Copper Accumulation in *Leucaena leucocephala* by *Mycorrhizae Glomus* Sp. Zac–19 in *Symbiosis* with *Rhizobium*

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***Abstract*—** *The Jose Antonio Alzate dam is the main man-made reservoir along the Lerma River in central Mexico. The water in this dam is heavily contaminated with organic and inorganic residues including copper and other heavy metals. For many years, people living in the vicinity of the dam use sediments as fertilizers, resulting in heavy soil contamination. This study focuses on the use of native Leucadena leucocephala, a small, fast-growing mimosid tree cultivated under greenhouse conditions in an attempt to reduce the levels of copper. The plant was inoculated with a fungus of the Glomus sp. Zac-19 species that in a symbiotic manner increases the decontaminating properties of L. leucocephala. The study was carried out in three stages, starting with sequential extractions to assess the geochemical distribution of copper. Next, L. leucocephala was grown under controlled conditions using a factorial statistical model adding two known doses of* Cu^{2+} *and a third plantlet grown under normal conditions to be used as control. The plant growth was followed at random during 30, 60, 90 and 180 days to assess the level of copper bioaccumulation in leaves, stem and in the whole plant. The bioaccumulation index was evaluated using the initial six fractions, with fraction five revealing a direct relationship between the levels of copper in plant and sediment. Due to the relation of fraction V with humic material, it was finally considered for the calculation of the bioaccumulation index. Copper accumulation in stems was greater than in leaves and the metal concentration decreased with time. The percentage of arbuscules, vesicles and complete colonization was affected by high doses of* Cu^{2+}*,inhibiting the growth of stem and leaves of L. leucocephala. These results suggest that the plant can be useful for the biological removal of copper in contaminated sediments or soils.*

Keywords*— *copper speciation, polluted soil, phytoremediation, Glomus sp.,Rhizobium, Leucaena.

I. INTRODUCTION

Anthropogenic activities such as agriculture, industry, urbanization and logging have alarmingly deteriorated extensive ecological areas throughout Mexico (López-Galván *et al*, 2011) Figure 1. The use of urban wastewater and sediments as fertilizers is a common practice in arid and semi-arid regions of developing countries and in areas where the human population is constantly increasing, as in the areas along the Lerma River in central Mexico (Avila *et al*, 2007). The Upper Lerma Basin is in the State of Mexico, with the Tejalpa and Temoaya rivers as its tributaries. These rivers are a depository of urban and industrial wastewater that are heavily contaminated with fertilizer and pesticide residues and large quantities of sedimentable materials that eventually end at the José Antonio Alzate dam (from here on referred to as the dam), with the inevitable deterioration due to accumulation of pollutants (Barceló-Quintal *et al*, 2013, Avila-Perez *et al*, 2011, Barceló *et al*, 2005). The dam sediments have a high content of organic matter and are used as fertilizers (Barceló, *et al*, 2012; Pedroza-Benitez *et al.*, 2010; Avila *et al.*, 1999; Smith, 1991). The soil in the region is mainly vertisol of the phaeozem type. The aquifers in this basin sit on basaltic rocks and the sediments are mainly made up of and esite, basalt, pyroclastic, lahar and alluvial materials. Corn is the main crop grown in the surrounding fields and copper sulfate is used to avoid the growth of pathogenic fungi adding Cu^{2+} ions to those from industrial effluents. Phytoremediation is a possible via to directly curb the excess of Cu in these soils through absorption, sequestration or storage of the pollutant or by indirect methods using microorganisms such as bacteria or fungi that act in symbiosis with the plant for removal of pollutants (Gonzalez-Chavez *et al.*, 2002).

Immobilization of metals by plants can be done with plants such as *L. leucocephala*, which is also an excellent host for arbuscular endomycorrhizal fungi (AMF; Gardezi *et al*, 2010, Gardezi, *et al*, 2011, George *et al.*, 1994, Smith and Read, 1997). The symbiotic association between AMF and the roots of plants is prevalent in nature. The symbiosis occurs in approximately 80% of vascular plant species in all terrestrial biomes (Smith *et al.*, 2010).The soil availability of phosphorus and AMF interact influencing the productivity of a plant community by mediating compensatory effects among plant species and functional groups, (Yang, et al, 2014).

Leep (1981) reported that plants have limited ability to minimize cooper absorption depending on accumulation of the element in soil. The *Rhizobium* strain plays an important role in the adsorption of nutrients (Gardezi et al, 1990). The importance of arbuscular symbiosis in crop production and natural ecosystems has been related to their ability to process mineral nutrition in soils, especially in soils lacking nutriments with low mobility, such as phosphorus, copper and chromium (Gardezi et al 2005, Gardezi et al, 2003). *Rhizobium* bacteria show resistance to metals, but they reduce its biological capacity to fix nitrogen (Gardezi et al, 2005).

The benefit of the dual inoculation of AMF and *Rhizobium* for biological decontamination in crops such as legume trees grown in soils contaminated with heavy metals has been widely studied (Rachid and Abdellah Kajji, 2015, Davies *et al.*, 2001; Dahilin *et al.*, 1997; Dev *et al.*, 1997; Tarafdar and Rao, 1997; McGrath *et al.*, 1994). Olson (1983) points out those proteins immobilize metals through binding. For example, anionic polysaccharides attract metallic cations and form insoluble sulfides of Cu, Cd, Pb, Cr and Hg. A basic parameter in phytoremediation is the bioacumulation index (BI), which can be estimated using equation (1).

$$\mathrm{BI} = \frac{metal\ in\ plant\left({}^{mg}\!/_{kg} \right)}{metal\ in\ soil\left({}^{mg}\!/_{kg} \right)} \qquad (1)$$

Kabata-Pendias and Pendias (1992) established that for some heavy metals the BI is in the 0.1 – 1.0 range. Although trace amounts of copper are considered essential for plants and animals, but it is considered to be harmful in larger quantities. Copper is associated with organic matter insoil, iron and manganese oxides, silicates and other minerals. Organic molecules bind Cu through carboxyl groups forming stable bonds. In plants, Cu is absorbed as the hexaaquo ion $[Cu(H_2O)_6]^{2+}$in acidic soils and as $Cu(OH)_2$ in neutral or alkaline soils (Lepp1981). The source of Cu in soils is mainly due to the use of fungicides, insecticides and fertilizers (Barceló-Quintal *et al*., 2012).

II. MATERIALS AND METHODS

The study area is located in the State of Mexico, in central Mexico. Sampling was conducted along the Upper Lerma River Basin, which has the Tejalpa and Temoaya rivers as tributaries. These are agricultural areas around the Alzate dam. Soil samples were taken at a depth of 0-30 cm, Figure 1.

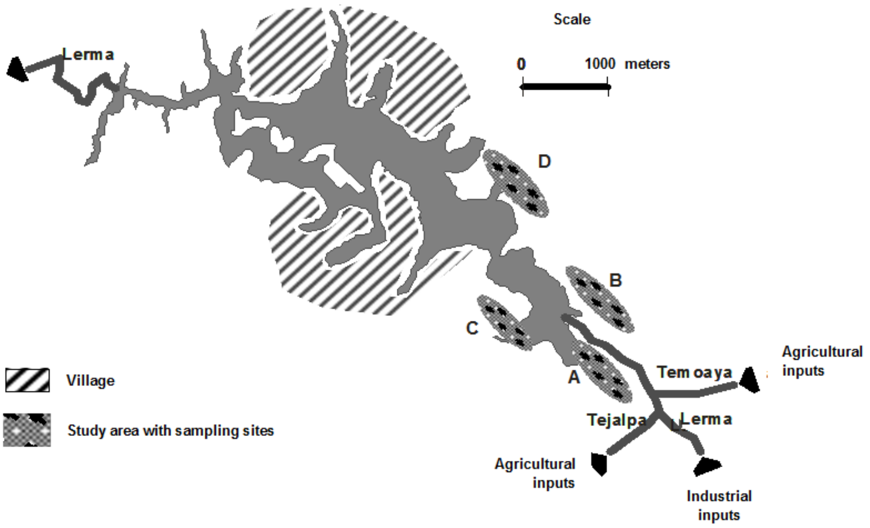

FIGURE 1. AREAS OF SOIL SAMPLING SITES

The collected samples were analyzed by a diffractive X-Ray (DRX) method using a Siemens D-500, 30 KV, 20 mA,IX10 sensibility X-ray apparatus. A scanning electron microscope (SEM, Phillips XL30), connected to a microanalysis equipment EDAX-DX4, was used for the characterization of soils. The Bouyocus method (1951) was used for texture analysis of soils. A Sensor Hanna HI 1618 with a Hanna JO98150interface was used for the determination of pH. The electrical conductivity

(EC) was determined using a LabQuestVernier. The Kjeldahl method was used for the determination of nitrogen and the Dickman and Bray molybdenum blue method was used for the determination of phosphorus. The total carbon content of organic matter (TOC) was determined using a MULTI N/C 3000 Analitik Jena equipment. Soils were dried by lyophilization (Virtis, 2KBTES-SS)and pretreated with 30% hydrogen peroxide in a bath at a constant temperature of 60˚C, the foam was removed and then digested with HF and HNO_3 (Baker, analytical grade) in a CEM Mars 5 microwave and the digest used for measuring chromium, copper, magnesium and potassium by atomic absorption using a Thermoelemental SOLAAR M6 spectrometer.

The seeds of *Leucaena leucocephala* were sterilized with sodium hypochlorite at 2% (Gardezi *et al.*, 2000), hydrated during 48 h and pregerminated in trays using inert material as substrate. The plants were transplanted in pots with 3 kg soil and inoculated with 10 g of 36-160 spores of *Glomus* sp. Zac–19. For the *Rhizobium* bacterium tests 1.5 mL of the TAL 995 strain having a density of 10^9 cells/mL were applied to plants after 10 days cultivation.

In this experiment a randomized block design was used with factorial arrangement (2x2x2x3), with four replications, as follows: sterilized and non-sterilized soils with- and without mycorrhiza; with- and without *Rhizobium,* at 0, 20 and 200 mg/kg Cu^{2+}. The Tukey method was used for evaluation of the agronomical results, setting the significance at $p<0.05$.

The experiment lasted 180 days, from planting until harvesting. The parameters evaluated were: metal speciation in soils and in inoculated soils with20 and 200 mg/kg Cu^{2+}. Leaves and stem samples were analyzed using atomic absorption spectrophotometry at 30, 60, 90, and 180 days. Other variables were plant height, number of branches (NB), stem diameter, (SD), number of leaves (NL), number of leaflets (NLf), leaf length (LL), leaf width (LW), root length (RL), root volume (RV), number of nodules (NN), dry weight of nodules (NDW), dry weight of leaves (DWL), stem dry weight (SDW), root dry weight (RDW), leaf area (LA, cm^2) total percentage colonization and the Cu and Cr content in the leafs and stems as well as the percentages of nitrogen, phosphorus and potassium in steams and leafs.

The geochemical distribution of Cu was determined by a combination of the Tessier et al(1979), Calmano and Förstner (1983) and Solomon y Förstner(1980) methods. For speciation ofCu^{2+} in sediment the sequence and reagents were by Fraction 1 (F1),to pH 7 ammonium acetate1M; Fraction 2 (F2), to pH 5 sodium acetate 1M; Fraction 3(F3), to pH 2 hydroxylamine chloride 0.1 M; Fraction 4 (F4) ammonium oxalate0.2M with oxalic acid0.2M; Fraction 5 (F5) with hydrogen peroxide and Fraction 6 (F6) with a mixture of hydrofluoric acid and nitric acid concentrated 1: 2 respectively.

Soils were treated with 0, 20 and 200 mg/kg Cu^{2+} and were sampled after 30, 60, 90 and 180 days of growth and the level of Cu in leaves and stems were assessed at each stage of growth. The samples were dried at 60 °C. The ashes were wet digested with concentrateHNO_3 (Ultrex II Baker) in a microwave oven (MARS 5, CEM). Copper was determined by atomic absorption using a SOLAAR M6, Thermo elemental spectrometer.

III. RESULTS AND DISCUSSION

Analyses of chemical and physical properties of soils at 0-5 (H1),5-10 (H2) and 10-30 cm(H3) horizons are shown in Table 1.One of the three horizons is classified as sandy clay and the remaining two as sandy loam. The composition of soil was 18% sand, 18% loam and 64%, clay, without significant differences among the horizons. The pH was determined with a potentiometer according to the procedures for soil and water. The soils were moderately acidic with pH values ranging from 5.4 to 5.7. The electrical conductivity was 0.42, 0.62 and 0.30 m/Scm, the total nitrogen was 0.22, 0.08, and 0.07% and the available phosphorus content is found for each horizon as 40.0, 50.0 and 40.0 mg/kg^{1}for depths of H1, H2, and H3, respectively.

The organic matter content, determined by the Walkley-Black method was relatively high for the first two soil horizons, reaching values of 6.13 % and 7.26 %, and moderately low 2.59% for H3.Potassium and magnesium were also determined in all horizons. The geochemical distribution of $Cu^{2+}$is shown in Figure 2.

TABLE 1
SOIL CHEMICAL ANALYSIS OF SEDIMENTS FROM THE ALZATE RESERVOIR

Soil depth (cm)	pH	Electrical Conductivity. (m/Scm)	Nitrogen (%)	Phosphorus (mg/kg)	Potassium (mg/kg)	OM (%)	Copper (mg/kg)
0-5	5.7±0.1	0.42±0.05	0.22±0.05	40±2	400±12	6.13 ± 0.1	2.5 ± 0.102
5-10	5.4±0.1	0.62±0.06	0.08±0.01	50±1	500±13	7.26 ± 0.1	3.0 ± 0.082
10-30	5.6±0.1	0.30±0.04	0.07±0.01	40±1	300±11	2.59 ± 0.1	3.0 ± 0.114

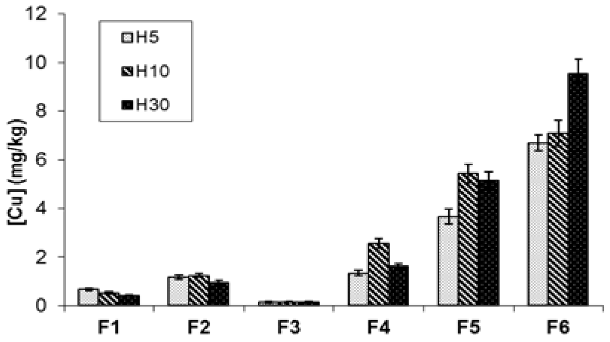

FIGURE 2: GEOCHEMICAL DISTRIBUTION OF COPPER AT H1,H2 AND H3 HORIZONS

Figure 2 shows the mobility of Cu (II) in the three horizons. Both H2 and H3 strongly concentrated in fraction F6. This can be attributed to colloidal crystallization of materials such as metal hydroxides, and the mineralization of organic carbon, which initiates location changes from dissolved or amorphous states to the crystalline state in the deeper layers of soil.

Copper at first can be adsorbed or bound to colloidal material in H1 and H2. The greater Cu^{2+} concentration is located in fraction 5 associated with organic matter, primarily humic materials. The agronomic variables (Table 2) show no significant effect of sterility of the soil, except for the presence of Rhizobium nodules, which in sterile soil they are scarcer, but larger.

There was also an increase in abundance of endomycorrhiza and Rhizobium, as well as increased development of the *Leucaena* host, modifying all the studied morphological characteristics. The concentrations of Cu^{2+} in the soil have different impacts on observed characteristics of *Leucaena*.

Figure 3 shows the percent of total colonization as arbuscules and vesicles. The highest percentage of total colonization was in sterile soil treated with *Glomus sp. Zac*-19 without Cu and without Rhizobium. Native soil treated with *Glomus sp. Zac*-19- and 200 mg/kg Cu, presented the lowest total colonization. Overall, there is a higher percentage of total root colonization of copper-treated plants inoculated with endomycorrhizal fungi, while treatments with 200 mg/kg Cu showed the lowest percentages of total colonization, indicating the possibility of toxicity in the soil-root system, but this does not necessarily imply a continuous bioaccumulation of metal on the ground.

The results also show that at 200 mg/kg Cu there was a marked decrease in the percentage of arbuscules (Figure 3).In the case of percentage of vesicles, treatments 6, 23, 9, 25 and 26 showed the lowest percentage while treatment 5 (Glomus sp. Zac-19 sterile soil without Rhizobium with a dose of 20 mg/kg Cu), had the highest percentage. Doses without copper and with 20 mg/kg favored a higher percentage of vesicles compared to the values of this variable in treatments with 200 mg/kg Cu.

For example the height and number of nodules of plants have somewhat greater values with 20 mg/kgCu, as compared to 0 mg/kg Cu, but the values with 200 mg/kg are notably lower (Table 2). This result suggests that plants grown with addition of 20 mg/kg might be beneficial for *Leucaena*.

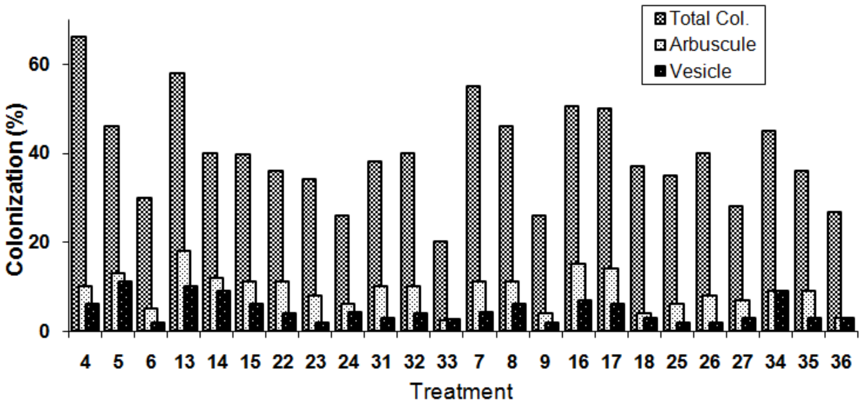

FIGURE 3. COLONIZACIÓN MICORRÍZICA EN L. LEUCOCEPHALA PARA LOS DIFERENTES TRATAMIENTOS

TABLE 2
AGRONOMIC CHARACTERISTICS OF PLANTS AT THE END OF THE GROWTH.

Soil Factors and Levels	Plant Height (cm)	Stem Diameter (cm)	Number of Branches	Root Length (cm)	Root Volume (cm^3)	Number of Nodules	Dry Wt of Nodules (mg)
Sterile	59.333 a	7.889 a	3.622 a	33.292 a	160.63 a	4.19 b	0.0218 a
Non-sterile	59.264 a	7.597 a	3.616 a	31.694 a	151.29 a	5.75 a	0.0234 a
HSD	**1.362**	**0.432**	**0.107**	**1.686**	**10.27**	**0.70**	**0.0053**
Without *Glomus*	51.250 b	5.542 b	2.915 b	26.60 b	109.15 b	4.32 b	0.0169 a
With *G.* Zac- 19	63.688 a	9.104 a	4.019 a	35.71 a	181.85 a	5.72 a	0.0268 b
HSD	**1.999**	**0.634**	**0.157**	**2.475**	**15.08**	**1.03**	**0.0078**
Without *rhizobium*	56.583 b	7.028 b	3.422 b	29.89 b	135.38 b	2.29 b	0.0076 c
With *rhizobium*	62.014 a	8.458 a	3.814 a	35.09 a	176.54 a	7.64 a	0.0376 b
HSD	**1.362**	**0.432**	**0.107**	**1.686**	**10.27**	**0.70**	**0.0053**
0 mg/ kgCu	61.729 b	8.2500 b	3.771 b	33.48 b	162.19 b	6.03 a	0.0245 a
20 mg/ kg Cu	65.021 a	9.229 a	4.044 a	36.42 a	179.77 a	6.41 a	0.0335 b
200 mg/ kg Cu	51.146 c	5.750 c	3.041 c	27.58 c	125.92 c	2.45 b	0.0096 c
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HSD = honest significant difference. Means with the same letters in the same column are statistically equal (P: 0.05).

The concentration of Cu^{2+} was determined in soil before planting (0 days, Figure 3). The soil was then inoculated with *Rhizobium* and *Glomus sp.Zac-19*, and 0, 20 mg/kg or 200 mg/kgCu^{2+} as $CuSO_4$.

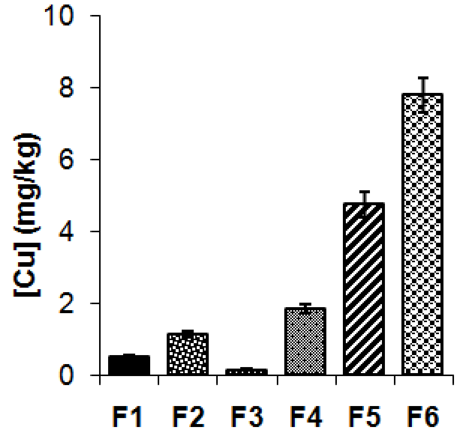
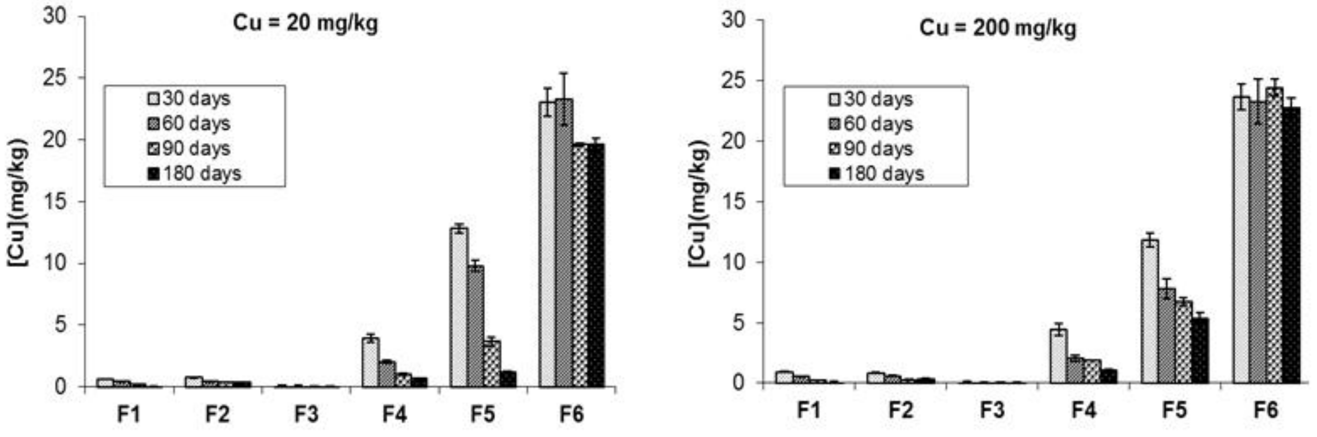


FIGURE 3. NORMAL AVERAGE GEOCHEMICAL DISTRIBUTION OF CU IN SOIL

After 30, 60, 90 and 180 days, the plants were harvested and analyzed for the percentage dry weight of Cu (II) in soil, stems, and leaves. In the plant growing with 20 mg/kg added Cu the geochemical distribution of the element changes with the time. The mobile forms of Cu^{2+} (F1 and F5), are less abundant with time (Figure 4a,b), but the addition of 20 mg/kg of Cu^{2+} show a similar distribution, except that more copper is accumulated in F5 after 90 and 180 days (Figure 4a).



(a) **(b)**

FIGURE 4. GEOCHEMICAL DISTRIBUTION AFTER 30, 60, 90 AND 180 DAYS IN PLANTS GROWN IN SOILADDED WITH 20 MG/KG CU (A) AND 200 MG/KG CU (B)

The Cu distribution pattern in fraction 6 is similar and was not changed by a 10-fold increase of Cu, but it did show a decline over time in fraction 5.There are two possible explanations: one, that in F5 the plant is rapidly assimilating Cu and two, that copper is chemically mobile in the presence of colloidal clay, oxyhydroxides, Fe, Mn, or organic matter present in humic materials (Pedroza, *et al*, 2010).

In the 200 mg/kg treatment there was a somewhat widespread distribution of Cu. Marquenie Van der Werff and Ernst, (1979) found that Cu forms the $[Cu(H_2O)_6]^{+2}$, which by hydrolysis forms a suspension of $Cu(OH)_2$ on the surface of the soil. This hydroxide is distributed in fraction 1 and can be lost by rain or irrigation (Bloomfield and Saunders, 1977). Considering that the relative availability of Cu in the soil is determined by the plant's response, the abundance of humic materials and the permeability of the plasma membrane of the root. The first fractions F1 and F2 can be lost by rain or irrigation, since the metal is absorbed physically but not chemically.

Figure 5 shows the concentrations in the leaves and stem at 30 to 180 days in the presence of *Glomus* sp. Zac-19 and *Rhizobium.* The stem accumulates more Cu than the leaves. Without addition of Cu the accumulation of Cu in leaves is barely perceptible during the first 30 days of growth but it is quite notable at 90 to 180 days compared to that at 30 to 60 days. This could result in less bioaccumulation in the 60 to 90 days period, or it could result in greater availability of Cu in the soil, as seen previously in the speciation diagram, Figures5 and 6.

The pattern of accumulation was similar in stem and leafs. The variation of Cu concentration in leaves (Figure 6a) was 0.67±0.06 to 3.82±0.10 mg/kg from 30 to 180 days and 4.28 ±0.40 to 12.00± 0.17 mg/kg in stems during the same period, when the total Cu in these organelles was 2.48±0.23 to 7.91 ±0.13 mg/kg.

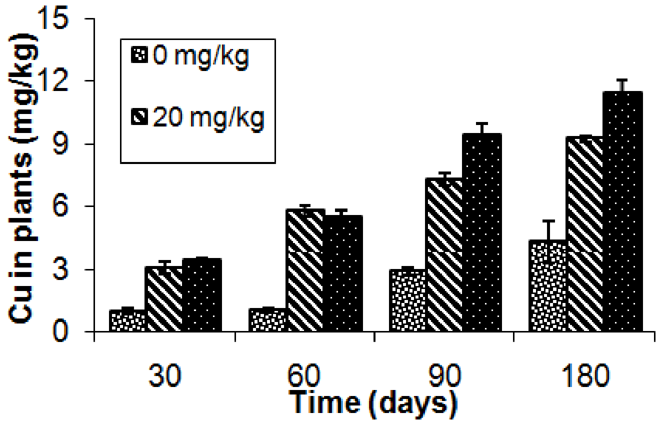


FIGURE 5. CU(II) ACCUMULATION IN *L.LEUCOCEPHALA* IN SYMBIOSIS WITH *GLOMUS* SP. ZAC 19 AND *RHIZOBIUM*. 0-200 MG/KG CU ADDED TO EACH POT

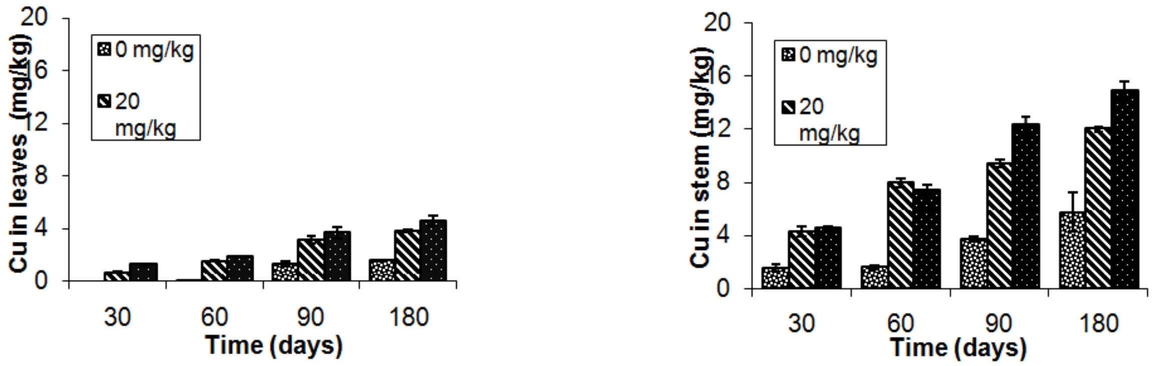


FIGURE 6. CU(II) ACCUMULATION IN LEAVES (A) AND STEMS (B) OF *L.LEUCOCEPHALA* IN SYMBIOSIS WITH *GLOMUS* SP.ZAC 19 AND *RHIZOBIUM*. 0-200 MG/KG CU ADDED TO EACH POT

Treating with 200 mg/kg Cu, its concentration in leaves increased from1.27±0.04 to 4.61±0.39 and from 4.57±0.08 to 14.89±0.70 in stems at 30 and 180 days, respectively (Figure 6b). As described in the Introduction, bioaccumulation in leaves and stems was estimated using George's Eq. 1 (1994).

Fraction 6 was not included in the calculation of Cu in the whole plant as this element it is not is metabolically involved. For the calculation of Cu in the whole plant only F5 was considered because it is more relevant to the metabolism of the plant and in the soil (Boyd *et a*l. 1981). In F5 a considerable amount of Cu is coordinated to organic molecules forming strong bonds between the metal and humic acids, reducing the chemical mobility of Cu but increasing the possibility for assimilation by the plants(Fassbender and Bornemiza , 1987; Bornemiza and Peralta, 1981, Korte *et al.*,1976).

Figure 7a gives the bioaccumulation index in leaves, stem and root, taking into consideration all fractions. Figure 7b gives the BI for fractions F1 to F5, because the plant does not absorb Cu in F6.Finally in Fig. 7c only F5 was considered because of the chemical interaction of Cu with humic materials.

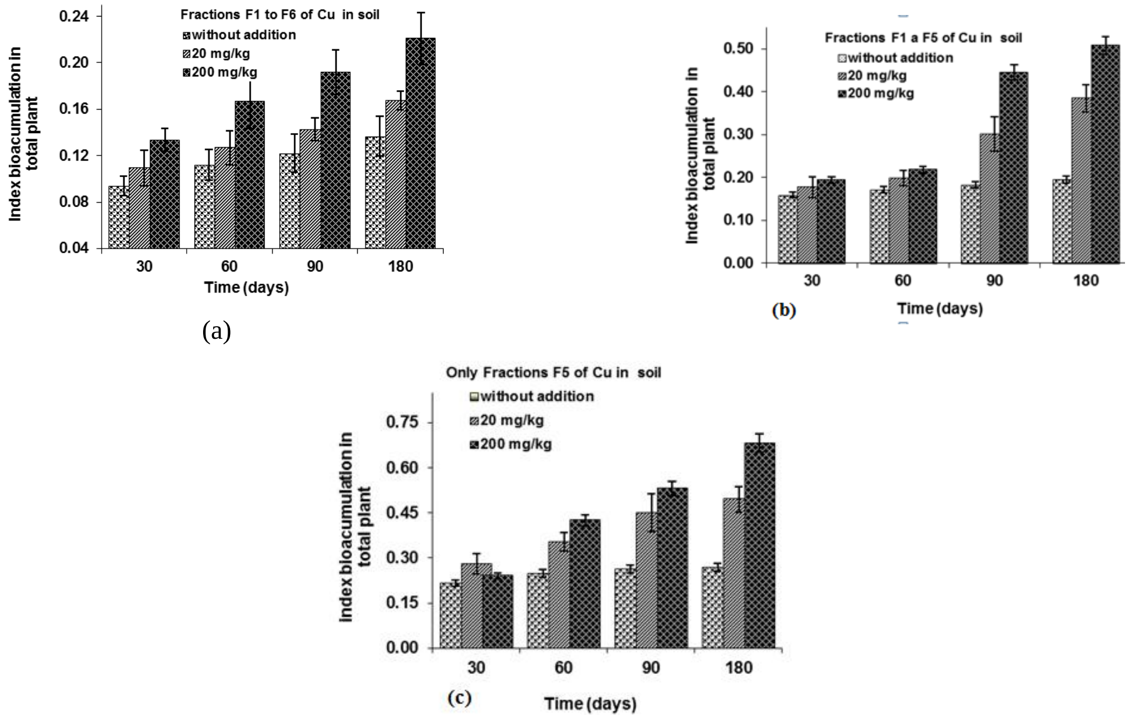
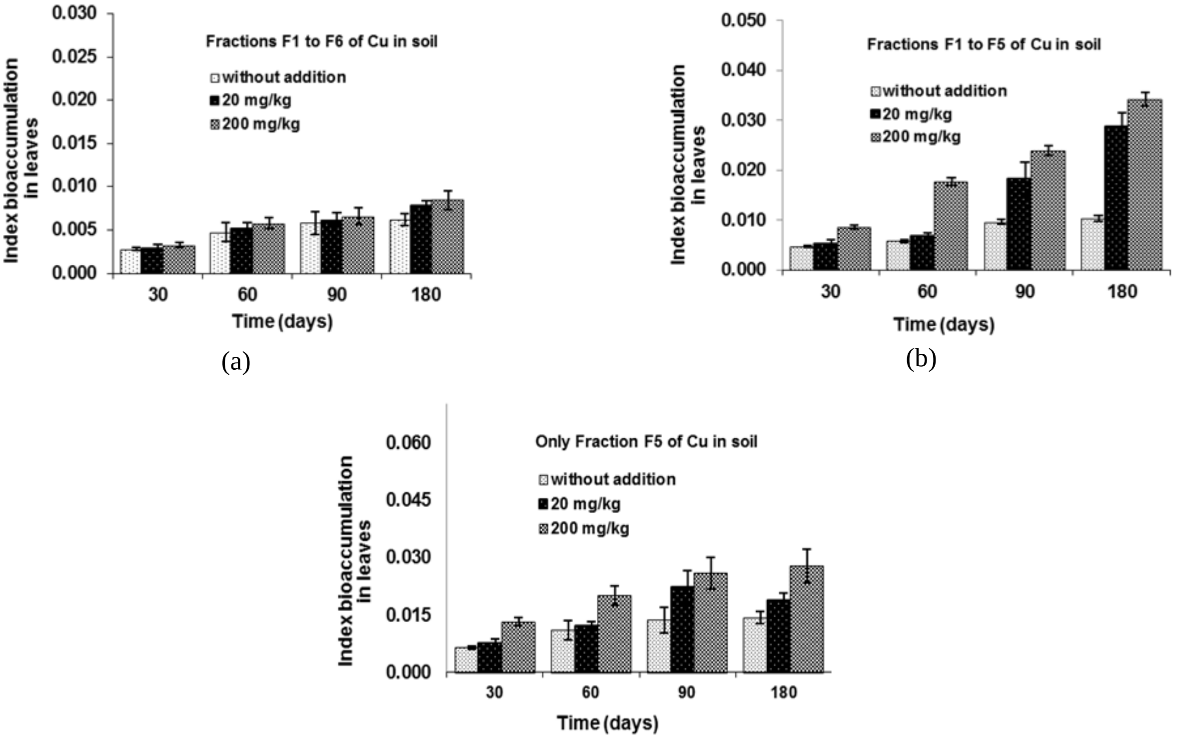


FIGURE7. COPPER BIOACCUMULATION INDEX (BI) IN *LEUCAENA*. (A) FRACTIONS 1-6 (B) FRACTIONS 1-5 (C) ONLY FRACTION 5

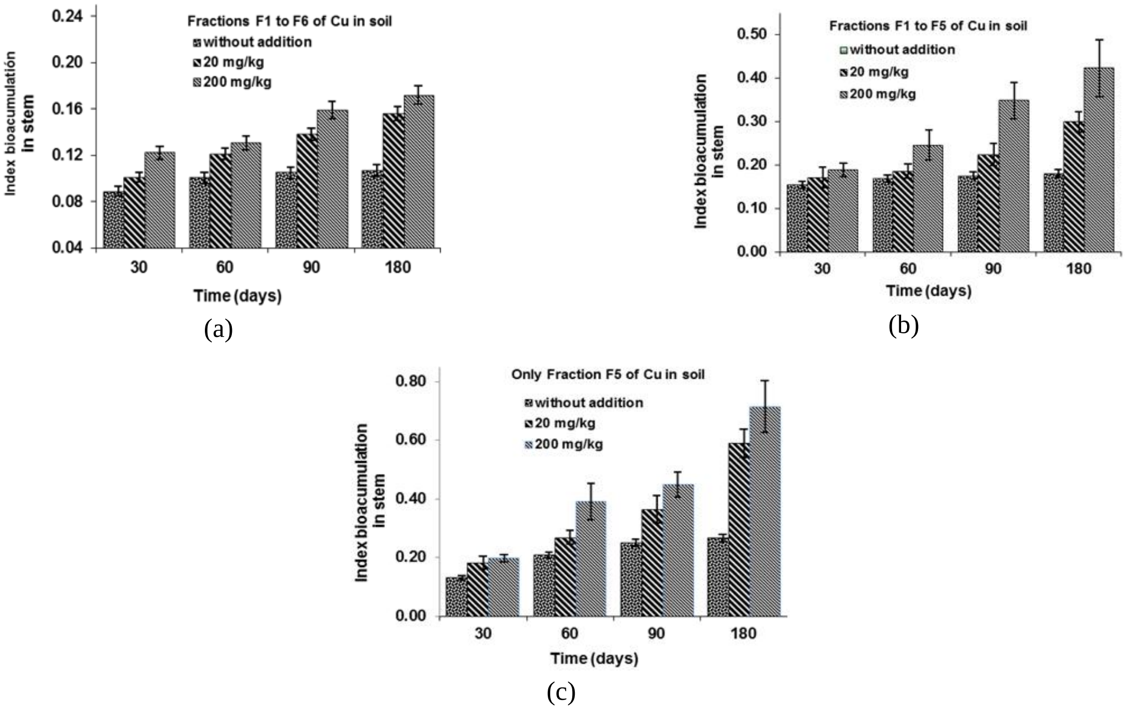
The BI in leafs was consistently smaller than in stem, but it did show an increase of growth with time, Figs. 8 and 9. In particular at 30 days almost no bioaccumulation was detected in the leaves. The BI declined to 0.174 or lower in fractions F1 to F5 even though additional Cu^{2+} was added to the soil. In other cases the BI was greater at the last sampling: going from undetectable in F1 to 1.561 mg/kg and for F5 alone it went from not detected to 3.26mg/kg. Addition of 20mg/kg Cu caused the largest BI after 180 days (F1-F5 and F5). A decline of the capacity to absorb Cu2+ when 200 mg/kg Cu was added, in agreement with studies reporting that the plant saturates and reduces its ability for metal accumulation.



(a) (b)

(c)

FIGURE8. COPPER BIOACCUMULATION INDEX (BI) IN LEAVES OF *LEUCAENA* (a) ALL FRACTIONS (b) FRACTIONS 1-5 (c) ONLY F5



(a) (b)

(c)

FIGURE 9. COPPER BIOACCUMULATION INDEX (BI) IN STEMS OF *LEUCAENA* (A) ALL FRACTIONS (B) FRACTIONS 1-5 (C) ONLY F5

IV. CONCLUSION

Soil components such as feldspar, halloysite and goethite have surfaces with greater potential of adsorption of humic materials. This allows greater Cu assimilation. Also, the soil is slightly acidic, with average pH = 5.7, which can promote mobility of Cu^{2+}.Copperappears to be toxic to *Leucaena leucocephala*, *Rhizobium* and mycorrizal fungi at concentrations above 200 mg/kg. In aerial parts, the leaves accumulate less Cu than the stem. This is important for the use of *Leucaena leucocephala* as a food source for ruminants. The mycorrhizal fungus *Glomus* Sp. Zac-19 in symbiosis with *Rhizobium* can enrich the soil with nitrogen and phosphorus and reduce soil erosion and other soils of the region. The geochemical distribution or speciation of Cu in the soil allows determination of the fixed fraction of Cu and its bioavailability with greater precision.The results of this study support the idea of using *Leucaena leucocephala* doubly inoculated with *Rhizobium* and *Glomus* sp.Zac-19 for bioremediation of agricultural soils with levels of copper that do not surpass the threshold for copper toxicity in such symbiotic system.

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