## Effects of Sewage Sludge Application and Arbuscular Mycorrhizal Fungi (*G. mosseae* and *G. intraradices*) Interactions on the Heavy Metal Phytoremediation in Chrome Mine Tailings.

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**Abstract**
The soil is crucial habitat for huge variety of organism and substrate for plants to supply nutrient and water. Currently, mining activities spoilt the soil with heavy metals and give serious damage to the environment. This study investigates the effects of arbuscular mycorrhizal fungi (AMF) interactions and sewage sludge application on heavy metal phytoremediation in mine tailings. The phytoremediation process was enhanced by the substantial symbiotic relationship between Arbuscular Mycorrhizal Fungi (AMF) and the hyper accumulator plant, sunflower. Sewage sludge was applied to mine tailings to boost the phytoremediation processes by promoting growth of plants. In the study, greenhouse experiments were performed with eight different pot sets prepared in three replicates. The pots were filled with mine tailing soil and different sewage sludge doses of 20 and 30 g/kg soil. One half of the sludge added pots were inoculated with *Glomus mosseae* and the rest with *Glomus intraradices* to determine the effects of two different AMF species in the remediation of heavy metals from mine tailing soils. Sunflower seeds were planted into the pots, as a hyperaccumulator plant and grown for three months to evaluate the role of sewage sludge application in AMF and host plant interrelation and plant heavy metal uptake. AMF association increased the metal uptake of the plants. The sewage sludge amendment improved the growth of plants. However, it supressed the AMF colonization in the roots of sunflowers. Combined *Glomus mosseae* and 20 g/kg sludge amendments resulted with the highest metal uptakes and glomalin contents.

Keywords: Phytoremediation, Arbuscular Mycorrhizal Fungi, AMF, sewage sludge, mine tailings, heavy metal

### 1. Introduction

The soil, having vital importance for the natural environment is the foundation of the food system. It is a habitat for the huge variety of organisms. Soil regulates the water flow and transition of the chemicals between atmosphere and earth [1].Soil pollution becomes substantial problem for human beings, living and non-living entity as the soil is a non-renewable resource. The organic fraction of pollution can be degraded by soil microorganisms but, being the major problem of inorganic components, the heavy metals need immobilization or physical removal. While trace amount of some heavy metals are essential for plants, the high concentrations are toxic as they form free radicals inducing oxidative stress. Furthermore, the toxic heavy metals can engage in the host plants pigments or enzyme disrupting their function [2]. Soil remediation is needed for removal of toxic heavy metals to retrieve the soil supply efficiently. Phytoremediation is a low-cost, feasible, green technology using metal-accumulating plants to remove toxic metals, pesticides, and other hazardous materials from soil and water.

Arbuscular Mycorrhizal Fungi enables host plants to promote stabilization of trace elements in rhizosphere of plants through the symbiotic relationship, favouring phytostabilization and phytoextraction [3]. AMF sets up intimate hyphal network of fungi with the host plant root which can be more than 100 meter hyphae per cubic meter. These special hyphal networks enable the host plant nutrient (predominantly phosphate), water and heavy metal uptakes [4]. *Glomus mosseae* and *Glomus intraradices* are different species of Arbuscular Mycorrhizal Fungi.

The municipal wastewater sludge (sewage sludge) containing phosphorous and nitrogen can be reused for soil amendment purposes after a proper treatment. They increase the water holding capacity of the soils and supply nutrients. Therefore, in remediation of the mine tailing soils, sewage sludge application is thought to improve the phytoremediation by conditioning the soil and promoting the growth of plants while reducing the amount of sludge to be disposed to environment. Lambert and Weidensaul applied sewage sludge to soybean crops and observed that sewage sludge application suppressed the arbuscular mycorrhization in soybean crops, probably due to the presence of toxic compounds [5]. In literature, there are limited studies dealing with the effects of sludge application on the phytoremediation of mine tailing soils in relation to growth and nutrition of mycorrhizal plants used for heavy metal removal.

The aim of this study is to investigate the effect of different AMF species associations and sewage sludge addition on phytoremediation of mine tailing soils. In the study, the chrome mine tailing soil having very high heavy metal (Cr, Al, and Fe) content were ameliorated by phytoremediation enhanced by *Glomus mosseae* or *Glomus intraradices* association and two different doses of sewage sludge application.

## 2. Materials and Methods

### *2.1 Study area*

The pot experiments were conducted in a greenhouse for a three-month period between August and October 2018. This period of the year is characterised by mean monthly maximum temperatures between 23 and 30 $^{0}$C and mean monthly minimum temperatures between 12 and 20 $^{0}$C.

### *2.2 Mine tailing soil and sewage sludge*

The mine tailing soil used in the research was taken from a chrome mine in Kütahya, Turkey. Tailing soil samples were collected from different parts of the mining area and mixed before planting to simulate the mining area. The sewage sludge was taken from an advanced wastewater treatment plant in Istanbul, Turkey. The soil pH ranged between 7.9 and 8.5. The bulk density of mine tailing soil was measured to be 1.7 g/cm$^3$. The soil with high bulk density has become highly compacted when irrigated. The heavy metal concentrations in sewage sludge and soil samples were measured by using ICP-OES equipment after digesting the samples according to EPA 3051[6]. Elemental analyses were performed by using an Elemental Combustion System (Costech ECS 4010) to determine elemental ratios of carbon (C), nitrogen (N), hydrogen (H) and sulphur (S) in the soil and sewage sludge samples used in the study. Total Kjeldahl Nitrogen (TKN) of the sludge samples were determined by Nessler Method 8075 HACH. PhosVer 3 Ascorbic Acid Method 8048 was used for determining the orthophosphate concentration of the sewage sludge samples after digesting the samples according to Nessler Method 8075 HACH [7].

**Table 1:** The metal contents of Cr mine tailing soil and sewage sludge used in the study.

| Sample (mg/kg) | Cr | Mn | Fe | Ni | Cu | Zn | Al | Cd | Pb | Si | Co | Mo |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Mine Tailing Soil** | 1218 | 672 | 26424 | 1585 | 8.84 | 17.1 | 29273 | 0 | 3.73 | 446 | 44.4 | 1.37 |
| **Sludge** | 709 | 461 | 86321 | 411 | 676 | 1815 | 72886 | 4.01 | 34 | 363 | 7 | 3 |

**Table 2:** C, H, N and S content of soil and sewage sludge samples.

| % | Mine Tailing Soil | | | Sewage sludge | | |
|---|---|---|---|---|---|---|
| **C** | 3.7 | 2.8 | 2.57 | 30.5 | 31.3 | 31.1 |
| **N** | 0 | 0.1 | 0.1 | 5.5 | 5.6 | 5.53 |
| **H** | 1 | 1 | 0.6 | 4.3 | 4.4 | 4.3 |
| **S** | 0 | 0 | 0 | 0.5 | 0.5 | 0.5 |

**Table 3:** TKN and phosphorous concentration in the sewage sludge sample.

| Sewage Sludge (mg/kg) | TKN | $PO_4$ | $P_2O_5$ |
|---|---|---|---|
| | 21641.53 | 41779.075 | 31177.14 |

***2.3. Plant and growth conditions***

In this study, sun flowers were used as hyper accumulator host plants. Initially surface of the sun flower seeds were sterilized with %70 ethanol and distilled water for three times. Eight different pot sets were prepared in three replicates. The control pots contained only mine tailing soil. The rest of the pot series contained mine tailing soil amended with sludge and inoculated with AMF species of *Glomus mosseae* or *Glomus intraradices* Contents of the pot sets were given in Table 1. The plants were watered weekly with approximately 100 – 150 ml distilled water to eliminate any metal contamination. The pot experiments were conducted in three replicates.

**Table 4:** Contents of the pots

| No | Pots Sets | Mine Tailings | Sludge | AMF |
|---|---|---|---|---|
| 1 | M (control) | 1 kg mine tailings | - | - |
| 2 | MMos | 1 kg mine tailings | - | *G. mosseae* |
| 3 | MS20 | 1 kg mine tailings | 20 g dry sludge | - |
| 4 | MS20Mos | 1 kg mine tailings | 20 g dry sludge | *G. mosseae* |
| 5 | MS20Int | 1 kg mine tailings | 20 g dry sludge | *G. intraradices* |
| 6 | MS30 | 1 kg mine tailings | 30 g dry sludge | - |
| 7 | MS30Mos | 1 kg mine tailings | 30 g dry sludge | *G. mosseae* |
| 8 | MS30Int | 1 kg mine tailings | 30 g dry sludge | *G. intraradices* |

***2.5. Plant sampling and analyses***

In this study, plant growth in other words plant biomass production was followed by measuring dry shoot weight of plants. Plant shoots were separated from the roots and placed in the paper bag to dry at 70 $^0$C for 2 days. After two days, the plant shoots were weighed and powdered to have homogenous samples. The samples were digested according to EPA 3052 ($HNO_3$:$H_2O_2$; 9:1) then metal characterization was determined by using ICP-OES equipment [8]. The plant roots were put in 50 ml falcon tubes with 70% ethyl alcohol and keep them at +4 $^{0}$C. The roots mycorrhization (colonization) rates were determined by light microscope after being dyed with trypan blue [9].

The soils associated with the roots of the plants were separated from the roots carefully and kept in -20 $^{0}$C for determination of glomalin protein. The glomalin related protein was determined according to Bradford assay. The glomalin contents of the soil samples were measured by using UV-160A SHIMADZU spectrophotometer at 595 nm wavelength after being extracted and dyed with Bradford Reagent [10].

## 3. Results and Discussion

### *3.1. Plant Growth and Mycorrhizal Colonization*

Figure 1 shows the plant shoot weights in the pots. The highest plant growth, measured in terms of plant dry shoot weight, was obtained in the pots amended with *Glomus mosseae* inoculation and 20 g/kg sewage sludge addition. Considerable differences on plant biomass were found when 20 g/kg sewage sludge was added to mine tailing soil. However; 30 g/kg sludge addition decreased the shoot biomass in comparison with 20 g/kg sludge addition. Turk observed that mycorrhizal symbiotic relationship supressed the plant growth in soils having optimum phosphorus concentration due to the carbon competition between the host plant and the mycorrhizal fungi [11].

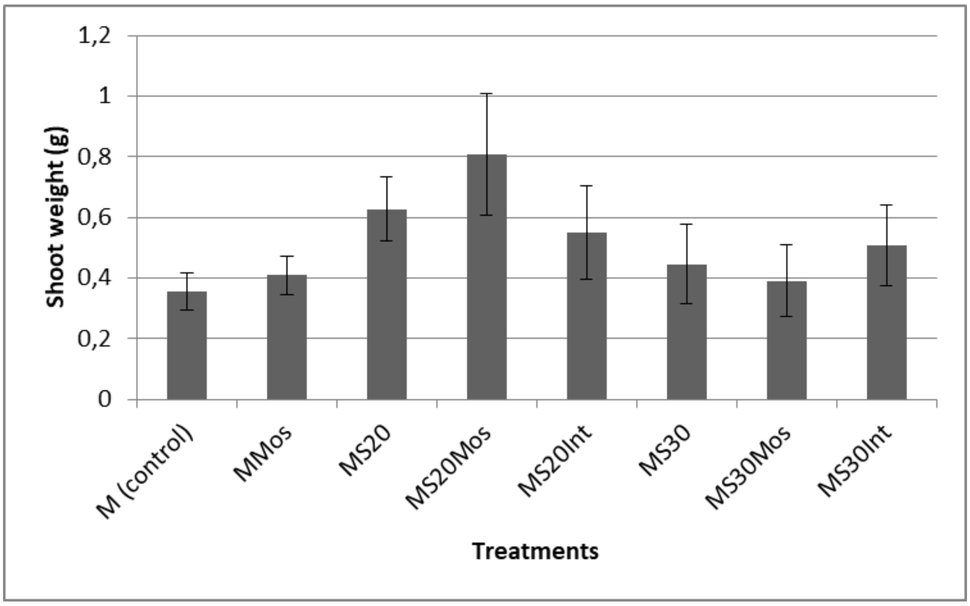


**Figure 1:** Plant shoots dry weight in the pots.

Furthermore, Lambert reported that the activated sludge amendment inhibited the mycorrhizal plant responses, like plant growth and uptake of Cu, P and Zn, the most probably due to the high concentration of NH3[5].

The nutrient deficiency, high heavy metal concentration, and especially the high bulk density of the Cr mine tailing soil restricted the root and plant growth of sunflowers. The root weights of the plants couldn't be determined since there was not enough plant root growth in the pots. The bulk density of the mine tailing soil was too high for sunflower lateral roots to grow healthy. The limited root samples were used in mycorrhizal colonization determinations. Supportively, McKenzie reported that soils having a bulk density higher than 1.6 g/cm$^3$ cause to restriction in plant and root growth by eliminating movement of air and water through the soil [12]. The physical properties of the soil such as bulk density, compactibility, water retention capacity etc. affect the ability of the AMF to spread and form a hyphal network in the substrates. In the same way, Gaur and Adholeya observed differences in AMF association and plant biomass production for the substrates of similar particle size under similar growth conditions and host plant [13].

AMF association and sludge application enhanced the plant growth measured in terms of shoot dry weight.

**Table 5:** Mycorrhizal colonization and plant dry weight (DW) of sunflower

| Pot sets | Mycorrhizal Colonization (%) | Plant Shoot DW (g pot−1) |
|---|---|---|
| **M (control)** | **7.4± 0.4** | **0.36±0.06** |
| **MMos** | **45± 14.5** | **0.41±0.062** |
| **MS20** | **5.66±2.84** | **0.628±0.11** |
| **MS20Mos** | **28.56±9.64** | **0.807±0.2** |
| **MS20Int** | **6±2.11** | **0.551±0.155** |
| **MS30** | **4.3±0.99** | **0.455±0.13** |
| **MS30Mos** | **6.24±0.77** | **0.391±0.12** |
| **MS30Int** | **6.9± 6** | **0.509±0.13** |

Three months after the inoculation of *G.intraradices* and *G.mosseae*, the roots of sunflowers showed considerable differences that were established fungal intraradical structures. The highest mycorrhizal colonization rate of 45% was observed in the pot MMos, containing *G.mosseae* inoculated plants. However, the second highest mycorrhizal colonization rate of 28% was obtained in the pot including 20 g/kg sludge and *G.mosseae* inoculated plants. The lower mycorrhizal colonization rates were obtained in the *Glomus intraradices* inoculated plants. There were no considerable

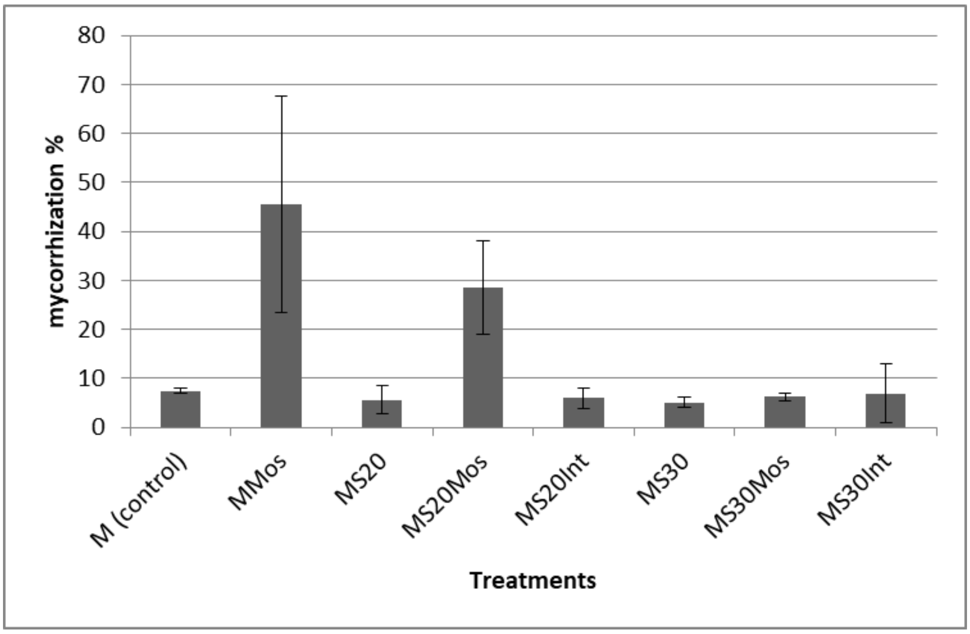


**Figure 2:** Mycorrhizal colonization rates in the pots.

differences between control pots and the pots inoculated with *Glomus intraradices*. The application of 30 g/kg sewage sludge to the pots supressed the mycorrhizal association. This can be attributed to the high nutrient, phosphorous and nitrogen, content of the sewage sludge.

### *3.2. Glomalin Related Protein*

The mycorrhizal association increased the glomalin protein concentrations in the soil. Gadkar and Rillig reported that mycorrhizal colonization released the glomalin proteins. *G.mosseae* was found to be more effective than *G. intraradices* [14]. Supportively, Tunali showed that *G.mosseae* was effective species for sunflower mycorrhizal colonization [15].

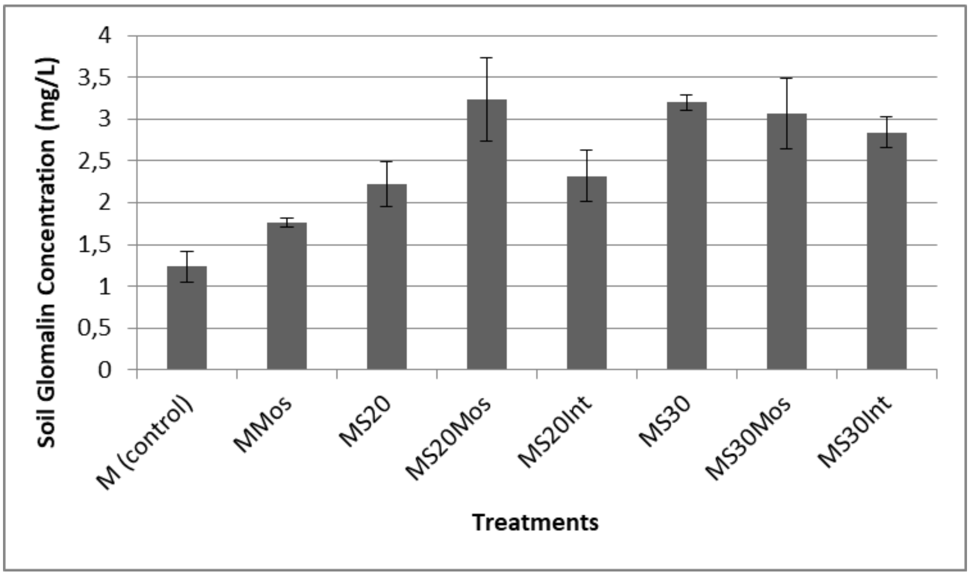


**Figure 3:** Glomalin related protein contents of the soils in the pots.

In this study, glomalin related soil protein was increased further with the application of sewage sludge. The highest glomalin protein was observed in plants grown in mine tailing soil amended with 20 g/kg sludge application and *Glomus mosseae* inoculation. A typical activated sewage sludge contains 224 – 462 mg protein $g^{-1}$ VSS [16].

### *3.3. Heavy metal uptakes*

There were considerable differences in shoot heavy metal uptakes between non-inoculated plants and *G. mosseae* inoculated plants except 30 g/kg sludge applied plants. Especially, *G. mosseae* association without sewage sludge application increased the total metal uptakes in plant shoots. The municipal sewage sludge application improved the growth of plants by supplying nutrients and increasing water holding capacity of the soil. However, the applied dose of sludge affected the plant metal uptakes. The application of 20 g/kg sludge considerably improved the metal uptakes of *G. mosseae* inoculated plant, while 30 g/kg sludge caused a decrease. This can be explained with the increased soil nutrient concentration with the increasing sewage sludge application rate leading to a negative effect on AMF association. Sullivan reported that increased sewage sludge application decreased the AMF association. Combined 20 g/kg sludge application and *Glomus Mosseae* inoculation resulted with the highest heavy metal uptakes [17].

Chrome extraction by sunflower shoots were given in Figure 4. Chrome accumulation increased by 100% with the mycorrhizal association (in pots MMos) and 34% with sludge addition (in pots MS20) respectively. When the mine tailing soil was amended with both mycorrhizal association and 20g/kg sludge addition, Cr uptake of sunflowers increased by 225%. The results of the study are in agreement with those Amna, AMF increases the Cr uptake in sunflower (*Helianthus annus.)* [18].

**Table 6:** Cr, Mn, Ni, Cu, Zn, Al, Pb, Co, Mo, Fe and Si uptakes in shoots of sunflower in microgram

| Metal | Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | M(control) | MMos | MS20 | MS20Mos | MS20Int | MS30 | MS30Mos | MS30Int |
| **Cr** | 6.15±0.7 | 12.47±3.8 | 8.25±6.94 | 20.01±11.44 | 11.42±4.6 | 9.29±3.63 | 4.06±1.24 | 4.07±1.55 |
| **Mn** | 23.74 ± 3.5 | 37.27 ± 7.2 | 52.87 ± 25.7 | 49.84±6.02 | 48.62±8.32 | 44.75±11.68 | 34.17±9.82 | 48.36±7.53 |
| **Ni** | 18.26 ± 4.5 | 35.71 ± 10,7 | 28.78±20.2 | 47.04±17 | 30.88±10.6 | 26.34±9.6 | 12.93±1.1 | 17.41± 4.76 |
| **Cu** | 4.96±0.7 | 7.35±0.8 | 10.01±0.6 | 18.37±2.4 | 10.03±1.4 | 10.1±0.7 | 9.77±3.2 | 11.51±0.8 |
| **Zn** | 20.44±2.2 | 23.37±3.4 | 41.07±4.7 | 73.60±4.3 | 38.07±2.2 | 35.46±3.7 | 34.44±9.3 | 43.18±5.3 |
| **Al** | 160.98±16.7 | 256.88±29.5 | 216.72±45 | 281.13±87.5 | 153.08±56 | 149.07±19.5 | 120.66±31.9 | 120.16±29.5 |
| **Pb** | 0.56±0.02 | 0.95±0.26 | 0.54±0.1 | 0.75±0.43 | 0.32±0.15 | 0.51±0.26 | 0.29±0.13 | 0.30±0 |
| **Co** | 0.74±0.23 | 1.87±1.4 | 1.45±0.96 | 1.92±0.72 | 1.43±0.5 | 1.43±0.58 | 0.71±0.04 | 0.82±0.131 |
| **Mo** | 1.27±0.3 | 1.50±0.24 | 1.62±0.35 | 2.21±0.23 | 1.46±0.1 | 1.26±0.2 | 0.83±0.13 | 1.12±0.15 |
| **Fe** | 444.45± | 855± | 625.81± | 976.05± | 622.8± | 537.4± | 276.43± | 314.25± |
| **Si** | 133.55±5.67 | 153.12±33 | 190.22±35 | 209.48±42 | 116.18±10.5 | 91.5±13.3 | 89.14±8.8 | 103.09±16.6 |

Nickel uptakes of sunflowers were higher in pots MS20Mos and MMos. Mycorrhizal association increased the Ni accumulation in the plant shoots. Citterio observed that *Glomus mosseae* colonization increased the Nickel uptake in stem and leaf of inoculated plants grown in contaminated soil [19].

Mn uptakes in plant shoots were improved with both AMF association (in pots MMos) and sludge addition (in pots MS20) by 57% and 122% respectively. The combined AMF association (*G. mosseae*) and 20g/kg sludge addition (in pots MS20Mos) resulted with 108% increase in plant uptake of Mn. Sludge application to mine tailing soil, without mycorrhizal association, increased the Mn uptakes in sunflower shoots. High phosphorus content of sludge was thought to increase the solubility of manganese. Shuman reported that Mn uptake increased with phosphorus level of the soil in non-inoculated plants [20]. Mycorrhizal association caused a slight decrease in Mn accumulation in the plants grown in sludge amended pots (in pots MS20Mos). Liu, A *et al*. observed that Mn accumulation was lower in the inoculated plants than non-inoculated in the presence of high micronutrient concentrations [21]. Furthermore, Arines *et al*. reported that Mn accumulation was decreased with the AMF inoculation. They also showed that *Glomus* species caused to an increase in the population of Mn-oxidising bacteria [22]. In this study, both sludge addition and *G. mosseae* inoculation may have increased the Mn-oxidising bacteria population.

The mine tailings contained 15.5 ppm zinc; however, the sewage sludge zinc concentration was 676 ppm. That's why the Zn uptake of sludge added plants were much higher than other plants. The highest plant zinc uptake was observed in pot MS20Mos amended with *G. mosseae* and 20 g/kg sludge. The mycorrhizal association enhanced the plant zinc uptake. Accordingly, Giasson showed that zinc plant content was higher in plants inoculated with *G. mosseae* or *G. intraradices* [23]. Christie pointed that mycorrhizal colonization was increased the root uptake but decreased the shoot accumulation compared to non-mycorrhizal plants [24].

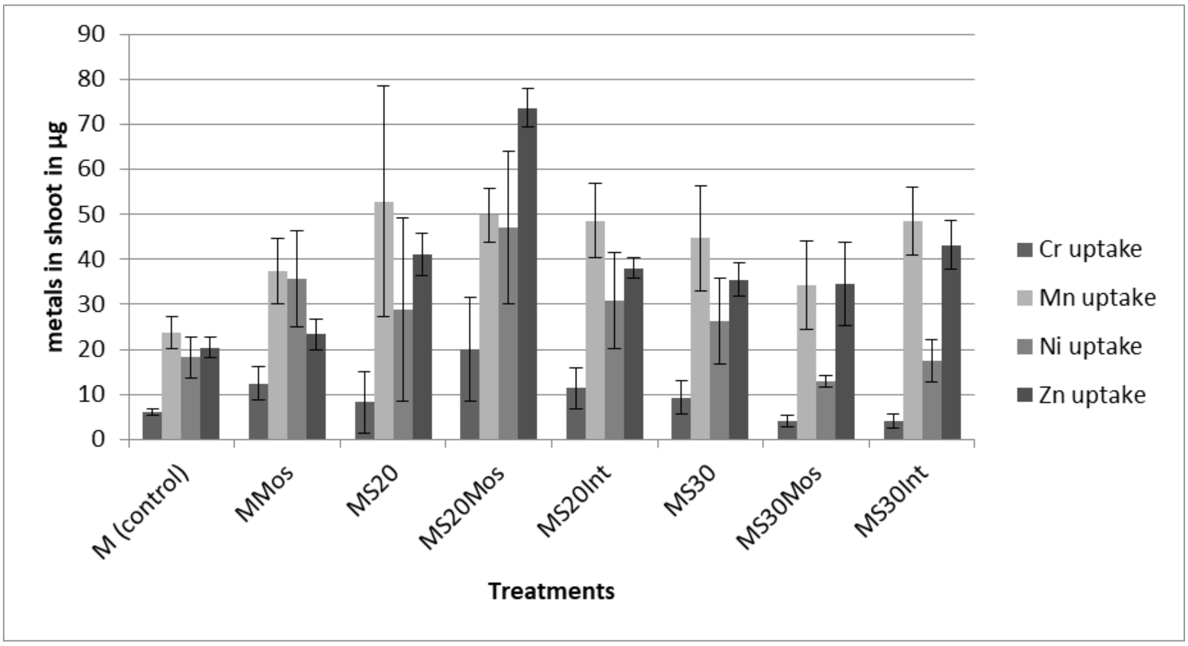


**Figure 4:** Cr, Mn, Ni and Zn uptake of plants.

In the same way, plant shoot Cu uptakes were found to be high in AMF inoculated plants The MS20Mos plants had the highest Cu uptake. The mycorrhizal colonization favoured the Cu uptake especially in the presence of *Glomus Mosseae*. Zinc and copper concentrations were high in the AMF inoculated plants.  Zn and Cu can be absorbed and translocated by the arbuscular hyphae and transmitted to host plants [25]. Contrarily, Joner found that Cu uptakes were %50 low in the inoculated clover shoots [26].

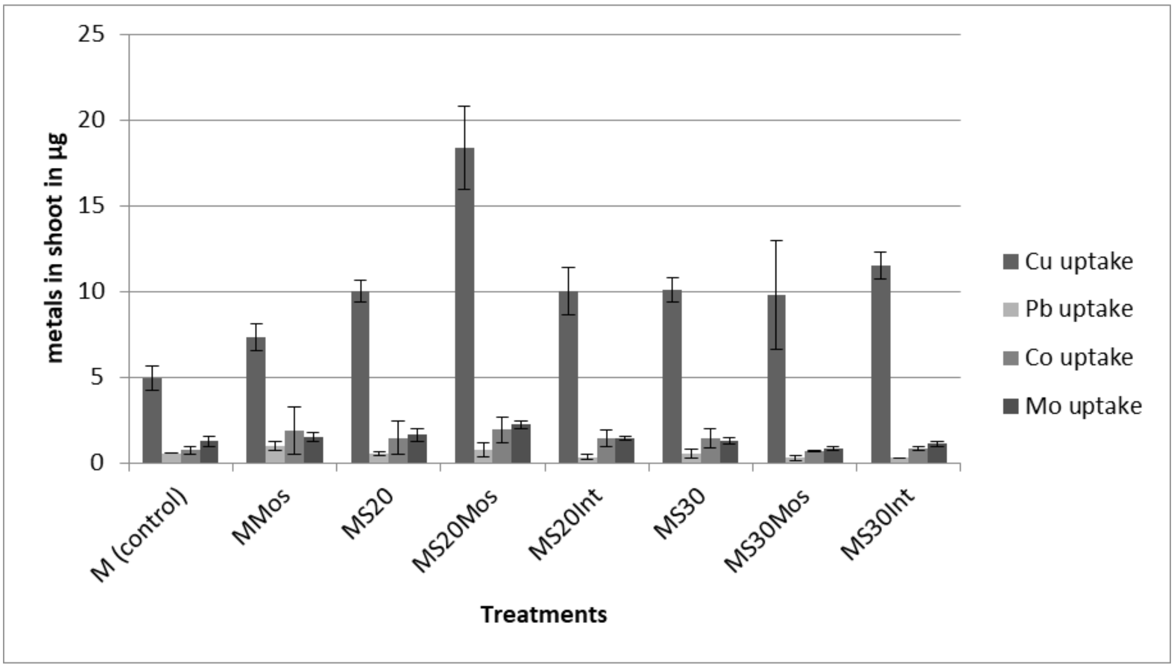


**Figure 5:** Cu, Pb, Co and Mo uptake of the plants.

Tough Pb concentration was really low in the shoots and the soil pH was so high, plants Pb accumulations were low in shoots [27].

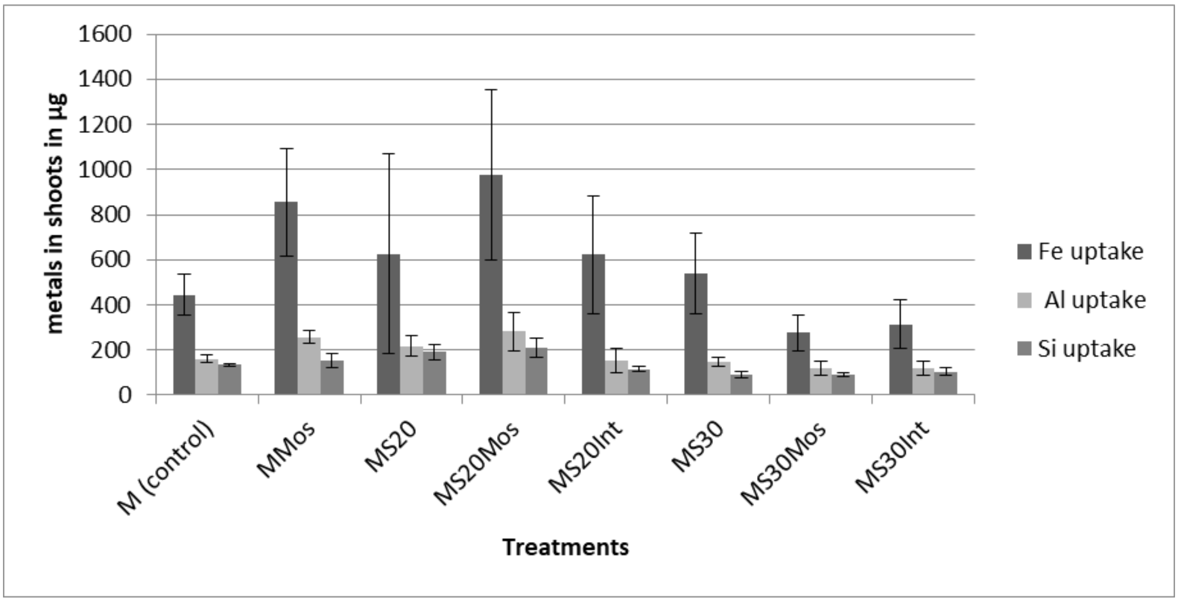


**Figure 6:** Fe, Al and Si uptake of plants.

Cobalt and molybdenum accumulations in plant shoots were also low. This can be attributed to the low cobalt and molybdenum concentrations in the chrome mine tailing soil and sewage sludge. Mo, Co and Pb uptakes were higher in the inoculated (mycorrhizal) plants.

Iron, Aluminum and Silisium accumulation in the host plant shoots were high in the 20 Mos and the Mos treatment. Clark and Zato confirmed that Si accumulation was higher in the *Glomus mosseae* inoculation in maize plants [28].

**4. Conclusion**

The sewage sludge application improved the growth of plants by supplying nutrients and increasing water holding capacity of the soil. AMF association improved the efficiency of phytoremediation by increasing the metal uptake of the plants. *G.mosseae* was found to be more effective than *G. intraradices* for sunflower mycorrhizal colonization.

The combined AMF and 20 g/kg sludge amendments resulted with the highest plant heavy metal uptakes and phytoremediation efficiency. The correlation between glomalin accumulation and certain metals uptake in plants shoot indicates the fact of metals sequestration by mycorrhizal fungi in the presence of glomalin.

The higher sewage sludge application rate of 30 g/kg led to a negative effect on AMF association due to increased soil nutrient concentration. The mycorrhizal symbiotic relationship of *Glomus mosseae* supressed the plant growth in soils having optimum phosphorus concentration due to the carbon competition between the host plant and the mycorrhizal fungi.

Soil bulk density may have crucial importance for phytoremediation efficiency with AMF association.

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