Egypt. J.Microbiol. **39**, No. 1-2, pp. 67-79 (2004)

Phytoremediation of Chromium Polluted Soil

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HEAVY metal-contaminated land is an important environmental, health, economic, and planning issue in Egypt. Phytoextraction involves use of plants to remove metals from soil. In a greenhouse experiment, *Zea mays*, *Helianthus annuus* and *Sorghum bicolor* plants were grown in tannery effluent polluted soils and non-polluted reference soils. After 8 weeks of growth, the plants were harvested and the dry weight and the content of Cr were determined. The relationship between mycorrhizae and plants indicates that the percentage of mycorrhizal colomization in all plant species grown in un-polluted soils were higher than plants grown in polluted soil. Roots of all three plant species growing on both soils possessed Arbuscular mycorrhizal (AM) colomization in their roots and AM propagules in the associated rhizospheres. High Cr contents adversely affected the number and diversity of AM species. The order of Cr foliar accumulation was *Z. mays>S.bicolor>H. annuus*. The effect of AM fungi on heavy metal uptake is dependent upon the initial soil metal concentration. The uptake of heavy metals by *Z. mays*, *H. annuus* and *S. bicolor* was affected by the colonization of roots with (AM) fungi.

Keywords: Arbuscular Mycorrhiza (AM) fungi, Maize, Phytoextraction, Sorghum, Sunflower, Tannery effluent.

Discharge of Cr waste from industrial applications such as leather tanning, textile production, electroplating, metallurgy, and petroleum refinery has led to large-scale contamination of land and water (Kamaludeen *et al.*, 2003). Compared with traditional techniques for removal and disposal of soil contaminants, phytoremediation is a low cost alternative approach for reducing the level of pollution in soils. In phytoremediation advantage is taken of the ability of specific plant species to accumulate in their biomass metals that they extract from polluted soils during growth (Chaney *et al.*, 2000; Ma *et al.*, 2001; Abou-Shanab *et al.*, 2003a). This technology is gaining popularity in reducing metal load in the contaminated medium. It is considered an environmentally

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friendly technology, is less destructive to soil biota, and is a cheaper alternative. Unfortunately, high levels of heavy metals are still toxic to these metal-tolerant plants, which can lead to low levels of plant biomass, and, therefore, inefficient phytoremediation (Cunningham *et al.*, 1995). Progress in the field is hindered by a lack of understanding of the many complex interactions in the rhizosphere and plant-based mechanisms, which allow metal translocation, and accumulation in plants.

Soil microorganisms are known to play a key role in the mobilization/ immobilization of metal cations, thereby changing their availability to plants (Burd *et al.*, 2000; Guan *et al.*, 2001; Abou-Shanab *et al.*, 2003b). Among the rhizospheric organisms involved in plant interactions with the soil milieu, the arbuscular mycorrhizal (AM) fungi deserve special attention. About 95% of the world's plant species belong to characteristically mycorrhizal families and potentially benefit from AM fungus-mediated mineral nutrition (Smith and Read, 1997; Cumming and Ning, 2003; Liao *et al.*, 2003). Glomalean fungi are believed to play a fundamental role in biogeochemical element cycling (Jeffries and Barea, 1994). Early phytoextraction studies have focused on metallophytes from predominantly nonmycorrhizal plant families, *e.g.*, *Brassicaceae* or *Caryphyllaceae*, so AM have not been considered important agents for phytoremediation practices (Kümar *et al.*, 1995; Lovely and Coates, 1997).

The present study was undertaken to determine the effect of Cr-rich tannery effluent on the diversity of AM fungi in the rhizosphere of maize (*Zea mays*), sorghum (*Sorghum bicolor*), and sunflower (*Helianthus annuus)* and to assess the extent of mycorrhizal colonization of roots of these plants and populations of mycorrhizal fungal propagules in their associated rhizospheres. Metal accumulation in plant tissues was also assessed.

Material and Methods

Soil sources and characterization

Soil samples were collected from tannery effluent polluted soil and on the nearby noncontaminated reference site at Max, Alexandria, Egypt. The soil was throughly mixed and air-dried at room temperature, then crushed and sieved to remove rocks and undecomposed organic materials. Soil mechanical analysis was carried out by the pipette method according to Black *et al.* (1982). The water-holding capacity was determined using the funnel and folded filter paper methods (Alef and Nannipieri,1995). Soil pH was electrometrically determined after mixing 1 g of soil with 2.5 ml water for about 5 min, to allow ionic exchange to reach equilibrium prior reading (Black *et al.*, 1982). Organic carbon content was measured by the rapid titration method (Nelson and Sommers, 1986). Cation exchange capacity (CEC) was determined by the method of Thomes (1982). Total metals in soil were determined by digesting 500 mg of soil in a mixture of concentrated HCl / HNO_3, 4:1, v / v (McGrath and Cunliffe, 1985). Water extractable metals were measured by shaking 10 g (dry wt) moist field soil for 2 h in 20 ml deionized water (Angle *et al.*, 1993). Samples were

filtered and acidified with HNO_3 before analysis. Metal concentrations in the acid digest and extracts solutions were analysed by flame atomic absorption spectrophotometry (AAS).

Pot experiment

Air-dried soils about 2 kg were loaded into plastic pots (18 cm in diameter and 13 cm in depth). Plant selection was based on the ability to achieve hyperaccumulator status for at least one metal. Sunflower, maize and sorghum have been proven to be effective in removing heavy metals from contaminated sites (Chen and Cutright, 2001; Madejon *et al.*, 2003). Seeds of maize, sorghum and sunflower were obtained from Agricultural Experimental Station, College of Agriculture, University of Alexandria. They were sown in plastic pots with four replicas for each treatment, tannery effluent polluted and reference non-polluted soils. Plants were grown for eight weeks in greenhouse illuminated with natural light. The moisture content of each pot was maintained at 70 % water holding capacity by weighing the pots two times per week. Deionized water was added as needed. Nutrient solutions of KNO_3, $(NH_4)_2SO_4$ and K_2HPO_4 / KH_2PO_4 were added as needed to all pots at the same time and in identical amount (Evans *et al.*, 1972).

Plant harvest and analysis

After eight weeks, plants were gently removed from soil. Shoots and roots were separated. Plant shoots and roots were washed with deionized water, rinsed, and dried at 70 ^{0}C for 3 days. Shoot and root length were measured, and then dry plant samples were weighed and ground using a Wiley Mill. Two grams or less of milled plant matter was digested using a mixture of concentrated HCl/HNO_3 (4:1, v/v) (McGrath and Cunliffe, 1985). Metal concentrations were determined using flame atomic absorption spectrophotometry.

Mycorrhizal study

Mycorrhizal root colonization: For assessment of percent mycorrhizal colonization, an aliquot of washed root material (2 g fresh weight) was collected from each pot and the roots were cut into approximately 1cm length. Washed roots were cleared and stained to determine arbuscular mycorrhizal (AM) colonization (Brundrett *et al.*, 1996). Briefly, roots were cleared with KOH (1.8 M) heated to 80°C for 30 min. After rinsing with three changes of deionized water, the roots were rinsed with HCl (0.3 M) and stained for 30 min in trypan blue staining solution (0.5 g trypan blue per l of 1: 1:1 solution of glycerin, lactic acid, and deionized water). Roots were destained using several changes of tap water. Percent colonization was determined on the top, middle, and bottom portions of the roots with the grid-line intersect method (McGonigle *et al.*, 1990).

AM fungi spore counts: Mycorrhizal spores were extracted by blending 10 g of each soil sample in 100 ml of tap water for 20 seconds to release intraradical spores. These samples were wet sieved onto a 37 μm sieve, re-suspended in deionized water and extracted at the interface of a 70 % sucrose/water gradient

following centrifugation at 1700 rpm for 3 minutes (Daniels and Skipper, 1982). The spore suspension was diluted with water, and intact spores were counted on 30 random fields of view per Petri dish by microscopy to obtain the relative abundance per species and the total spore numbers g^{-1} fresh weight soil. Spores were counted under the dissecting microscope and grouped according to morphological characteristics. Permanent slides were prepared for each different spore morphotype with polyvinyl-alcohol and polyvinyl-alcohol plus Melzer's solution (1:1). Different morphtypes of AMF spores were identified on the basis of spore size, color, wall structure, and hyphal attachments to the species level according to (Walker, 1983; Schenk and Perez, 1990).

Results and Discussion

Physical and chemical analysis of soils used in this study are presented in Table 1. The tannery effluent polluted soil is a sandy loam, while the reference soil was a sandy clay loam. The pH and organic matter values of the tannery effluent-polluted soil were 6.8 and 4.4 %, respectively. Total and water-extractable chromium concentrations were higher in the tannery effluent polluted soil (16865 and 13.6 mg Cr/kg soil, respectively) as compared with total and water extractable Cr in the reference soil (176 and 1.2 mg Cr/kg soil, respectively). The concentration of Cr was high compared to the values generally observed in agricultural soils and considered to be toxic according to Kabata and Pendias (2001). These results agree with Khan, (2001) who found that the DTPA-extractable and total concentrations of Cr in the soil from the polluted site was significantly higher than the corresponding values in the reference unpolluted soil.

TABLE 1. Physical and chemical characteristics of reference and tannery effluent polluted soils.

Soil properties	Reference soil	Tannery effluent soil
Sand %	54	75
Silt %	24	14
Clay %	22	11
Soil texture	Sandy clay loam	Sandy loam
Organic matter %	2.6	4.4
Soil pH (1-2.5 soil water ratio)	6.9	6.8
CEC (mequiv/100g)	43.5	34.8
Total Soil Cr (mg kg^{-1})	176	16865
Water extractable Cr (mg kg^{-1})	1.2	13.6

In this study variations in metal content in soils were observed. These were also recorded in the extractable metal content. This can be attributed to the behavior of trace metals in soils that depends not only on the level of contamination, as expressed by the total content, but also on the form and origin

of the metal and the properties of the soils (Chlopecka *et al.*, 1996). The total Cr (16 865 mg/kg) in the tannery effluent-contaminated soil found in the present study is considerably higher than that reported by Raju and Tandon (1999) in the sludge formed as a result of basic tanning activities (970 µg/kg) in India and also higher than the total Cr (630 µg/kg) in the tannery effluent polluted soils in Pakistan (Khan, 2001). Raju and Tandon reported elevated concentrations (1200 mg/kg) of Cr in plants growing on this sludge. The cation exchange capacity of soil is a major factor in determining the extent to which heavy metals are adsorbed by the solid-phase constituents and hence the extent of their solubility. CEC was higher in reference soils compared to the polluted soils, 43.5 and 34.8 meq/100 g dry soil, respectively (Table 1). In general, soils with high CECs can adsorb larger amounts of heavy metals than can soils with a low CEC (Chaney *et al.*, 1977).

The presence of high levels of metals in soils exerts pressure on plant species leading to the selection of specific flora (Shallari *et al.*, 1998). Certain metals are essential to biological activity. However, all metals, whether essential or nonessential, will tend to show toxicity at certain levels (Valix and Loon, 2003). Data in Table (2) shows that shoot and root elongation and their dry weights of *Z. mays, S. bicolor* and *H. annuus* grown in tannery effluent-polluted soil were reduced compared with plants grown in unpolluted reference soil. These differences in soil metal levels suggest a strong selection pressure on the plants tested. The Cr concentration in the shoot and root of maize plants was markedly higher for tannery effluent polluted soil than for reference soils (Table 3). The order of Cr accumulation was maize > sorghum > sunflower. Mean values of Cr accumulation (mg/kg plant dry weight) in the shoots and the roots of the three plants from contaminated soils showed that, relatively higher amounts were retained by their roots. This agrees with the previous results indicating that the translocation of Cr from the root to the shoot is rather slow. Chromium tends to accumulate in root tissues and does not normally correlate well with the extractable amounts in soil (Soon and Abboud, 1993). Shallari *et al.* (1998) found that the high pH value (8.2) for a soil at Prrenjas, Albania, affected the availability and metal uptake by plants grown in soils containing 3865 mg Cr/ kg dry soils. He found that the maximum Cr concentrations in *Euphorbia myrsinites* was 9 mg Cr/kg dry weight. Metal concentrations in aerial parts of hyperaccumulators greatly exceed those of roots (Baker *et al.*, 2000). This may also be partly due to the method of extraction used in this study. Shewry and Peterson (1976) showed that acid ammonium oxalate was a better exractant of soil Cr. It extracts a form of Cr, which is not readily plant available and is different from that extracted by water. Maize displayed higher levels of Cr accumulation as compared with the sorghum and sunflower, 7356.8, 6191.6 and 2508 mg/kg^{-1} dry weight, respectively of plant species were grown on the tannery effluent polluted soils. On the contrary, the present study did not reveal any correlation between Cr content in dried plant tissue and the water extractable Cr in the soil.

TABLE 2. Shoot and root elongation, and dry weights of plants grown in tannery effluent polluted soil and unpolluted reference soil.

Plant species	Elongation (cm)		Dry weight (g)	
	Shoot	Root	Shoot	Root
Zea maize (Polluted)	41.5	40.25	0.43	0.31
Z. maize (Reference)	76.6	45.6	3.0	1.4
Sorghum bicolor (Polluted)	27.5	41	0.09	0.03
S. bicolor (Reference)	73.5	48.7	1.16	0.25
Helianthus annuus (Polluted)	49	14	0.79	0.06
H. annuus (Reference)	77	27	3.94	0.22

TABLE 3. Chromium concentrations, numbers of mycorrhizal spores and percent of mycorrhizal root colonization of the plant growing on the tannery effluent-polluted soil and reference unpolluted soil .

Plant species	Cr Concentration (mg/kg dw)		AM infection (%)	AMF spores/ 100 g dry soil
	Shoot	Root		
Zea mays (Polluted)	1066.8	6290	23.3	68.8
Z. mays (Reference)	230	159	40.6	396.3
Sorghum bicolor (Polluted)	791.6	3370	43.0	172.9
S. bicolor (Reference)	554.8	46.1	56.6	496.5
Helianthus annuus (Polluted)	163	2345	3.2	0.0
H. annuus (Reference)	85.3	163.9	27.0	140

Khan 1999; Khan *et al.*, 2000 and Khan, 2001 demonstrated that plants growing on the tannery effluent-contaminated soils were mycorrhizal and that their mycorrhizospheres contained arbuscular mycorrhizal (AM) fungal spores. The previous studies show that these fungi have evolved heavy metal tolerance and that they may play a role in the phytoremediation. In this study a higher number of AM fungi propagules were recovered from the reference site as compared to the contaminated site (Table 3). The percentage of mycorrhizal colonization of plant roots and mycorrhizal spore numbers were higher in unpolluted reference soil as compared to the tannery effluent polluted soil. A host plant effect on AM fungi was observed. AM endophyte spore frequency was lower under sunflower, probably due to the lower density of infectable roots, than under maize and sorghum. The sorghum plants showed the highest number of spores. This may reflect the rapid root growth rate of this plant species, which may allow earlier contact between host and fungus. The high incidence and intensity of colonization in sorghum may be a manifestation of greater

mycorrhizal dependency, attributed in part to a high phosphorus requirement (Bever *et al.*, 1996). The percentage of mycorrhizal root colonization of the three plants ranged between 27-56.6% in unpolluted soil and 3.2-43% in polluted soil. The total number of arbuscular mycorrhizal (AM) spores in the rhizosphere of all tested plants grown on unpolluted soils was higher than plants grown on tannery effluent polluted soils. The number of AM fungal spores in unplanted non-polluted soil and Cr-polluted soil was lower than in the rhizosphere of polluted and /or nonpolluted soils. In general the percentage of AM fungal colonization is positively related with the numbers of AM fungal spores in soils.

The composition of the AM fungal population in the various host plants rhizospheres, as affected by the different polluted and unpolluted soils is shown in (Fig. 1). Heavy metal pollution may suppress or even kill sensitive parts of the plant and soil microbial communities and lead to shift in their functional diversity and structure (Chaudhry *et al.*, 1999). *Glomus* sp. was the most common AM fungal species in the rhizospheres of sorghum, maize, *and* sunflower, 244, 195, and 54 spore per 100g soil, respectively grown on unpolluted soils, but its population decreased in the rhizosphere of the same plants grown on tannery effluent polluted soils. In Cr-contaminated soils *G. mosseae* seemed to be the AM fungal species with the best ability to sporulate, becoming the most common species in the rhizospheres of maize compared with other five AM fungal species.

The soil samples harbored six species of arbuscular mycorrhizal (AM) fungi (Fig. 1). Five of them belonged to the *Glomus* genera in Glomaceae and other one belonged to genera in the Acaulosporaceae family. Most of the AM endophyte spores found at polluted and unpolluted soils were closely related to *Glomus fasciculatum*, *G. macrocarpum*, *G. mosseae*, *G. clarum*, an un-described *Glomus* spp. and *Acaulospora* spp. The most frequent species were *Glomus mosseae*, *G. clarum* and the undescribed *Glomus* species, which in total accounted for 90% of the samples. Less frequent isolates were *Glomus fasciculatum* (7%), followed by *G. macrocarpum* and *Acaulospora* sp. (3%). Khan (2001) reported that the AM fungai community varied, i.e. trees growing on the unpolluted reference site are exposed to a wide variety of AM fungi such as *Glomus*, *Scutellospora*, and *Acaulospora*, whereas those on the contaminated site contained only *Gigaspora* sp. in their rhizospheres. Raman and Sambandan (1998), on the other hand, reported the numerous AM fungai genera were present in tannery effluent-polluted soils of Tamil Nadu, India. The AM fungal propagules never disappeared completely in the tannery effluent polluted soils, suggesting adaptation of these indigenous AM fungai to Cr. Koomen *et al.*, (1990) and Loth (1996) reported the changes in the diversity and abundance of AM fungal populations due to heavy metal content. Other authors found no correlation between the concentration of heavy metals (Zn, Cu, Cd, Ni, etc.) and AMF fungal populations (Weissenhorn and Leyval, 1994).

Glomus macrocarpum and *Acaulospora* sp. were very sensitive to the presence of metals in soil, and its propagules practically disappeared in

contaminated soil, while *G. clarum* and *Glomus* sp. maintained a consistent density in all soils independent of soil polluted. *G. mosseae* showed another pattern, increasing its density in polluted soil. It is noteworthy that *Glomus* sp. was abundant in sorghum, maize and sunflower rhizospheres.

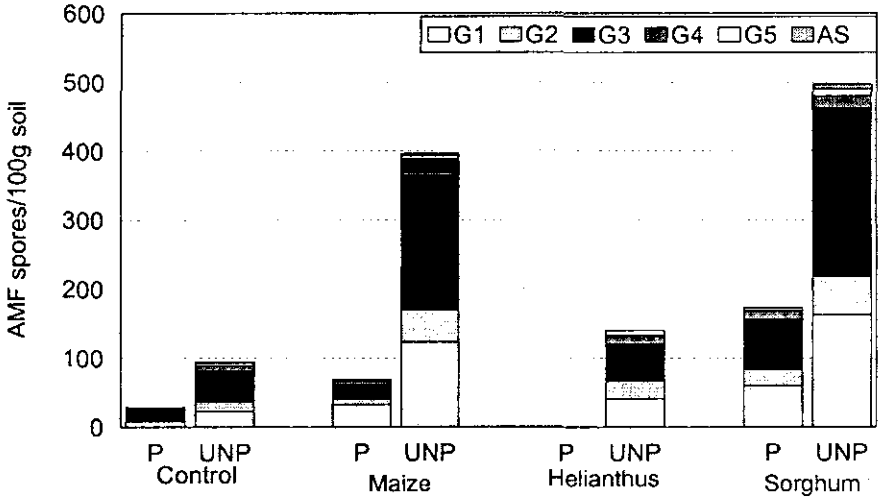

Fig . 1. Arbuscular mycorrhizal species abundance and diversity after 8 weeks of trap culture.
G1= *Glomus mosseae*, G2= *G. clarum*, G3= *Glomus* spp, G4= *G. fasciculatum*, G5= *G. macrocarpum*, AS= *Acaulospora* spp, P= Polluted, UNP= Unpolluted .

Khan (2001) found a typical infection pattern of *Glomus* sp. in the roots of *Dalbergia sissoo* growing in Cr-contaminated soils but a lake of spores of *Glomus* sp. in the associated rhizospheres in the present study indicates the potential error of using AM fungal spore surveys to extrapolate the root colomization. PCR fingerprinting and ribosomal small subunit (SSU) sequences from both AM root and AM fungal spore samples need to be investigated to reveal genetic variations within AM fungal species and genera (Clapp *et al.*, 1999; Regvar *et al.*, 2003). However, the mycorrhizal population response to heavy metal toxicity as well as the biochemical and molecular basis of tolerance is still poorly understood (Turnau, 1993).

Conclusion

Tannery effluent polluted soils reduced shoot and root biomass, as well as percentage mycorrhizal root colonization of *Zea mays*, *Helianthus annus* and *Sorghum bicolor*. Metal uptake by plant was affected by the colonization of roots with (AM) fungi. *Z. mays* shows much greater accumulation of, and tolerance to Cr than the other plant species studied. The order of Cr foliar accumulation was *Z. mays* > *S. bicolor* > *H. annuus*. Our results suggest *Z. mays* plants could be used to remediate Cr-contaminated soils in situ within short time frame.

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(*Received* 25/12/2004;
accepted 29/1/2005)

المعالجـــة النباتيـــة للتربـــة الملوثـــة بالـــكروم

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اصبح تلوث التربة بالمعادن الثقيلة بمصر من الموضوعات الهامة من الناحية البيئية والصحية والاقتصادية. وللاستفادة من قدرة النبات على امتصاص الملوثات فقد تم تنمية نبات الذرة الشامية والذرة الرفيعة (العويجة) وعباد الشمس في الصوبة على تربة غير ملوثة وأخرى ملوثة بالمخلفات السائلة الناتجة من دباغة الجلود. وبعد ثمانية أسابيع من الزراعة تم حصد النباتات وتقدير الوزن الجاف ومحتوى الكروم بأنسجتها. وأوضحت العلاقة بين الميكوريزا وتلك النباتات أن النسبة المئوية لأستعمار النباتات بتلك الفطريات كانت أعلى في التربة الغير ملوثة مقارنة بالتربة الملوثة كما أظهر الوزن الجاف للجذور والمجموع الخضري لتلك النباتات نفس الشيء. أظهرت جذور النباتات الثلاثة في كلا النوعين من التربة الإصابة بالميكوريزا (AM) ووجود جراثيمها في المنطقة المحيطة بجذور هذه النباتات. وكان للمحتوى العالي من الكروم تأثير عكسي على عدد وتباين أنواع الميكوريزا. ويمكن ترتيب النباتات تنازليا من حيث قدرتها علي تجميع الكروم داخل أنسجتها كالآتي الذرة الشامية ، الرفيعة وعباد الشمس.

وتلقى الدراسة الضوء على دور الميكوريزا فى عملية التخلص الحيوي من الكروم في التربة الملوثة ووجد أن تأثير فطريات الميكوريزا على امتصاص العناصر الثقيلة داخل النبات يعتمد على المحتوى المبدئي للعنصر في التربة. كما أن تجميع هذه النباتات محل الدراسة للعناصر الثقيلة يعتمد على نسبة استعمار فطريات الميكوريزا جذورها.