal concentration. The increase in mycorrhized plants tolerance to

HM was observed in different species such as maize, barley and rye (Hildebrandt et al., 1999; Gaur and Adholeya, 2004).

Moreover, it was observed that many plants accumulate proline when were treated with toxic concentrations of heavy metals (Bassi and Sharma, 1993; Costa and Morel, 1994; Schat and Vooijs, 1997). The effect of HM on proline synthesis is contradictory. Some authors describe an increase in the intracellular concentration of proline in the presence of high concentrations of metals (Costa and Morel, 1994; Schat and Vooijs, 1997). Kavi Kishor et al. (1995) suggest that proline might protect plants from metal toxicity. Schat and Vooijs (1997) observed that metal-induced proline accumulation does not occur until damage has been caused; hence plants would not be protected against stress.

Rodriguez and Redman (2005) reported that proline protects fungal cells against abiotic stresses such as UV light, heat, salt, and hydrogen peroxide. Rodriguez et al. (2004) proposes that mutualistic fungi allow symbiotic plants to perceive stress more quickly than nonsymbiotic, resulting in the rapid activation of plant biochemical reactions that mitigate the impacts of stress. However, the mechanisms that conferred stress tolerance are poorly defined.

In our work, proline synthesis increased concomitant to Cr increase, in agreement with Bassi and Sharma (1993) and Costa and Morel (1994), however with different responses in leaves and roots and in none inoculated and inoculated plants.

In the roots of non-inoculated plants proline concentration increased with increasing Cr concentration, while it decreased in leaves. Though, in the roots of inoculated plants proline concentration decreased with increasing Cr concentration, while it increased in leaves, in agreement with data previously described for many species; this would respond to a modification in the partition of free Cr in plants.

This leaf values were higher and root values were lower in *G. intraradices* than in *G. mosseae*, due probability to a higher percentage of mycorrhized-roots. However, Porcel and Ruiz-Lozano (2004) demonstrated the accumulation of higher proline levels in soybean mycorrhizal roots and lower contents in mycorrhizal shoots compared to non-mycorrhizal plants under drought conditions. A more detailed analysis of both root and shoot samples during stress is necessary to be performed (Pinior et al., 2005).

Mycorrhizal fungi, in some cases increase absorption and accumulation of HM in roots and in other cases they favor HM translocation to the aerial part of the plant, this would explain the different behavior of mycorrhized and non-mycorrhized plants.

It is known that Cr is poorly mobile, so that in inoculated plants mycorrhizal fungi could be a protective barrier avoiding the translocation of metal towards the aerial part of the plant. Hence, the lower uptake of Cr in aerial part allows the proline metabolism to operate more efficiently, while in roots the high concentration of Cr can affect metabolism of this protecting amino acid. Although increase in HM concentration raised the cell proline content, inhibition of proline accumulation was evident beyond a certain threshold of the metal. Thus, in accordance with Mehta and Gaur (1999), high concentrations of heavy metals are inhibitory to proline biosynthesis, and the threshold sensibility change from organ and the mycorrhizal can modified the responses.

In conclusion, increase in Cr concentration modifies root colonization by mycorrhizal fungi, reduces height, leaf area, dry weight and other growth parameters, and modifies chlorophyll, protein and proline contents. Mycorrhization increases pepper plant tolerance to high Cr concentrations in the soil, modifying proline metabolism to make the response more efficient and confirming the hypothesis proposed.

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