

EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI INTERACTIONS AND  
SEWAGE SLUDGE APPLICATION ON HEAVY METAL  
PHYTOREMEDIATION IN MINE TAILINGS

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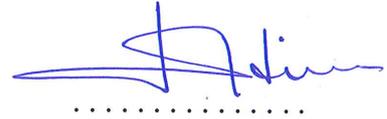
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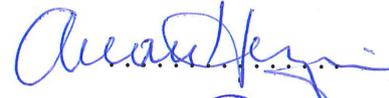
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## ABSTRACT

### **EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI INTERACTIONS AND SEWAGE SLUDGE APPLICATION ON HEAVY METAL PHYTOREMEDIATION IN MINE TAILINGS**

Phytoremediation can be enhanced by the substantial symbiotic relationship between Arbuscular Mycorrhizal Fungi (AMF) and the hyperaccumulator plants (sunflower). AMF set up intimate hyphal network of fungi with the host plant root and enables nutrient, water and heavy metal uptakes. Additionally, application of sewage (wastewater) sludge as a soil amending material improves the phytoremediation efficiency by supplying nutrients and promoting the growth of plants. This study aims to investigate the effects of AMF interactions and sewage sludge application on heavy metal phytoremediation in mine tailings. The samples of chrome tailings having very high heavy metal (Cr, Al, and Fe) contents were ameliorated by phytoremediation enhanced by inoculation of *Glomus mosseae* or *Glomus intraradices* and application of three different doses of sewage sludge and buffer soil. The results showed that AMF inoculation improved the efficiency of phytoremediation by increasing the metal uptake of plants. The sludge application improved the growth of plants. The combined *Glomus mosseae* and sludge (20 g/kg) amendments resulted with the highest plant heavy metal uptake and phytoremediation efficiency. This study offers confirmation on the role of the plant biomass and phytoextraction efficiency interactions in Cr mine tailings as well as mycorrhizal associations and the contribution of the sewage sludge to the plants growth. The outcome of the study confirms that Glomalin related protein rose with the mycorrhizal associations. Meanwhile, the sewage sludge also helped the plant to propagate glomalin protein.

## ÖZET

### ARBUSKÜLAR MİKORİZAL MANTARLARIN VE ATIKSU ÇAMURU EKLENMESİNİN KROM MADENİNİN FİTOREMEDİASYON İLE TEMİZLENMESİNE ETKİLERİ

Maden atıklarına uygulanan fitoremediasyonun verimi, Arbuscular Mikorizal Mantarlar (AMF) ile hiperakümülator bitkiler (ayçiçeği) ile kurduğu önemli simbiyotik ilişki ile geliştirilebilir. AMF, bitki kökü ile iç içe mantar yapısı kurar ve besin, su ve ağır metal alımını sağlar. Bunlara ek olarak, atık su çamurunun maden toprağında büyüyen bitkilere ekstra besin sağlayarak bitkilerin büyümesini teşvik ederek fitoremediasyon verimini artırır. Bu çalışma, AMF etkileşimlerinin ve atıksu çamuru uygulamasının, maden atıklarında ağır metal fitoremediasyonu üzerindeki etkilerini araştırmayı amaçlamaktadır. Çok yüksek ağır metal (Cr, Al ve Fe) içeriğine sahip krom madeni atıkları, *Glomus mosseae* veya *Glomus intraradices* inokülasyonu ve üç farklı dozda atıksu çamuru ve tampon toprağı tatbik edilmesiyle güçlendirilmiş ve fitoremediasyon ile iyileştirilmiştir. Sonuçlar, AMF inokülasyonunun, bitkilerin metal alımını artırarak fitoremediasyonun etkinliğini arttırdığını gösterdi. Çamur uygulaması bitkilerin büyümesini artırdı. *Glomus mosseae* inokülasyonu ve çamur (20 g / kg) eklenmesi, en yüksek bitki ağır metal alımını sağladı. Ayrıca en yüksek fitoremediasyon verimi de bu bitkilere aitti. Bu çalışma, bitki biyokütlelerinin ve fito-ekstraksiyon verim etkileşimlerinin Cr madeni atıklarının yanı sıra mikorhizal birliktelikler üzerindeki rolünü ve atık su çamurunun bitkilerin büyümesine katkısı hakkında bir onay sunmaktadır. Çalışmanın sonucu, Glomalın ile ilişkili proteinin, mikorhizal ilişkilerle ortaya çıktığını doğrulamaktadır. Bunun yanında, arıtma çamuru da toprağın glomalın proteininin çoğaltmasına yardımcı olmuştur.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	iii
ABSTRACT .....	iv
ÖZET .....	v
TABLE OF CONTENTS.....	vi
LIST OF FIGURES .....	viii
LIST OF TABLES .....	xii
LIST OF SYMBOLS/ABBREVIATIONS.....	xiii
1. INTRODUCTION .....	1
2. LITERATURE REVIEW .....	4
2.1. Pollution of Heavy Metals.....	4
2.2. Soil Remediation.....	4
2.2.1. Physical Soil Remediation .....	5
2.2.2. Chemical Soil Remediation .....	5
2.2.3. Biological Soil Remediation .....	6
2.3. Phytoremediation.....	6
2.3.1. Mechanism of Phytoremediation .....	7
2.3.2. Hyperaccumulator Plants.....	12
2.4. Mycorrhizal Associations.....	14
2.4.1. Arbuscular Mycorrhization.....	15
2.5. Glomalin Related Soil Protein (GRSP).....	18
2.6. Phytoremediation of Mine Tailings .....	19
2.6.1. Cr Toxicity of Plants and Its Uptakes.....	20
2.7. Land Application of Sewage Sludge.....	21
2.7.1. Sludge Application in Remediation of Mine Tailings.....	22
3. MATERIALS AND METHODS .....	24
3.1. Sample Collection.....	24
3.1.1. Sampling of Mine Tailings .....	25
3.1.2. Sewage Sludge Sampling .....	26
3.2. Germination Tests.....	27
3.3. Elemental Analysis .....	27
3.4. TKN and P analysis .....	27
3.5. Greenhouse set up and pot experiment .....	28

3.5.1. First Run .....	29
3.5.1. Second Run .....	30
3.6. Metal Analysis .....	32
3.6.1. Digestion Method for the Soil and Sludge .....	32
3.6.2. Digestion Method for the Plants .....	32
3.6.3. Metal Analysis of All Samples .....	32
3.7. pH Measurements .....	32
3.8. Mycorrhization Rate Counts.....	33
3.9. Determining Glomalin Related Protein.....	34
3.10. Statistical Analysis.....	35
4. RESULTS AND DISCUSSION .....	36
4.1. Metal Analysis of the Mine Tailings, Buffer Soil and Sewage Sludge .....	36
4.2. Elemental Analysis .....	37
4.3. Plant Growth.....	37
4.3.1. The First Run of Greenhouse Experiments.....	38
4.3.2. The Second run of Greenhouse Experiments.....	41
4.4. Root and Shoot Weights.....	42
4.4.1. The Sunflowers Planted In the First Run of Greenhouse Experiments.....	42
4.5. Mycorrhization Rates .....	46
4.6. Glomalin Analysis .....	48
4.7. Metal Analysis .....	50
4.7.1. First Run Plantation.....	50
4.7.2. Second Plantation.....	57
4.7.3. Cluster Analysis of Second Run Plantation.....	64
5. CONCLUSION .....	71
REFERENCES.....	72

## LIST OF FIGURES

Figure 2.1. Schematic illustration of phytoremediation approaches .....	8
Figure.2.2. Engineered rhizofiltration system.....	10
Figure 2.3. Schematic representation of phytoextraction of metals from soil .....	11
Figure 2.4. Schematic image of hyperaccumulators that is diverse from non-hyperaccumulators.....	13
Figure 2.5. Structural features of ECM roots of arbuscular mycorrhizal (AM) or gymnosperms or angiosperms .....	15
Figure 2.6. Schematic description of morphological properties of arbuscular mycorrhiza fungal metabolism .....	17
Figure 3.1. The flow chart of the experimental study .....	24
Figure 3.2. Turkey Map. Kütahya is highlighted in red .....	25
Figure 3.3. The first sample of Cr mine tailings .....	25
Figure 3.4. The second sample of Cr mine tailings.....	26
Figure 3.5. The second sample of Cr mine tailings.....	26
Figure 3.6. The root samples in the steam bath.....	33
Figure 3.7. The photo of the aligned roots on the lam.....	34
Figure 4.1. The average heights observed in the first run of greenhouse pot experiment.....	38
Figure 4.2. The average heights of sunflower ( <i>Helianthus annuus</i> ) plants after 11 weeks .....	39

Figure 4.3. Sunflower plants in the second week of the first run plantation .....	39
Figure 4.4. Sunflower plants in the fourth week of the first run plantation.....	40
Figure 4.5. Sunflower plants in the seventh week of the first run plantation .....	40
Figure 4.6. Sunflower plants grown in the sewage sludge in the seventh week .....	41
Figure 4.7. Sunflower plants in the fourth week of second run plantation.....	41
Figure 4.8. Insect and lice problems on the sunflowers .....	42
Figure 4.9. Fresh weights of plant shoots obtained in the first greenhouse experiment .....	43
Figure 4.10. Dry weights of plant shoots obtained in the first greenhouse experiment .....	44
Figure 4.11. Dry weights of plant roots obtained in the first greenhouse experiment .....	45
Figure 4.12. Dry weights of plant shoots obtained in the first run of greenhouse experiments .....	46
Figure 4.13. The average mycorrhization rates in the second run plantation .....	47
Figure 4.14. The roots of MMos plant have blue staining hyphae indicate AMF existence .....	47
Figure 4.15. The roots of MS10Mos plant have blue staining hyphae indicate AMF existence .....	47
Figure 4.16. The roots of MS20Mos plant have blue staining hyphae and arbuscules indicate AMF existence .....	48
Figure 4.17. Calibration curve of BSA standards .....	49
Figure 4.18. Glomalin concentrations in the amended and un-amended mine tailings .....	49

Figure 4.19. Chromium accumulation in the shoot of the first run plantation.....	51
Figure 4.20. Manganese accumulation in the shoot of the first run plants .....	51
Figure 4.21. Nickel accumulation in the shoot of the first run plants .....	52
Figure 4.22. Copper accumulation in the shoot of the first run plants .....	53
Figure 4.23. Zinc accumulation in the shoot of the first run plants .....	54
Figure 4.24. Aluminum accumulation in the shoot of the first run plants .....	54
Figure 4.25. Iron accumulation in the shoot of the first run plants .....	55
Figure 4.26. Silicon accumulation in the shoot of the first run plants.....	55
Figure 4.27. Metal accumulation in plant shoots grown in sewage sludge .....	56
Figure 4.28. Metal accumulation in plant shoots grown in sewage sludge .....	56
Figure 4.29. Metal accumulation in plant roots grown in sewage sludge .....	57
Figure 4.30. Metal accumulation in plant roots grown in sewage sludge .....	57
Figure 4.31. Chromium accumulation in the shoot of the second run plants .....	58
Figure 4.32. Manganese accumulation in the shoot of the second run plants .....	59
Figure 4.33. Nickel accumulation in the shoot of the second run plants.....	60
Figure 4.34. Zinc accumulation in the shoot of the second run plants .....	60
Figure 4.35. Copper accumulation in the shoot of the second run plants.....	61

Figure 4.36. Iron accumulation in the shoot of the second run plants.....	62
Figure 4.37. Silicon accumulation in the shoot of the second run plants .....	63
Figure 4.38. Aluminum accumulation in the shoot of the second run plants .....	63
Figure 4.39. Hierarchical dendrograms for clustering of metals with respect to their uptake by sunflower .....	65
Figure 4.40. Hierarchical dendrograms for clustering of metals with respect to their uptake (Redrawing of Figure 4.39 focusing on the grouping of only Fe) .....	66
Figure 4.41. The evaluation of plant Cr uptake by Hierarchical cluster analysis on dendrogram....	69
Figure 4.42. The evaluation of plant Ni uptake by Hierarchical cluster analysis on dendrogram ....	70

## LIST OF TABLES

Table 3.1. TKN and phosphorous concentration in the sewage sludge sample .....	26
Table 3.2. Contents of first run of the greenhouse experiments .....	30
Table 3.3. Contents of the second run of the greenhouse experiments .....	31
Table 3.4. Composition of MSR medium.....	31
Table 4.1. Mine Tailings, Buffer Soil and Sewage Sludge Heavy Metal Concentrations (ppm) .....	36
Table 4.2. Mine Tailings, Buffer Soil and Sewage Sludge Elemental analysis.....	37
Table 4.3. Absorbance values of indicated BSA samples at 595 nm.....	48
Table 4.4. Meaning of terms used in cluster analysis.....	67

## LIST OF SYMBOLS/ABBREVIATIONS

<b>Symbol</b>	<b>Explanation</b>	<b>Unit</b>
$\mu\text{l}$	Microliter	
<b>Abbreviation</b>	<b>Explanation</b>	
AM	Arbuscular Mycorrhiza	
AMF	Arbuscular Mycorrhizal Fungi	
BAS	Branch Structures	
BSA	Bovine Serum Albumin	
$\text{Ca}(\text{NO}_3)_2$	Calcium Nitrate	
CSS	Composted Sewage Sludge	
ERM	Extraradical Mycelium	
ECM	Ectomycorrhiza	
EDTA	Ethylene Diamine Tetraacetic Acid	
NaFeEDTA	Ferric Sodium EDTA	
GRSP	Glomalin Related Soil Protein	
HCl	Hydrochloric Acid	
KCl	Potassium Chloride	
$\text{KH}_2\text{PO}_4$	Potassium Dehydrogenase phosphate	
$\text{KNO}_3$	Potassium Nitrate	
KOH	Potassium Hydroxide	
$\text{MgSO}_4$	Magnesium Sulfate	
Mos	<i>Glomus mosseae</i>	
MSR	Modified Strullu-Romand	
ICP-OES	Inductively Coupled Plasma - Optical Emission Spectrometry	
Int	<i>Glomus intraradices</i>	
IRM	Intraradical Mycelium	
TKN	Total Kjeldahl Nitrogen	

## 1. INTRODUCTION

The soil is the important dynamic ecosystem that contains microbes and larger organisms that perform many crucial mechanisms turning dead and decaying matter as well as minerals into plants body (Bot and Benites, 2005). Furthermore, its geochemical sink helps to purify the contaminants in the environment naturally and control the transport of chemical elements and substances (Lu, et al., 2012).

By the rising population, the activities of industry, agriculture and mining have increased many toxic chemicals and heavy metals released into the environment, causing heavy metal contamination and chemical pollution. Especially, heavy metal pollution in soil comes from industrialization, agriculture, urbanization, natural constituents of earth crust, and the mining activities (Xu et al., 2014). These toxic substances have variety of toxic effects on living organisms in food chain by bioaccumulation and bio-magnification. The soil is mostly used for the disposal site for a number of heavy metal wastes which needs to be refined (Jadia and Fulekar, 2009). As a result, humans have great concern on the heavy metal contamination in soil due to its great negative effect on human health and environment (Xu et al., 2014). So that, it has become substantial problem for human beings, living and non-living entities as the soil is a non-renewable resource. Accordingly, soil treatment is fundamental to retrieve soil supply efficiently. These contaminations can contain both organic and non-organic components. The major problem of inorganic components seems to be heavy metals. The organic pollution can be degraded by soil microbes but heavy metals need immobilization or physical removal (Gosh and Singh, 2005). Cadmium, copper, lead, chromium, zinc, and nickel are crucial heavy metal pollutants especially in the areas with high anthropogenic pressure (USEPA, 1997). While trace amount of some heavy metals essential for plants, these high concentrations are toxic as they form free radicals inducing oxidative stress. Furthermore, the toxic heavy metals can engage in pigments or enzymes disrupting their function (Gosh and Singh, 2005). Soil remediation is needed for removal of toxic heavy metals to retrieve the soil supply efficiently.

There are three kinds of soil remediation techniques. These methods can be used in different combinations with one another to diminish the pollution to a non-toxic and tolerable level (Jadia and Fulekar, 2009). Although these methods are efficient for remediation, they require having much time and money (Luu et al., 2009). Moreover, because they produce huge amounts of waste to be disposed of, these methods are generally hazardous to the natural soil environment (Cunningham et

al., 1995). Currently, phytoremediation is found to be low-cost, feasible and green technology using metal-accumulating plants to remove toxic metals, pesticides, and other hazardous materials from soil and water (Cluis, 2004).

Mine tailings are the by-products of mine activities. The mine tailings structure was not appropriate for plants to be raised. Low nutrient levels, high bulk density and extreme pH values restrict growth of the plants and body masses which have the significant effect on phytoextraction. Therefore, in phytoextraction process some supportive amendments are essential to remediate mine tailings efficiently with the phytoextraction methods.

In phytoremediation process, different soil microorganisms help to remediate contaminated soil efficiently. Arbuscular Mycorrhizal Fungi (AMF) are crucial soil organisms that enable host plants to stimulate symbiotic relationship in combined soil and root area (rhizosphere) (Cabral et al., 2015). After having association with the host plant, AMF form mycelium that is also called hyphae. These hyphae support the host plant nutrient (predominantly phosphate), water and heavy metal (Parniske, 2008). AMF also require plants to endure drought conditions and their special product glomalin increase the soil aggregation capacity and carbon content in the soil. These mechanisms influence the stability and the soil carbon systems (Singh, 2012).

In recent years, because of the increasing population and industrialization, wastewater quantities and sludge productions are getting higher. The enhancement in wastewater treatment technologies is improving the quality of effluent; however, sewage sludge problems arise. Therefore, sewage sludge disposal becomes an important problem in cities. Use of sludge in heavy metal phytoremediation might be an alternative disposal method (Rulkens, 2007).

The sewage sludge is used for soil amendment as it has the organic matter, N, P and other plant nutrients which increase the plant growth and yield (Hernández et al., 1991). The wastewater (sewage) sludge containing phosphorous and nitrogen can be reused for soil amendment purposes after a proper treatment, and it is the better alternative way to incineration and disposal to the landfill since it is cost-effective and has benefits for plants (Kidd and Monterroso, 2007). They increase the water holding capacity of the soil and supply nutrients. Therefore, in remediation of the mine tailings, 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.

In literature, there are limited studies dealing with the effects of sludge application on the phytoremediation of mine tailings 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 tailings. In the study, the chrome tailings having very high heavy metal (Cr, Al and Fe) content were ameliorated by phytoremediation enhanced by different species of AMF (*Glomus mosseae* or *Glomus intraradices*) associations and three different doses of sewage sludge application.

The objectives of the study are as follows:

- to determine the effect of arbuscular mycorrhiza in the remediation of heavy metals from mine tailings
- to evaluate the role of sewage sludge application in AMF and host plant interrelation and plant heavy metal uptake
- to investigate the effect of sewage sludge and AMF on the growth of the host plant.

## 2. LITERATURE REVIEW

### 2.1. Pollution of Heavy Metals

Heavy metals are the natural parts of the earth's crust. The geo-chemical cycles and biochemical balance of the heavy metals have been altered drastically by human activities. Therefore, large areas of soil are polluted with highly concentration of metals such as heavy metals which are mainly the end product of the municipal and modern agricultural systems, industrial processing, automobiles and mining activities (Ismail et al., 2013), and they tend to become severe threat to living organisms' health for their long-term existence in the surrounding areas (Subhashini and Swamy, 2013).

The basic characteristics of the heavy metals are an atomic density and number which are higher than  $5\text{g cm}^{-3}$  and  $20\text{ cm}^{-3}$  respectively (Alloway and Ayres, 1997; Jadia and Fulekar, 2008). The metals that are highly prevalent are Cd, Cr, Cu, Hg, Pb, and Zn. (Zovko and Romic, 2011).

There are two basic sources of heavy metals, one of them well-known as a natural source. The Natural source appeared by lithogenic process of weathering of parent materials contains heavy metals inherently. These are usually not toxic and considered trace. Other source nominated as anthropogenic sources: heavy metal contamination results from human activities, such as mining, smelting, electroplating, energy and fuel production, power transmission, intensive agriculture, sludge dumping, and melting operations. The mobility of the heavy metals which comes from the anthropogenic sources in the soil can be more mobile and bioavailable than the natural sources of heavy metals from anthropogenic sources. The mining activities and processing metals are the primary sources of surrounding environment heavy metal contamination (Laghlimi et al., 2015).

### 2.2. Soil Remediation

There are several soil remediation processes. Among them heavy metal remediation can be done through physical, chemical and biological processes. These are grouped into two categories in-situ and ex-situ method (Baker and Walker, 1990).

Ex-situ method is required taking contaminated soil and remediating them at another place, and then taking them again to the resorted area. The conventional ex-situ methods applied for

remediating the polluted soils relies on excavation, detoxification and/or destruction of contaminant physically or chemically, as a result, the contaminant undergoes immobilization, solidification, incineration or destruction (Gosh and Singh, 2005).

Heavy metal remediation techniques are both expensive and technically complex. Commercially, there are three kinds of remediation techniques for removing heavy metals. These are physical, chemical and biological remediation (Yao et al., 2012).

### **2.2.1. Physical Soil Remediation**

In physical remediation, two techniques are used in soil remediation. These are replacement method and thermal desorption. In the replacement method, the contaminated soil separated from the contaminated area and clean soil is added to the contaminated soil. In other words, the contaminated soil is replaced by the cleaned soil (Li and Zhang, 2010). Another replacement technique is taking the contaminated parts of the soil and burying them in the soil. This method enables the soil's natural remediation. Furthermore, contaminated soil is covered by non-contaminated soil so as to dilute the contamination. However this remediation technique can be used feasibly only for small areas and costs much (Nicholson et al., 2003). The thermal desorption methods have shown an ability only to remove volatile heavy metals. These are mercury, cadmium, arsenic and zinc. According to this procedure, the contaminated soil is heated by steam, microwave and infrared radiation. The heavy metals are collected with vacuum negative pressure or carrier gas to remove the volatile metals. This method has limited applicability in remediation process since it is expensive and inconvenient (Yao et al., 2012; Sidhu, 2016).

### **2.2.2. Chemical Soil Remediation**

Chemical leaching, chemical fixation, electrokinetic remediation and vitrification are the main chemical remediation methods (Yao et. al, 2012). In the chemical leaching, the contaminated soil is washed with water, reagents, fluids and gases that allow the heavy metals to become a substance of leachate coming from the soil. The heavy metals extracted by this method are rescued from the leachate by chelating agent, surfactant, and inorganic solvent, etc. (Tampouris et al., 2001). Chemical fixation involves decreasing the heavy metals mobility and converting them into insoluble form by adding some reagents (HF, HCl, HNO<sub>3</sub>, EDTA, etc.) However, adding chelating reagents can change the soil structure and it may have detrimental effect on soil microbes particularly soil beneficial microorganisms (Zhou et al., 2004). High voltage is used for heavy metal

removing in the electrokinetic remediation. It is suitable for low permeability soil and not demolishes the natural environment (Luo et al., 2004). Furthermore, it is low cost and easy to manage the system. In vitrification, the soil is heated to very high temperature (1400-2000°C) so as to volatilize or decompose (Sidhu, 2016).

### **2.2.3. Biological Soil Remediation**

Biological soil remediation is a green method which brings many multi-disciplinary aspects such as soil, plant and biochemistry expertise. This method includes phytoremediation, bioremediation and combining phytoremediation and bioremediation. In biological remediation, soil microorganism cannot degrade heavy metal content in the soil, but change the physical and chemical composition of the soil. Phytoremediation contains phytostabilization, phytovolatilization and phytoextraction.

## **2.3. Phytoremediation**

Phytoremediation processes are the cost-effective, non-destructive and environmentally friendly in situ technology that remediate, treat, stabilize or control the polluted soil effectively by using exploitation of natural plant physiological processes (Schwitzguébel and Khalvati, 2011; Zhang et al., 2010). In other words; phytoremediation is a general name, and humans have used it since 1991 to define utilization of plants to minimize the volume, mobility, or toxicity of contaminants in soil, groundwater, or other contaminated media (USEPA, 2000). In the phytoremediation, plants were used to remediate the surrounding areas. Anthropogenic pollutions comprising metals, pesticides, explosives, and oil were cleaned up by the plants which can also obstruct dissipation of pollutants from sites to other sites with wind, rain, and groundwater (Etim, 2012). In addition to these, it has aesthetic advantages and long term applicability.

The plant can break down or degrade organic pollutants or absorb and fix the heavy metals by acting as a strainers and catchers (Ecobiol, 2006). Phytoremediation includes extraction, degradation or fixation of the contaminants (Schwitzguébel and Khalvati, 2011). The wide range of plant species have been investigated and tested for their properties in the intake and accumulation of distinct heavy metals to determine the mechanism of metal trapping through the plant and cellular levels (Lone et al., 2008) Phytoremediation processes have an approach to facilitate a clean profit from the symbiotic relationship between host plants, microorganism, water and soil. It naturally exists in the terrestrial globe over millions of years in the earth ecosystem. In nature, the pollutant

can be detoxified and converted into neutral compounds due to plants uptake and growth system. In this procedure pollution can be safely removed from the soil through extraction and enzymatic processes. Plant-microbe interactions systems enable to recycle or sequester the organic compounds they encounter (Ecobiol, 2006).

The plants treat, as host plants, microbial life, providing them an optimal physical and chemical area. The host plants' roots and shoots give opportunities to microorganism to have wider surface structures to increase colonization and microbial activity. Furthermore; tropic climates is appropriate for the phytoremediation because hot climates enhance the plant growth and excite the microbial activity (Zhang et al., 2010).

### **2.3.1. Mechanism of Phytoremediation**

The mechanisms and potency of phytoremediation rely upon the kind of pollutant, bioavailability and soil properties (Etim, 2012). There are several phytotreatment mechanisms to decontaminate and remediate the polluted soil. The uptake of the contaminants occurs through the root system preventing the toxicity of the pollutant. The plant roots supply huge area to absorb and accumulate essential nutrients and water in company with non-essential contaminants. The plant roots induce changes in the rhizosphere with releasing organic and inorganic exudates. There is a strong relationship between the root exudates and microbial activity and microbial density. Furthermore, the root exudates influence the accumulation and stabilization of the soil particles around the roots and the availability of the contaminants through the changes in soil characteristics. The root exudates change chemical composition by releasing organic substances and increase in plant assisted microbial activity.

Phytoremediation consists of seven main topics. These are phytoextraction (phytoaccumulation), phytostabilization, phytodegradation, phytovolatilization, phytofiltration, rhizodegradation rhizofiltration. Different phytoremediation mechanisms are applied according to properties of contaminants and chemical nature of the soil. For example; phytodegradation, phytovolatilization and rhizodegradation are required to remediate organic pollutants. Phytoextraction, phytostabilization, rhizofiltration, and phytovolatilization are implemented for inorganic components. The schematic representation of phytoremediation strategies are shown in Figure 2.2 (Favas et al., 2014).

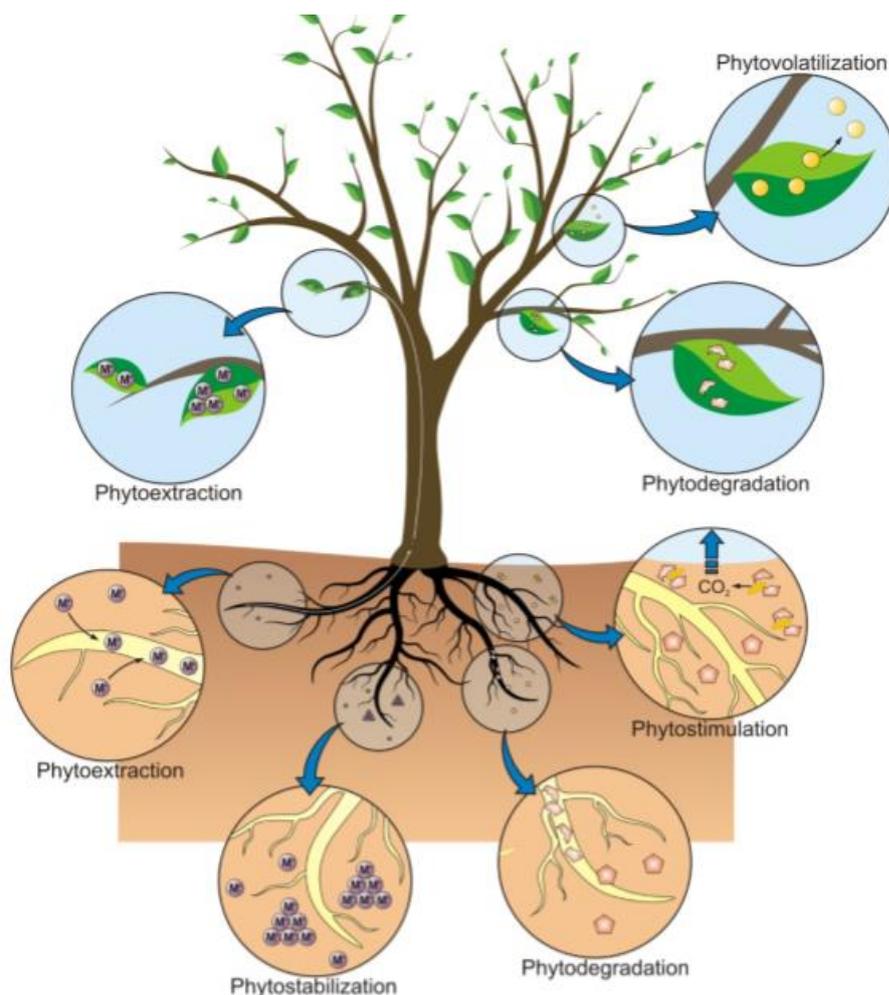


Figure 2.1. Schematic illustration of phytoremediation approaches (Favas et al., 2014).

**Phytostabilization** also called phytoimmobilization intends to immobilize metals and other inorganic contaminants in the soil, sediment, and sludge. Uptake and detoxifying, precipitation, combination system, or metal accumulation reduction result in phytostabilization. This process decreases contaminants mobilization and bio-availability of metal into the food chain and enhances the adsorption to the lignin of the cell wall of roots cells or soil particles, accumulation by roots and the precipitation on to the plant root. Beside these, the suitable soil amendments are used for phytostabilization process. The phytostabilization prevents erosion, runoff and leaching since it enhances the immobilization (Ecobiol, 2006; Schwitzguébel, Comino and Khalvati, 2011; USEPA, 2000). Lead (Pb) as well as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu) and zinc (Zn) are treated efficiently with phytostabilization (EPA, 2001).

This phytoremediation method is useful when the rapid immobilization is needed to protect ground and surface waters. Furthermore, phytostimulation isn't required to hazardous waste to be disposed of (Zhang et al., 2009). Nevertheless, the contaminants are not removed from the soil

environment entirely. This remediation technology is sometimes required extensive fertilization or soil amendments and the contaminants have to be monitored.

**Rhizodegradation** is also called phytostimulation. Rhizodegradation is based on the degradation of contaminants within the rhizosphere. Rhizosphere is the soil area that is directly affected by a plant's root system, related root secretions, and microorganisms. Microorganisms grown in the rhizosphere are responsible in the rhizodegradation (USEPA, 2000). The plants secrete sugars, amino acids, enzymes, and other compounds that may stimulate the growth of microorganism. Furthermore, the plants of the roots offer further surface area for microbes to grow on and a pathway for gas transfer from the surroundings. For this reason, microorganisms can be very common in the rhizosphere.

Rhizodegradation has been searched for treating various contaminated soil. It is useful for treating a wide variety of mostly organic chemicals, including petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, pesticides, polychlorinated biphenyls (PCBs), benzene, toluene, ethyl benzene, and xylene (Etim, 2012). In addition to that, due to the stimulation of microbial and fungal degradation by release of exudates/enzymes into the root zone, this mechanism is thought to be plant-assisted bioremediation (Zuang et al., 2005).

**Phytodegradation** (Phytotransformation) is based on degradation of the organic contaminants inside the plant cells with the presence of specific enzymes. The complex contaminants are transformed into simpler molecules used inside the plant tissues (Tangahu et al., 2011). Phytodegradation mechanism is similar with phytoextraction and phytovolatilization because it works if the contaminants' solubility and hydrophobicity fall into a certain acceptable range and they are taken up by plants. Phytodegradation has been observed to clean up organic contaminants, such as chlorinated solvents, herbicides, and munitions from the soil, sediment, or groundwater (EPA, 2001).

**Phytovolatilization** is based on the plant ability to absorb pollutant from the soil and convert them into volatilised form, and then to release them into the atmosphere. In other words, inorganic or organic contaminants can be transformed into volatilised forms by the plant and transpired them from the plant leaves. Phytovolatilization is generally used to decontaminate mercuric ion to convert into less toxic forms (Gosh and Singh, 2005).

Phytovolatilization is able to clean up contaminants' presence in soil, sediment, or water. The common element used to remediate the contaminant is mercury. Trichloroethene which is volatile organic and inorganic compounds are also cleaned up through the phytovolatilization (EPA, 2001).

In this phytoremediation mechanism transforms toxic substances into the less toxic contaminant (i.e., mercuric ion transforms into elemental Hg) and release them into the atmosphere. However, these compounds can be recycled by precipitation from the atmosphere and cause contamination in lakes and oceans, repeating the production of methylmercury by anaerobic bacteria (USEPA, 2000).

**Phytofiltration** (Rhizofiltration) technique relies on absorbing, accumulating and precipitating contaminants from aqueous medium through the plant roots. Phytofiltration is used for heavy metal and radioactive element contamination. This technique is similar to phytoextraction but it is used for hydroponic systems rather than soil because the effluents are filtered by the roots or other organs that absorb and concentrate contaminants (EPA 2001). Terrestrial plants are used in the rhizofiltration rather than aquatic plants having a special feature such as long term developing facility with having huge rhizosphere including vast amount of root hairs with large surface area. In order to encourage plants to form a large root system; first plants are watered with fresh water, later contaminated water is collected from a waste site and brought to the plants where it is substituted for their water source. After the roots take up the contaminated water and become satiated with contaminants, they are harvested (EPA, 2001). Figure 2.3 shows schematic representation of an aerated rhizofiltration system (Akpore and Muchie, 2010).

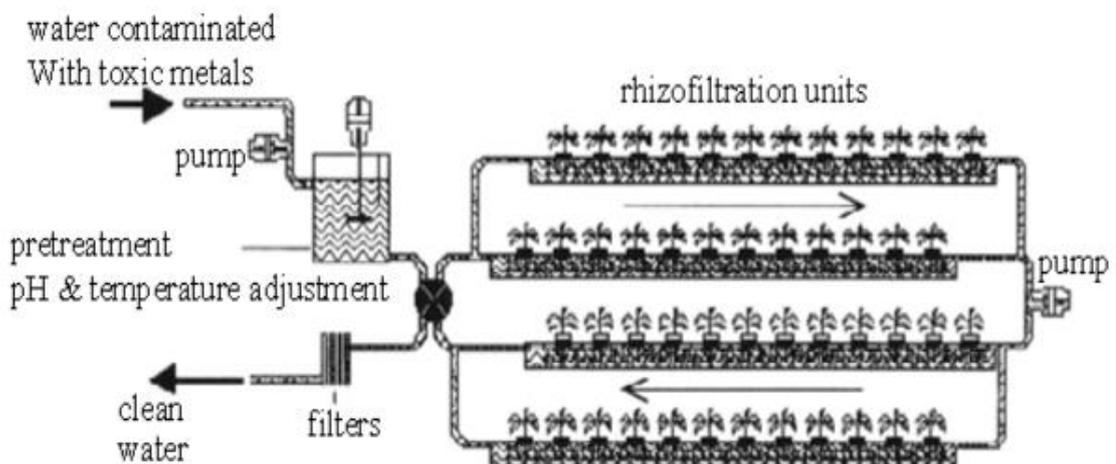


Figure 2.2. Engineered rhizofiltration system (Akpore and Muchie, 2010).

**Phytoextraction** (Pytoaccumulation) is the crucial phytoremediation mechanism. The soil contaminant is absorbed by the plant root. The roots translocate the contaminant into aboveground portions of the plant. The phytoextraction is mostly used for metal uptake from the contaminated soil. Many plant species have been investigated comprehensively to determine the hyperaccumulator plants having several mechanisms called extraction, exclusion and compartmentation (Favas et al., 2014). Metals like nickel, zinc and copper are the simplest candidate for removal by phytoextraction as a result of it has been demonstrated that they are preferred by a generality of plants (roughly 400) that uptake and absorb unusually large amounts of metals (Etim, 2012). The schematic representation of phytoextraction of metals from the soil is shown in Figure 2.4 (Favas et al., 2014).

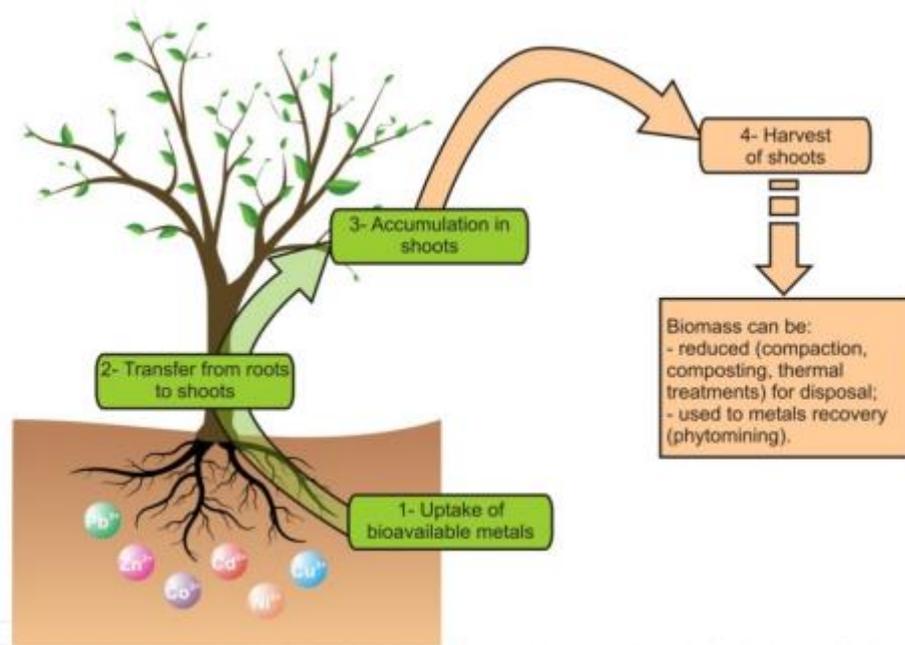


Figure 2.3. Schematic representation of phytoextraction of metals from soil (Favas et al., 2014).

Phytoextraction has several benefits: It is quite cheaper than other conventional methods. Another advantage is that the toxic pollutant is permanently extracted from the soil. Moreover, the amount of waste material that must be disposed of is widely minimized and in some cases, the pollutant can be retrieved from plant biomass (USEPA, 2000). However, several factors that limit metal phytoextraction process are as follows (Etim, 2012):

- The availability of the heavy metals by living organisms within the rhizosphere.
- Metal capacities of the roots

- Amount of “immobile” metal in roots
- Proportion of xylem loading/translocation to shoots
- Cellular tolerance to heavy metals

Phytoextraction mechanisms have several items to remediate the contaminated area feasibly (Brennan and Shelley, 1999):

- The plants must take heavy metals into their roots in large concentrations.
- The heavy metals must be translocated into the surface biomass by plants.
- A large amount of plant biomass must be produced by remediated plants.
- The remediated plants must have mechanisms to allow the detoxification and / or removal of high metal concentrations extracted in the above parts.

### **2.3.2. Hyperaccumulator Plants**

Phytoextraction needs host plants to uptake heavy metals. Hyperaccumulator plants are genetically different in terms of metal accumulation and metal tolerance. Hyperaccumulator plants used for their certain characteristic that have a high rate of growth and high production of above- and below-ground organs such as stems, leaves, and roots. Also, these plants can accomplish efficient translocation of heavy metals, endure to high concentrations of heavy metals biotic and abiotic stresses and be easily cultivated and harvested (Ali et al., 2013).

Hyperaccumulators can absorb heavy metals from soils through roots and translocate higher amounts to shoots and leaves. Contrastingly, metal uptake is decreased by the in ordinary plant species roots and the privileged metals are stored in the root vacuoles and constrained translocation into shoots (Poschenrieder and Coll, 2003). In other words, non-hyperaccumulators plants can take up heavy metals in their below-ground parts and cannot transmit them to shoots and leaves. Some non-hyperaccumulators can accumulate in the shoots and leaves in a limited amounts. Figure 2.5 shows the difference between hyperaccumulators and non-hyperaccumulators (Ghori et al., 2016).

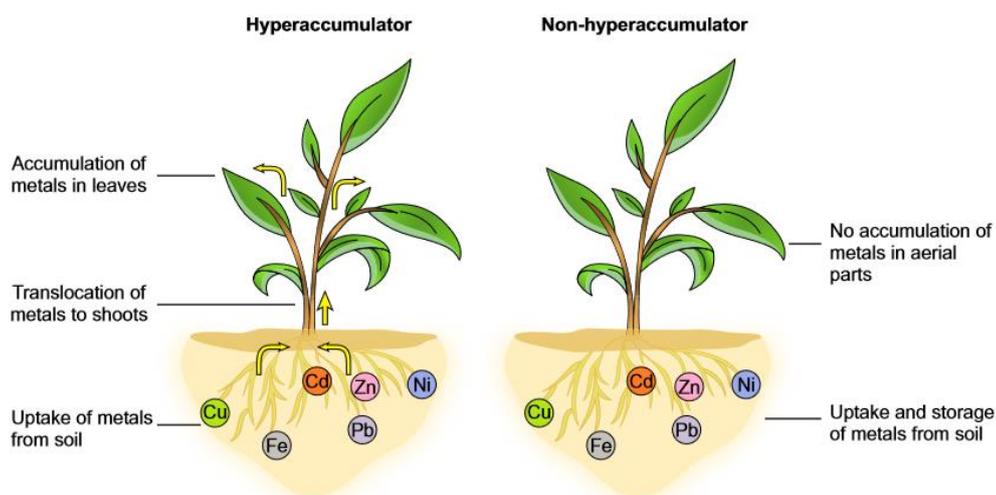


Figure 2.4. Schematic image of hyperaccumulators that is diverse from non-hyperaccumulators (Ghori et. al., 2016).

The hyperaccumulators can survive in the soil having abundance of heavy metals and accumulate huge amount of metals in comparison with other plants. After the growth period of the hyperaccumulator plant, the plant body and along with the contaminants are taken from the soil. The incineration is used to transform the plant body to ash in order to recover the heavy metals (Ecobiol, 2006; Schwitzguébel and Khalvati, 2011; Lone et. al., 2008). Production of biodiesel is also used after the hyperaccumulator plants harvest. Especially, oil-producing plant species are used to generate more biodiesel (Van Ginneken et. al., 2007).

Heavy metal concentrations of root and shoots ratio shows the translocation factors (TF). The hyperaccumulator plants have greater than one translocation factor. In other words, it is the capability of plants to uptake heavy metals. The ratio between the plant and soil heavy metal concentration is called bioconcentration factor (BCF). This is another factor to examine the plant accumulation potential (Ahmadpour et al., 2014). In the phytoremediation, plant biomass is crucial parameters because harvestable parts of the plants remove heavy metals from soils with the plant above-ground parts. However, the most of hyperaccumulator plants have low biomass production and heavy metals cannot be extracted from them.

In terms of the plants accumulation capacity, hyperaccumulators are divided into natural hyperaccumulators and transgenic hyperaccumulators. Natural hyperaccumulators have natural tendency to take up heavy metals in their shoots but its biomass decreases as they accumulate high concentration of toxic metals. Contrastingly, biotechnological techniques have been used to modify

transgenic hyperaccumulators to increase the metal accumulation ability of plants. Furthermore, its biomass can be higher than natural hyperaccumulators for greater metal absorption (Ghori et. al., 2016; Macek et. al., 2008). The scientists have observed that sunflower (*Helianthus annuus*), Indian mustard, tobacco, rye, spinach, and corn have shown significant capability to eradicate lead from water, mainly sunflower indicated with better ability (Etim, 2012). Sunflowers (*Helianthus annuus*) were able to remediate other toxic components. For example, radioactive pollution was cleaned up effectively from pond water by sunflowers (*Helianthus annuus*) in an assessment (EPA, 2001).

#### 2.4. Mycorrhizal Associations

Through the fossil records, the heritage of the earth confirms that arbuscular mycorrhizal interactions occurred 400 to 450 million years ago and they have crucial importance in the symbiosis with the host plants. Mycorrhizal interactions are formed by plant species which are known around 80% of them and arbuscular mycorrhiza (Smith and Read, 2008; Wang and Qiu, 2006).

Arbuscular mycorrhizal fungi are important species for the sustainable agriculture because they preform building a form of symbiotic relationship with agriculturally important food and bioenergy crops and treat them like biofertilizers and bioprotectors. There is another mycorrhiza species called ectomycorrhiza. They colonize with tree and shrub species and they have the key role in forest ecosystems (Finlay, 2008). Figure 2.6 shows the structural characteristics of arbuscular mycorrhizal (AM) or ectomycorrhizal (ECM) roots of gymnosperms or angiosperms.

Arbuscular mycorrhizal fungi (endomycorrhiza) and ectomycorrhizal associations act different structural feature in terms of their connection. In ectomycorrhizal roots, fungus penetrates intracellularly into the root cortex but in arbuscular mycorrhizal roots, it also penetrates both intracellularly and intracellularly into the root cortex. Figure 2.6 shows the fundamental structural differences between AM and ECM associations of angiosperms or gymnosperms (Bücking et. al., 2012). Meanwhile, research and recent finding emphasize that arbuscular mycorrhiza is the most important species of mycorrhizal association.

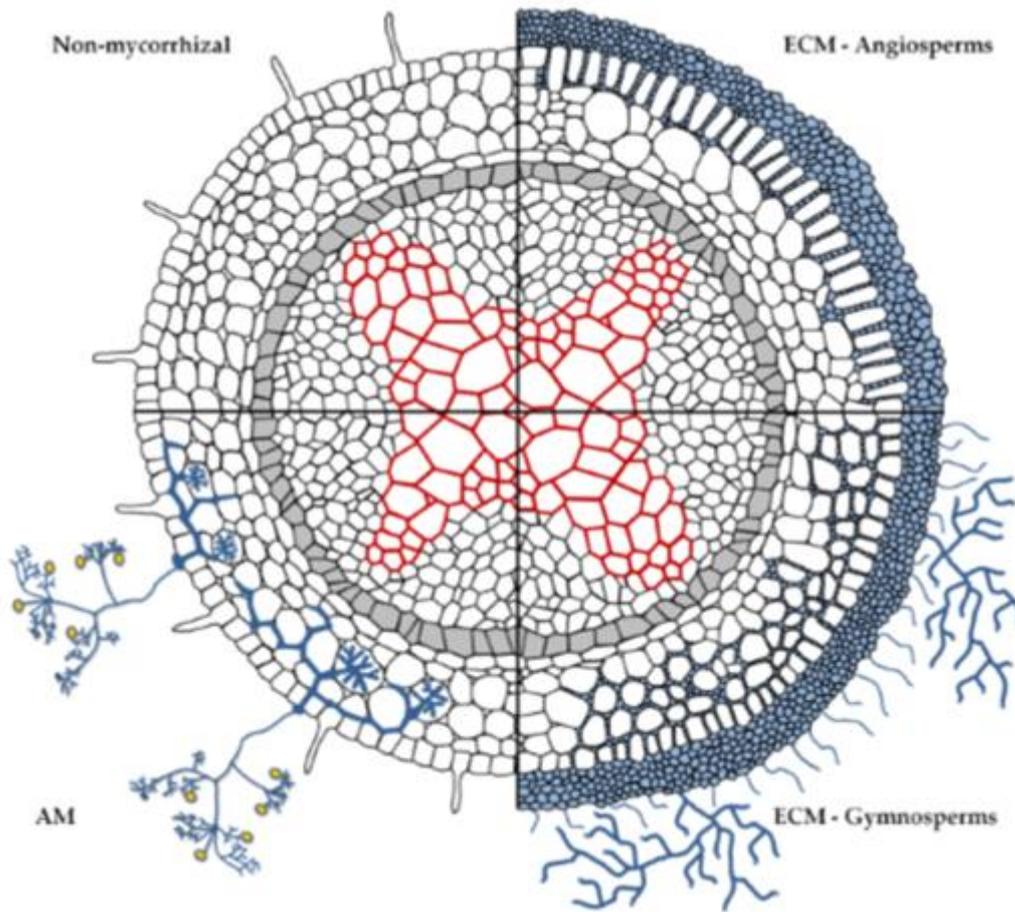


Figure 2.5. Structural features of ECM roots of arbuscular mycorrhizal (AM) or gymnosperms or angiosperms (Bücking et al., 2012).

#### 2.4.1. Arbuscular Mycorrhiza

Arbuscular Mycorrhizal Fungi (AMF) have the crucial role in shaping the planet by assisting primitive plant to colonize the soil (Parniske, 2005). Huge variety of plant species cannot complete their life cycle without mycorrhizal association; these plants are called ‘obligatory mycorrhizal’ plants and others are facultatively mycorrhizal (Smith et. al.,2003) AMF set 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) and water uptake (Khalvati et al., 2005). Arbuscular mycorrhizal fungi enhance the soil quality with the three main mechanisms: plant physiology, soil ecological interactions, and soil engineering (Rillig, 2004).

Arbuscular Mycorrhizal Fungi (AMF) spores can germinate without the presence of a host plant; whereas, autotrophic host plant is required to complete the life cycle of mycorrhiza and to

produce the next generation of spores. The hyphal branching and metabolic activity of the AMF rely on plant root exudates. Therefore, AMF are called asexual obligate biotrophs which means that fungi need a living host cell to feed the fungi without killing it (Bücking et. al., 2008; Tamasloukht, 2003). The host plants roots release secretion called strigolactones (branching factors) that stimulate extensive hyphal branching in AM fungi (Akiyama and Hayashi, 2006).

Arbuscular mycorrhizal fungi life cycle begins with propagates of the spores starting to grow as in the Figure 2.7.A The individual mycorrhizal growth is gone on for one or two weeks. Nevertheless, if mycorrhizal symbiosis doesn't actualize, the AMF inhibit their growth before the carbon storage is exhausted and re-enter the state of dormancy. Therefore, AMF gain several chances to regerminate and set up symbiosis with proper host plants. Figure 2.7.B shows that the AMF generate symbiosis with a host plant. A specific appressorium- the hyphopodium is observed on the root surface of these plants. Absorption of nutrients can be facilitated with a special hyphal branch composed using lobed cells by Hyphopodium is shown in Figure 2.8. Hyphopodium form fungal hyphae penetrating into the root. Apoplast is the entrance of the plants and the diffusional space between the plasma membrane and the cell wall in plant cells. AMF send their hyphae through the apoplast and penetrate into inner root cortical cells. Therefore, AMF form intraradical hyphae (Fig 2.7.C), arbuscules and lipid-rich vesicles.

AMF have storage organ accumulating phosphorous called vesicles. This organ might retain the fungi significant properties to deliver to the colonized plant if lack of phosphorous concentration occurs (Olsson et. al., 2011). Arbuscules invaginate the host plants cell membranes and set up extraradical mycelium (ERM) (Fig 2.7.D). These characteristic branch structures (BAS) increase the contact area to absorb nutrient, water from the soil and deliver them to the host roots. Furthermore, intraradical mycelium (IRM) releases nutrients into the interfacial apoplast and exchanges them against carbon from the host. Carbon storage is crucial for fungi to form and develop the ERM. By this way, the external AMF spores are generated on the BAS to fulfil their life cycle. In a very large numbers of AMF spores are developed (Fig 2.7.E) during the AM fungal sporulation phase. (Bago et al., 2000; Bücking et al., 2012).

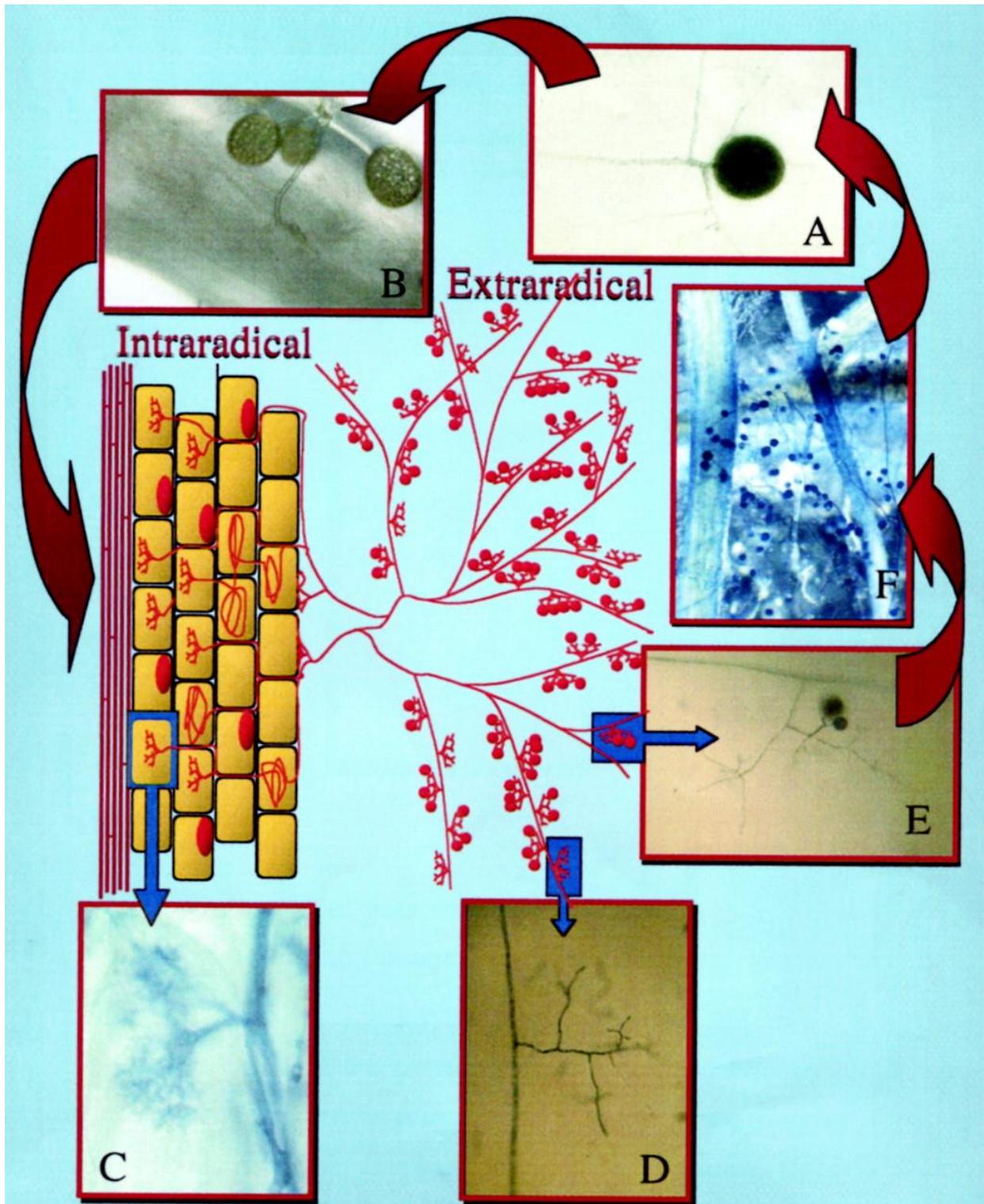


Figure 2.6. Schematic description of morphological properties of arbuscular mycorrhiza fungal metabolism, A, Soil-derived spores have a limited "saprophytic" growth and cannot complete their life cycle if they cannot find a host root to colonize. B, After successful colonization, the AM fungus differentiates intraradically establishing improved structures, the most descriptive being arbuscules (C). At the same time the fungus profusely develops an extraradical mycelium with dissimilar features structures: BAS (D), BAS-spores (E), and sleeping spores (E and F), hereafter closing its life cycle (Bago et al., 2000).

Mycorrhizal fungi generally establish symbiotic relationship which is classical mutualism. However, mycorrhiza sometimes can have negative effect on host plant (Johnson et. al., 1997).

## **2.5. Glomalin Related Soil Protein (GRSP)**

Soil structure is determined by the particle size and their arrangements with the pores (Hartge and Stewart, 1995.) Soil structures are important substance for the soil living organisms; therefore, soil structure influence soil-borne aspects of biogeochemical cycling processes and soil structures determine the soil erosion resistance (Paul and Clark, 1989).

Soil aggregation is an important soil process that governs soil structure to tolerate diffusion which is an essential factor in soil ecosystem and its effect. This brings an excellent possibility to capture and save water and nutrients in the soil for later releasing and use for plants (Drinkwater and Snapp, 2007). The soil aggregation is a complex process, because huge varieties of organisms and glues have responsibility like abiotic factors (such as wetting-drying and freeze-thaw cycles). Nevertheless, AMF are considered to have crucial importance in this process since AMF are ubiquitous and abundant soil organisms (Rillig, 2004). Furthermore, photosynthetic plant carbons are transformed into the AMF directly; therefore, plant and AMF don't need to compete for soil organic matter carbon. The hyphal growth of AMF has also contribution to stabilizing structures and AMF are considered to have important in longer-term aggregate stabilization because of the formation of the hyphae and their products (Miller et al., 2000).

Glomalin, which is a universal substance, is defined as glycoprotein generated by an actively growing AMF mycelium in the soil. This proteinaceous compound was extracted from the soil, and it constitutes the biochemical, physiochemical and biological mechanism of AMF amended soil (Purin and Rillig, 2007). The soil parameter and water aggregate stability are influenced by GRSP that contributes to soil organic matter (Gadkar and Rillig, 2006). Glomalin was first determined by United States Department of Agriculture during producing monoclonal antibodies reactive with AMF. The hyphae of AMF activate these antibodies (Nichols, 2003).

There is not much information available whether AMF hyphae can be breakdown of glomalin in the soil yet. This is due to obligatory feature of AMF where fungal mycelium and spores in soil providing glomalin through establishing plant carbon sink in the symbioses relationships (Steinberg and Rillig, 2003). There are two studies which explain the glomalin resources. Wrigth and co-workers (1996) observed that glomalin is released from the AMF into the soil where it enhances the

soil aggregation. However, Driver and co-workers (2005) were found that the glomalin protein correlated with AMF hyphae and spores. So that, glomalin comes through the releasing mainly from hyphae in the soil, not through the secretion. GRSP to soil aggregation remains correlative, and the mechanisms involved are still unclear. Rillig and Eviner (2002) advocated that creation of glomalin by AMF hyphae which is secondary effects of hyphae is tougher than the first effect of hyphae.

Glomalin is hypothesized to act as 'glue' with hydrophobic properties; however there is a lack of information which still appears in front of scientists regarding this feature of glomalin (Rillig and Mummey, 2006). Beside all of these opportunities, huge amounts of toxic heavy metals have been sequestered by glomalin related soil protein (GRSP). For example; each gram of the protein contains up to 1.88 mg of manganese, 0.08 mg of cadmium and 1.12 mg of lead. Therefore, GRSP has been recommended as a molecular mechanism for bioremediation of soils and sediments polluted with potential toxic metals (Chern et al., 2007).

Hammer and Rillig (2011) observed that *Glomus intraradices* mycelium increases the glomalin protein concentrations. Singh (2012) observed that propagation of GRSP in lands might be an actual significant mechanism for aggregate and degrade soil pollution caused by mining sectors. This can be a fact to utilize this technology in order for stabilize heavy metals and sustain environmental pollution.

## **2.6. Phytoremediation of Mine Tailings**

Mining activities have continued for decades because humans use the elements which are the output of the mining as foundations distributing simple raw material for economic movement. Therefore; uncontrolled mines and discarded of mines have occupied all around the world (Allan, 1995; Jordan and Abdaal, 2013).

Mine tailings are defined as by-products of mine activities. Mine tailings comprising with the mixtures of sands, fine grained solid material, water and significant quantities of heavy metal are generally collected in outdoor ponds without any refinement (Wang et. al., 2017). Mine tailings which contain noxious heavy metals have detrimental and vigorous effects on the environment health (Allan, 1995). Although excavating activities are done in the local areas; their influences can reach regions and earth (Jordan and Abdaal, 2013).

Natural hard rocks splintered in mine activities contain minerals that lead up to all-encompassing soil damage and changing the community of microorganisms. Moreover, mine spoil have an impact on plantation bringing about devastation of immense quantities of terrestrial soil (Sheoran, 2010). The mines spoils (mine tailings) consist of generally silt or sand sized particles and have nutrient levels that restrict biological growth (N, P, K), and include nearly not any organic matter (Mendez and Maier, 2008).

Because of the poor soil structure, high concentration of heavy metals and lack of nutrients of mine spoils, plant root growth is suppressed. Leaf chlorosis and lower biomass production are observed when heavy metals cause the restriction in root growth (Jadia and Fulekar, 2009). For example, the high concentrations of heavy metal soil are attributed to the increase in metal uptake by plant. This cause plant growth deficiency and this sometimes invites plant mortality (Schmidt, 2003).

In order to remediate mine tailings efficiently, the plants should be grown well and extraction of the host plant should be effective. Therefore, soil structure has to be improving in order to enhance its productivity and biological quality for plant biomass. This directly affects phytoremediation efficiency and performance. Several natural fertilizers are used as a soil amending material such as saw dust, wood residues, sewage sludge, animal manures, as these amendments stimulate the microbial activity which provides the nutrients (N, P) and organic carbon to the soil (Sheoran, 2010).

### **2.6.1. Cr Toxicity of Plants and Its Uptakes**

The Cr uptakes of the plants vary among species due to absorption, transportation and storage of metals (Zayed et al., 1998; Chandra and Kulshreshtha, 2004). While most plants store the majority of chromium in their roots, translocation to all other parts of a plant does occur. It was found that some plants have ability to transport Cr from root to shoot. The hyperaccumulator plants have the ability to accumulate over 1000 mg/kg Cr in their above ground parts. Moreover these plants can accumulate more Cr in their leaves than in roots. (MiningWatch, 2013).

Lethal effects to plants exposed to excess Cr include: reduced growth, decreased chlorophyll production producing yellow leaves, narrow leaves, small root systems, decreased or complete inhibition of seed germination, delayed growth, decreased seed yield, wilting and death (Dube et al., 2003; Zayed and Terry, 2003). High concentrations of chromium harms root membranes and a

capacity of the plants to absorb water. Excess chromium changes acceptance and translocation of essential elements such as nitrogen, iron, potassium, magnesium, manganese, phosphorous, calcium, sulphur, copper and zinc (Zayed and Terry, 2003; Dube et al., 2003; Gardea-Terresdey et al., 2005). Human health and nature may also be influenced by the chromium detrimental effects of plants health through the change in plant nutrient balances. Making toxicity predictions are challenging because the sensitivity and effects change between species.

Moreover, microorganisms living in the rhizosphere of the plants create soil structure and cycling organic matter and elements in the environment. The low concentrations with the chromium forms (Cr (VI) (1-10 mg/kg) and Cr-(III) (25-100 mg/kg)) may damagingly influence the number, type, health and activity of soil organisms and so effect the health of the soil ecosystem (Viti et al., 2006). The high concentrations of chromium are related to diminished microbe records and enzyme activity (Huang, 2008).

## **2.7. Land Application of Sewage Sludge**

The soil stability has the crucial importance on preserving cultivation productivity and decreasing erosion of the soil. Unfortunately, the physicochemical characteristics of mine tailings are not appropriate for a natural plant growth due to dynamics such as extreme pH, high salinity, low water holding capacity, high level of heavy metals, and they lack organic matter and fertility in soil (Wang et al., 2017).

Beside all of these, increased population, urbanization and industrialization have caused dramatic increase in the volume of waste water production. Increasing in number of waste water treatment plants have resulted in increment in production of sewage sludge. Huge amounts of sludge need to be disposed of or treated. Sludge application on land is the one way to solve the disposal problems (Wang et al., 2008). After banning the disposal of sewage sludge at the sea, the sewage sludge has been used on agricultural and mine tailings (Gove et al., 2001; Asensio and Kandeler, 2013). The amendment sewage sludge ameliorates the physical, chemical and biological properties of mine tailings through the addition of organic matters and plant nutrients (phosphorus and nitrogen) (Gove et al., 2002). Sewage sludge amendment also increases the diversity and biomass of microorganism, and their activity (Asensio and Kandeler, 2013).

Del Val and Azcon-Aguilar (1999) assessed how the diversity of arbuscular mycorrhizal fungi is affected by sludge amendment containing heavy metals in a long-term experiment. They used

five different types of treatments, which are of  $100 \text{ m}^3 \text{ ha}^{-1} \text{ yr}^{-1}$  low and high metal sludge and  $300 \text{ m}^3 \text{ ha}^{-1} \text{ yr}^{-1}$  low and high metal sludge and  $180 \text{ kg of N ha}^{-1} \text{ yr}^{-1}$  inorganic fertilizer. The total number of AMF spores decreased by high metal content, but the diversity of the AMF species increased with the  $100 \text{ m}^3 \text{ ha}^{-1} \text{ yr}^{-1}$  low metal sludge applications. They mention that AMF population is negatively interrelated with P concentrations of the soil because more than  $50 \text{ mg/kg}$  P content decreases AMF colonization, and AMF species could deal with the heavy metal stress and conclude their life cycles.

Jamali and Kazi (2009) investigated heavy metal accumulation in different varieties of wheat grown in soil amended with sewage sludge. It was observed that total and bioavailable fraction of heavy metals in sewage sludge amended soil increased.

### **2.7.1. Sludge Application in Remediation of Mine Tailings**

In mine tailings remediation generally phytoextraction and phytostabilization was used as practical mechanisms. Roger and Baker (2000) observed that metal uptake was able to be actualized with selected high biomass agronomic crops in conjunction with soil amendments application to the contaminated soils. Andrés and Francisco (2008) suggest that sewage sludge can remove or/and mobilize heavy metals from the mine tailings areas. However, Lambert and Weidensaul (1991) 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.

National Science Foundation Water Quality Center supported a project about mine tailings restoration with sewage sludge amending without any leachate decreasing the groundwater quality. Arizona University had observed that this remediation method didn't release toxic metals or additional nitrogen into the land because sewage sludge defined as biosolids have an affinity to link complex heavy metals within the mine tailings, constructing them less bioavailable. Moreover, the biosolids applied to the mine tailings, had raised the microbial population in the treated area. Figure 2.8 shows the conversion after amendment of sewage sludge ( $150 \text{ dry tons per acre}$ ) in mine tailings. The plants were able to grow in the treated mine tailings on the contrary to untreated mine tailings (McGinley, 2003).

Thorne and Kennedy (1998), examined sewage sludge and mycorrhizal effects on scar blue bunch wheatgrass in mine tailings. In this research, the soil was ameliorated by (i) different quantities of the sewage sludge ( $0, 6.2, 12.4, 24.5, 49 \text{ g of composted sewage sludge}$ ) (ii) inoculation

AMF (native, non-native), and (iii) different quantities of the sewage sludge plus AMF. The research was mentioned that composted sewage sludge stimulated the growth of the plant, and native AMF inoculation with composted sewage sludge (CSS) application provided the host plant greater biomass, and the root biomass of the host plant with only native AMF association was less in comparison to the other AMF treatments.

Amir and co-workers (2019) investigated that efficiency of sewage sludge amendment (1.5 ton dry weight of sludge/ ha) and three species of AMF colonization combination in the nickel mining-degraded areas. Lack of soil nutrients, heavy metal toxicity and depressed growth rate of the native plant species inhibited the ecological restoration. An endemic plant species of Myrtacea and mixed AMF isolation were used for the experiment. After 528 days of the growth period, dry weight of the plant colonizing with AMF was 4 times higher than the non-inoculated plants. Combined treatment of AMF inoculation and sewage sludge application increased the dry weight of plant more than seven times compared to untreated control plants. Amir and co-workers (2019) observed that sewage sludge application can remediate mine tailings with the help of mycorrhizal associations. They showed that mycorrhizal association after 528 days had been stimulated by sewage sludge in both inoculated and non-inoculated plants. They also reported that mycorrhizal associations reduce the translocation of heavy metals, but that the displacement of Cr is not affected by AMF inoculation and sludge addition.

### 3. MATERIALS AND METHODS

#### 3.1. Sample Collection

Greenhouse pot experiment was used to determine the level of metal uptake of the plants. Half of sun flowers were planted with AMF inoculation (*Glomus mosseae*, *Glomus intraradices*) to determine the effect of Arbuscular Mycorrhizal Fungi (AMF) on phytoremediation and plant growth. After the completion of 3 month growth period, the plants were harvested, and the plant metal uptakes were quantified.

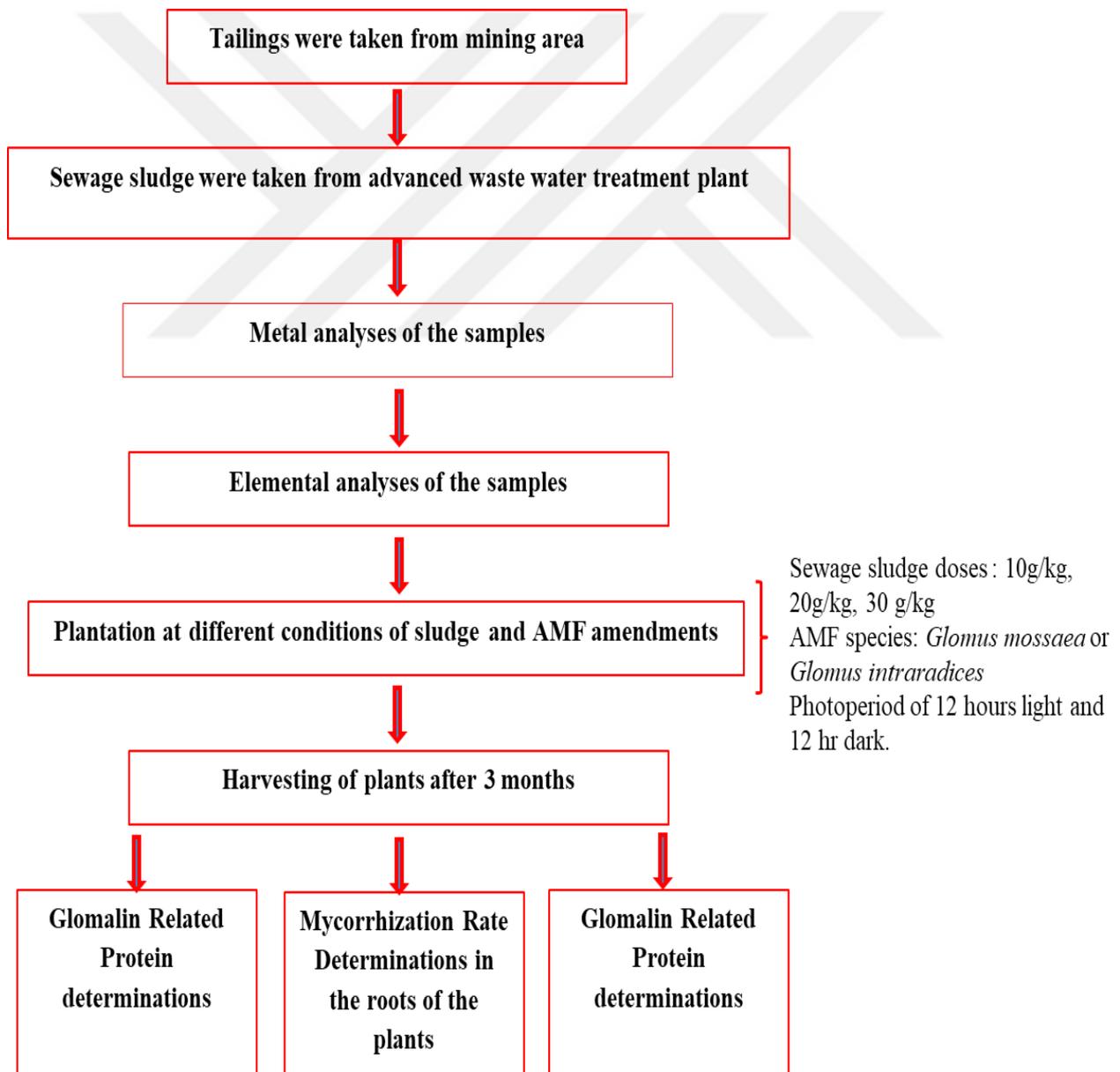


Figure 3.1. The flow chart of the experimental study.

### 3.1.1. Sampling of Mine Tailings

The experimental soil used in the research was taken from tailings of a Cr mine tailings in Kütahya, Turkey. The mine tailings pH ranged between 7.9 and 8.5. The bulk density of mine tailings was measured to be  $1.7 \text{ g/cm}^3$ .



Figure 3.2. Turkey Map. Kütahya is highlighted in red.

The samples of mine tailings were taken twice from Kütahya in 22.02.2018 and 16.06.2018. The first samples of mine tailings were shown in the Figure 3.2; it had very big stones and required buffer soil for plants to grow. The second samples of mine tailings were collected from different parts of the mining area and mixed before planting to simulate the mining area exactly as it is. Therefore, it was not required buffer soil.



Figure 3.3. The first sample of Cr mine tailings (photographed by F.Ece Sayın).



Figure 3.4. The second sample of Cr mine tailings (photographed by F.Ece Sayın).



Figure 3.5. The second sample of Cr mine tailings (photographed by F.Ece Sayın).

### 3.1.2. Sewage Sludge Sampling

The sewage sludge was taken from an advanced wastewater treatment plant in Istanbul, Turkey. The two kinds of experimental sludge (25% total solid and 98% total solid) were taken from the treatment plant. The sludge TKN and phosphate concentration were given in the Table 3.1.

Table 3.1. TKN and phosphate concentration in the sewage sludge sample.

Sewage	TKN	$\text{PO}_4^{-3}$	$\text{P}_2\text{O}_5$
Sludge (mg/kg)	21641.53	41779.075	31177.14

### 3.2. Germination Test

Sunflower (*Helianthus annuus*) seeds were supplied from commercial farming business located in Tekirdağ. Sunflower (*Helianthus annuus*) seeds washed with 70% ethanol and distilled water for one minute respectively for three times.

In order to monitor the seeds germination capacity, germination test were applied. Whatman No.4 filter papers were put inside 8 petri dishes. 4 of the petri dishes were filled with sewage sludge solution (0.15% w/v) until the full parts of the filter papers got wet. Four of the petri dishes were filled with sewage sludge solution (0.3% w/v) until the full parts of the filter papers got wet. 7 seeds were planted in each dish. Aluminum foil and black plastic were used to prevent seeds from sun light. Germination capacities of the seeds were monitored for duration of one week.

### 3.3. Elemental Analysis

20- 30 gr mine tailings, buffer soil and sewage sludge were dried in the oven at 103 °C. They were put into the desiccator for dry cooling. The dried samples were grinded with the blender to have homogenous powder samples.

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. The tin capsules were used for elemental analysis. After determination of the tare of the tin capsule, 1 mg sample was put into tin capsule and weighted with the analytical balance. The tin capsule was folded with the help of nippers and gotten the shape of a sphere. The samples were put into the Elemental Combustion System. It determined the percentage of carbon, nitrogen, hydrogen and sulphur elements.

### 3.4. TKN and P analysis

TKN concentrations 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.

For Total Kjeldahl Nitrogen (TKN) and orto-phosphate determination, the samples required digestion. Digesdhal Digestion Apparatus (Hach) was used for digestion. The blank was prepared with 25 ml deionized water and 0.25 g samples were weighted. The sample and 4 ml sulphuric acid was put into the digestion apparatus and heated at 440 °C. After the white fume was seen (it means that sample digestion started), the sample was heated for 4 minutes. Then, the sample was heated with 15 ml hydrogen peroxide for 1 minute and diluted with 100 ml deionized water (Company, 1992).

Nessler Method 8075 HACH was used for determining TKN. 1 ml of digested sample and digested blank were pipetted separate 25 mL mixing graduated cylinders and 8 N KOH dropwise added to each cylinder until the first blue color was seen. 1 N KOH was dropped to the each cylinder until the permanent blue color was reached. The graduated cylinders were filled to the 20 ml mark with deionized water. 3 drops of Mineral Stabilizer and Polyvinyl Alcohol Dispersing Agent were added into the sample respectively and inverted several times to mix. The graduated cylinders were filled to the 25 ml mark with deionized water put into 1 ml of Nessler's Reagent. After two minutes reaction times, first the blank was read then the sample.

PhosVer 3 Ascorbic Acid Method 8048 was used for determining the orto-phosphate. 1 ml of digested sample and 9 ml deionised water were pipetted to the cell. A content of one PhosVer 3 Phosphate Reagent Powder Pillow was added to the cell and a blue color develops with the phosphate existence. The sample was read at 492 PReact. PV AV. Program.

### **3.5. Greenhouse set up and pot experiment**

The greenhouse experimentation was set up with using Cr mine tailings which was taken from Kütahya, sewage sludge which was taken from the advanced treatment plant and buffer soil which had sufficient nutrients for plants to grow healthy.

In this study, sun flowers were used as 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 tailings. The rest of the pot series contained mine tailings amended with sludge and inoculated with AMF species. The greenhouse pot experiments were performed in two runs in different seasons.

### 3.5.1. First Run

10, 20 and 30 g (98% Total Solid) sewage sludge were weighted. 1.7 liters pots were used in the experiment. Roughly 1000 ml of pots filled with mine tailings and different quantity of sludge (10, 20, 30 g sludge) was added into the pot. Sunflower (*Helianthus annuus*) seeds were used in the experimentation. AMF species of *G. mosseae* (2.4 million spores) was inoculated before the seeds were planted. Six pots were filled with only sewage sludge and three pots were inoculated with *G. mosseae*. In order to ameliorate mine tailings structure, buffer soil was added. Heavy metal concentration in the buffer soil and the mine tailings were assessed to have background information about the soil, and to assess plant uptake accurately. Thirteen different treatments were applied to the experimental plots as follows:

3 parallels were set up. 39 pots were used in this experimentation. Irrigation was done three days in a week. First two weeks, 300 ml distilled water used for irrigation. Then, 150 ml distilled water was enough for the pots. After 2 months experiment, the plants grew and needed much water. In terms of weather condition, 200-250 ml of water was used. Nutrient was used in only two treatments. Three weeks after the plantation and 6<sup>th</sup> and 9<sup>th</sup> weeks, 50 ml nutrient solution and 50 ml distilled water were added to the pots. Contents of the pot sets were given in Table 3.2.

Table 3.2. Contents of first run of the greenhouse experiments.

No	Pots Sets	Mine Tailings (g)	Buffer Soil (g)	Sludge (g)	AMF Species	Nutrient Solution (ml)
1	M	1000	-	-	-	-
2	75%MMos	1000	333	-	<i>G. mosseae</i>	-
3	75%M.Nut	1000	333	-		100 ml
4	75%MNutMos	1000	333	-	<i>G. mosseae</i>	100 ml
5	75%MS10	1000	333	10	-	-
6	75%MS10Mos	1000	333	10	<i>G. mosseae</i>	-
7	75%MS20	1000	333	20	-	-
8	75%MS20Mos	1000	333	20	<i>G. mosseae</i>	-
9	75%MS30	1000	333	30	-	-
10	75%MS30Mos	1000	333	30	<i>G. mosseae</i>	-
11	25%M	333	1000	-	-	-
12	Sludge	-	-	1333	-	-
13	Sludge Mos	-	-	1333	<i>G. mosseae</i>	-

(75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution)

### 3.5.1. Second Run

In order to ameliorate mine tailings structure, sewage sludge was added. In the second run nutrient was used in all treatments. Especially, *Glomus intraradices* was used for 20 and 30 kg/year sludge additions to determine any differences between *G.mosseae* and *G.intraradices* colonization with the sewage sludge presence. 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. Contents of the pot sets were given in Table 3.3.

Table 3.3. Contents of the second run of the greenhouse experiments.

No	Pots Sets	Mine Tailings (kg)	Sludge (dry) (g)	Sludge dose (g/kg tailings)	AMF species
1	M (contr)	2 kg	-	-	-
2	MMos	2 kg	-	-	<i>G. mosseae</i>
3	MS10	2 kg	20 g	10	-
4	MS10Mos	2 kg	20 g	10	<i>G. mosseae</i>
5	MS20	2 kg	40 g	20	-
6	MS20Mos	2 kg	40 g	20	<i>G. mosseae</i>
7	MS20Int	2 kg	40 g	20	<i>G. intraradices</i>
8	MS30	2 kg	60 g	30	-
9	MS30Mos	2 kg	60 g	30	<i>G. mosseae</i>
10	MS30Int	2 kg	60 g	30	<i>G. intraradices</i>

M: Mine Tailings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition

Table 3.4. Composition of MSR medium (Fortin et al., 2002).

Elements	Concentration, $\mu\text{M}$
N( $\text{NO}_3^-$ )	3800
N( $\text{NH}_4^+$ )	180
K	1650
P	30
Ca	1520
Mg	3000
S	3013
Cl	870
Na	20
Fe	20
Mn	11
Zn	1
B	30
Mo	0.22
Cu	0.96

Modified Strullu-Romand (MSR) medium was used to prepare nutrient solution. The composition of the MSR medium is shown in Table 3.4. The type of chemicals used for the preparation of these medium were  $\text{MgSO}_4$ ,  $\text{KNO}_3$ ,  $\text{KCl}$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{Ca}(\text{NO}_3)_2$  and  $\text{NaFeEDTA}$ .

### **3.6. Metal Analysis**

#### **3.6.1. Digestion Method for the Soil and Sludge**

The soil and sludge samples were dried at  $105\text{ }^\circ\text{C}$  for 2 hours. 25 g samples of mine tailings and sewage sludge were weighted and digested in the microwave system. Sewage sludge, mine tailings and buffer soil samples were digested with 9 ml of nitric acid (65%) and 3 ml of hydrochloric acid (32%) under  $175\text{ }^\circ\text{C}$  for 10 minutes in Bergoff Microwave System, Germany (USEPA, 1996). All the samples were put into falcon tubes. The samples were diluted up to 50 ml falcon tubes with distilled water. The samples were stored at  $4\text{ }^\circ\text{C}$ .

#### **3.6.2. Digestion Method for the Plants**

Plant shoots were separated from the roots. The plant shoots were placed in the packet to dry at  $70\text{ }^\circ\text{C}$  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 ( $\text{HNO}_3\text{:H}_2\text{O}_2$ ; 9:1) under  $175\text{ }^\circ\text{C}$  in Bergoff Microwave System, Germany. Ramp time was 5.25 minutes and hold time was 15 minutes (USEPA, 1996).

#### **3.6.3. Metal Analysis of All Samples**

The digested plant shoots were taken with the 10 ml syringe and they were sieved with the 45 micron syringe tip filter. The samples were put into the falcon tubes with the volume of 15 ml falcon tube. The heavy metal concentrations in sewage sludge, mine tailings and plant body were measured by using ICP-OES equipment after digesting the samples according to EPA 3051 and 3052.

### **3.7. pH Measurements**

The mine tailings pH values were measured, before planting. 10 g of dried soil and 25 ml distilled water were mixed in the falcon tubes for 3 minutes and waited for 1 hour. The samples

were measured with the pH meter. pH of supernatant were measured with glass probe pH meter (WTW).

### 3.8. Mycorrhization Rate Counts

The sunflower (*Helianthus annuus*) plant roots were put in 50 ml falcon tubes with 70% ethyl alcohol and kept them at +4 °C.

The plant roots were packed in the tulle to restrain the loss of roots. They were bathed with 10% KOH solution for 5 hours in the steam bath at 60 °C. It is shown in the Figure 3.5 1% HCl solution was prepared to wash the roots. Before the staining process, the roots were washed with three times distilled water and 1% HCl solution respectively for a minute to clear out the roots. The 50 ml falcon tubes filled with 0.05% (w/v) trypan blue solution (1:1:1; lactic acid, glycerol and water) and the clean packed roots were put into the tubes. They waited for 12 days in dark area.



Figure 3.6. The root samples in the steam bath (photographed by F.Ece Sayın).

The packed roots were opened, and roots were washed with the distilled water to have orderly and separately put roots because the roots had stuck to each other. The colonization wouldn't be seen if the roots were stuck. The roots were ranked on lam and covered with coverslips. Colorless fingernail polish was used to attach lam and coverslip. The second run of the greenhouse experiments mycorrhization rates were determined by light microscope (Microscope Axio Observer.Z1, with EC Plan-Neofluar 20x/0.3 objective, and AxioCam MR5 camera) after being dyed with trypan blue (Phillips and Hayman, 1970). Mycorrhizal colonization refers to the percentage of root cortex occupied by the AMF (Amir et al., 2019).

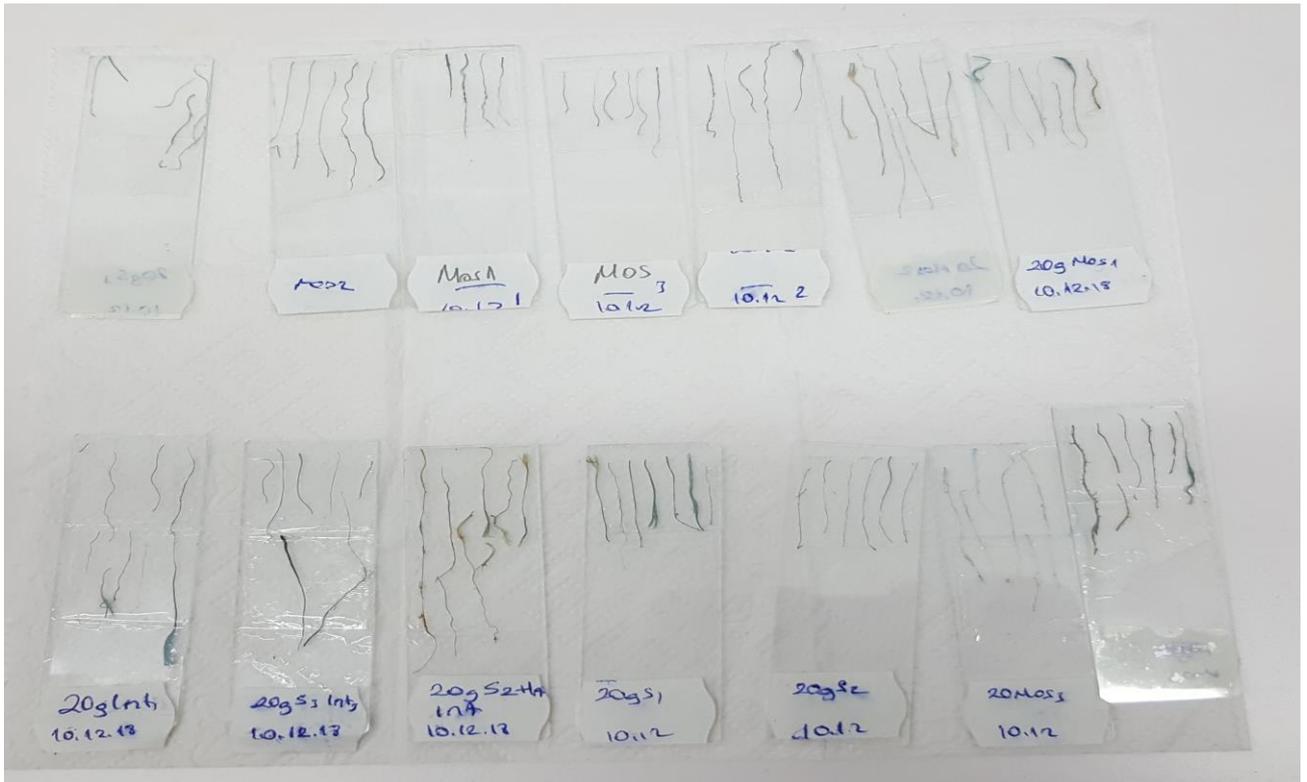


Figure 3.7. The photo of the aligned roots on the lam (photographed by F.Ece Sayın).

### 3.9. Determining Glomalin Related Protein

The soils associated with the roots of the plants were separated from the roots carefully and kept in  $-20^{\circ}\text{C}$  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 (Wright and Upadhyaya, 1996).

The samples were dried at room temperatures for 4-5 days before the experimentation. 2gr of soil was weighted and put in to the sterile falcon tube. 50 mM Citrate Buffer (pH=8) was prepared and 8 ml of the Citrate Buffer was pipetted to remaining soil in 50 ml falcon tubes. The samples were autoclaved at  $121^{\circ}\text{C}$  for 60 minutes. After autoclave, the falcons were centrifuged at 5000 rpm for 15 minutes. The supernatants were transferred into 15 milliliter sterile falcon tubes and kept at  $4^{\circ}\text{C}$  (Rillig, 2004).

Glomalin measurement was done with spectrophotometer at 595 nm and calibration curve was repaired with the standard solution.

This standard solution came from company (Protein Standard, P0834-10x1ml; Sigma; 2mg/ml; Lot SLBS3852) and should be prepared as 0, 0.2, 0.4, 0.6, 0.8, 1 mg/ml with distilled water and kept at 4°C. 100 µl of each dilution standard solution was added to the cuvette and added 3ml of Bradford solution to cuvettes. The cuvette was shaken and waited for 40 minutes. The samples were read at 595 nm wave length. Zero calibration was needed. It was filled just with 4ml distilled water.

### **3.10. Statistical Analysis**

For the statistical analysis, the uptake of heavy metals by sunflower in the different conditions was determined by using hierarchical cluster analysis. Cluster analysis was applied to data set using the nearest neighbour method and “Block” interval. SPSS 25 (IBM, 2017) software was used to conduct the cluster analysis

## 4. RESULTS AND DISCUSSION

This study investigated the effect of AMF association and sewage sludge addition on phytoremediation of Cr mine tailings. In the study, the Cr tailings having very high heavy metal (Cr, Al, and Fe) content were ameliorated by phytoremediation enhanced by different species of AMF (*Glomus mosseae* or *Glomus intraradices*) associations and three different doses of sewage sludge application. The greenhouse pot experiments were performed in two runs in different seasons.

### 4.1. Metal Analysis of the Mine Tailings, Buffer Soil and Sewage Sludge

The metal analysis of Cr, Mn, Fe, Ni, Cu, Zn, Al, Cd, Pb, Si, Co and Mo in the mine tailings, buffer soil and sewage sludge samples were performed by using ICP-OES (Perkin Elmer) method before the pot experiments were conducted. The heavy metal contents of the mine tailings, buffer soil and sewage sludge samples were summarized in Table 4.1. The concentration of Cr mine tailings was above the toxic level (Paustenbach et al., 1991). Chromium concentrations were high compared to other Cr ore tailings (283.89 ppm) (Panda et al., 2016). Iron and aluminum were revealed as the highest concentrations among these 12 metals. It was found that Cu and Zn wastewater sludge concentrations were higher than those of mine tailings and buffer soil.

Table 4.1. Mine Tailings, Buffer Soil and Sewage Sludge Heavy Metal Concentrations (ppm).

Metals (ppm)	Cr	Mn	Ni	Cu	Zn	Al	Cd	Pb	Co	Mo	Fe	Si
First Run Mine Tailings	948	598	1561	7.79	15.5	25243	0.2	3.7	41.1	0.1	23849	441
Second Run Mine Tailings	1218	672	1585	8.84	17.1	29273	0	3.7	44.4	1.4	26424	446
Buffer Soil	40.9	690	56.5	41.5	111	11617	0.1	50	12	1.7	20239	740
Sewage Sludge	709	461	411	676	1815	72886	4	34	7	3	86321	393

## 4.2. Elemental Analysis

Elemental analyses of the Cr tailings, buffer soil and sewage sludge samples were performed by using Elemental Combustion equipment. The results were given in the Table 4.2. Carbon and nitrogen content of the Cr mine tailings was found to be much lower compared to buffer soil. It was necessary to increase the nutrient content of Cr tailings to grow healthy plants leading to improvement of the phytoremediation. Therefore, sewage sludge was used to ameliorate the nutrient contents of Cr mine tailings.

Table 4.2. Elemental analysis results of mine tailings, buffer soil and sewage sludge.

Weight %	Mine Tailings	Buffer Soil	Sewage sludge
C	2.69	6.65	31.3
N	0.1	0.45	5.55
H	1	4.5	4.3
S	0	0	0.5

## 4.3. Plant Growth

In the first run of the greenhouse experiments (first plantation) performed in the period of 23.02.2018 – 25.05.2018, growth of plants was measured in terms of both dry weight and plant height. Heights of the sunflowers (*Helianthus annuus*) were measured weekly. Figure 4.1 shows heights of the plants. It was observed that the average heights of the sunflower (*Helianthus annuus*) plants grown in pots containing only mine tailings were extremely low compared to the other plants. These plants died at the end of 7<sup>th</sup> week indicating that the un-treated Cr-mine tailings were not suitable for the plant growth. Amendment of the Cr-mine tailings with buffer soil and different doses of sewage sludge helped the formation of suitable environment for growth of the plants. In the second run of the greenhouse experiments (second plantation) performed in the period of 24.09.2018- 20.12.2018, the plant growth was measured in terms of shoot dry weight. Plant heights were not measured to minimize the damages given to the leaves of the plants.

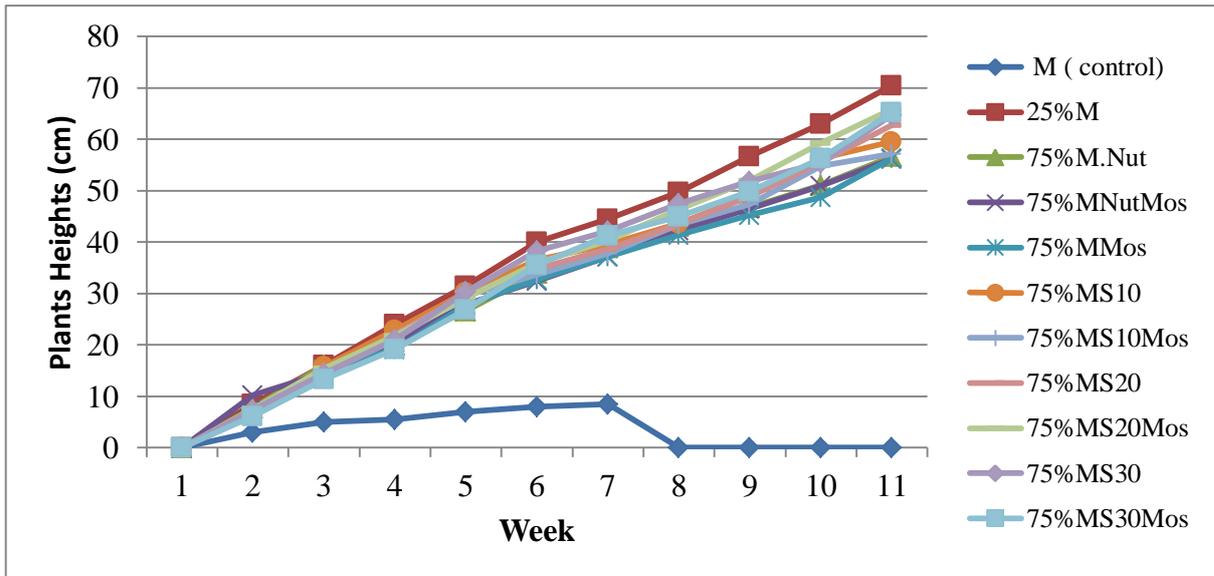


Figure 4.1. The average heights observed in the first run of greenhouse pot experiment.

#### 4.3.1. The First Run of Greenhouse Experiments

After germination period, sunflower (*Helianthus annuus*) seeds were planted in chrome mine tailings mixed by buffer soil, and some of mine tailings were treated with the different doses of sewage sludge application and AMF inoculation. After three months of planting period, sunflower (*Helianthus annuus*) plants were cut. There were not important differences in plants heights as shown in Figure 4.2 It was deduced that the sunflower height was not significantly affected by AMF association. Davies and co-workers (2001) also had revealed that mycorrhizal sunflower (*Helianthus annuus*) height was not different from non-mycorrhizal plants. However, it has been observed that the longest plant height was performed in plants growing in mine tailings mixed by 75% buffer soils. Moreover, the addition of 20 g and 30 g of sewage sludge increased the height of the plant grown in mine tailings slightly more than the addition of 10 g of sludge. Taking these results into account, the high nutrient content may have led to a slight increase in the height of sunflowers.

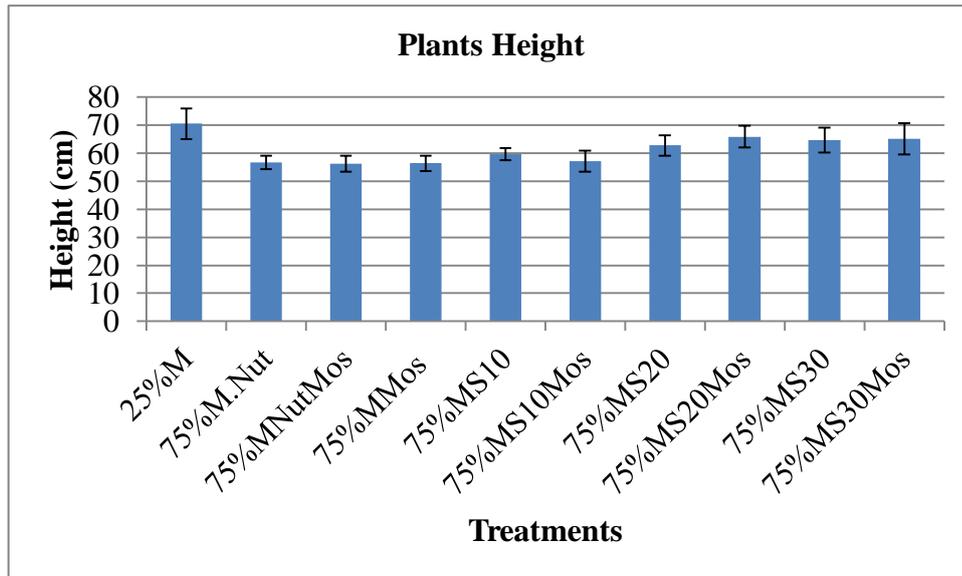


Figure 4.2. The average heights of sunflower (*Helianthus annuus*) plants after 11 weeks.

The general appearances of the plants were given in Figures 4.3, 4.4, and 4.5 in the order of the date.



Figure 4.3. Sunflower (*Helianthus annuus*) plants in the second week of the first run plantation. (photographed by F.Ece Sayın).



Figure 4.4. Sunflower (*Helianthus annuus*) plants in the fourth week of the first run plantation.

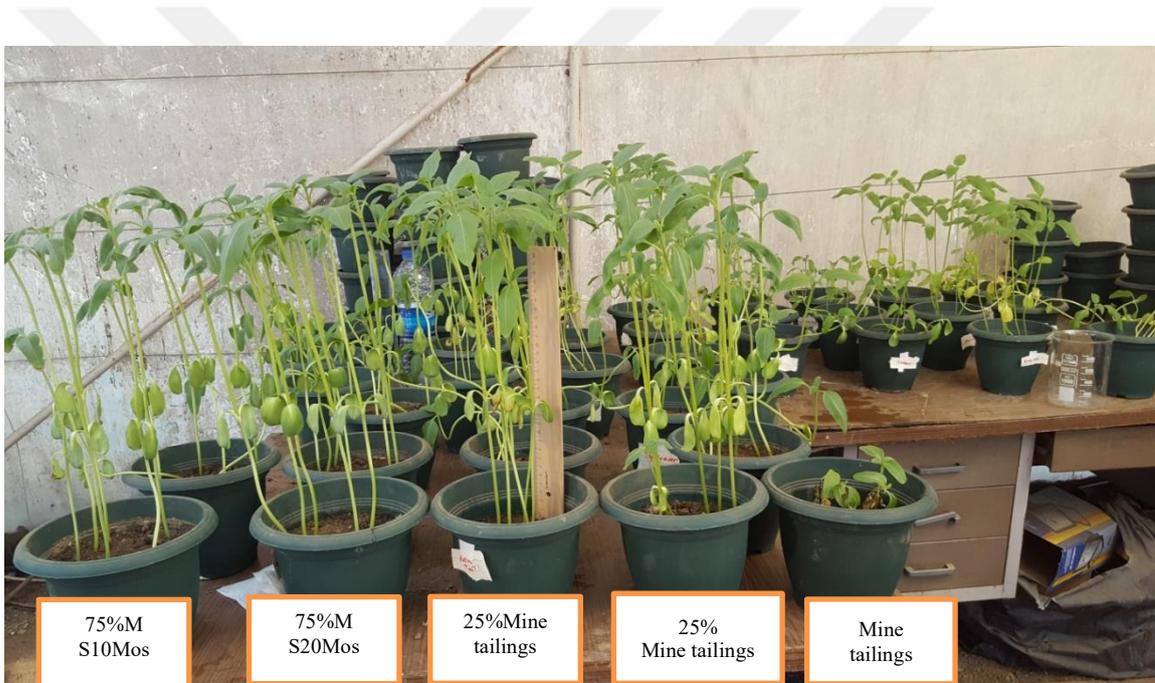


Figure 4.5. Sunflower (*Helianthus annuus*) plants in the seventh week of the first run plantation.

In the greenhouse experiments, some of the sunflowers were grown in the pots containing sewage sludge only. Half of them were inoculated with AMF. Figure 4.6 shows the plants in pots containing sewage sludge only. It has been observed that the germination was faster in the AMF inoculated plant seeds.



Figure 4.6. Sunflower (*Helianthus annuus*) plants grown in the sewage sludge in the seventh week.

#### 4.3.2. The Second run of Greenhouse Experiments

The next greenhouse study was initiated in 24 August 2018 (Figure 4.7). Due to some factors like high air humidity, high soil bulk density and nutrient deficiency of the mine tailings, the plants did not grow healthy and were not strong enough to resist the pests.



Figure 4.7. Sunflowers (*Helianthus annuus*) plants in the fourth week of second run plantation.

The white plant lice infested the sunflowers (*Helianthus annuus*) and caused their leaves to turn yellow, dry out and fall. Therefore; sunflower (*Helianthus annuus*) cultivation was repeated 4 times. Figure 4.8 shows the white plant lice taking shelters under the sunflower (*Helianthus annuus*) leaves.

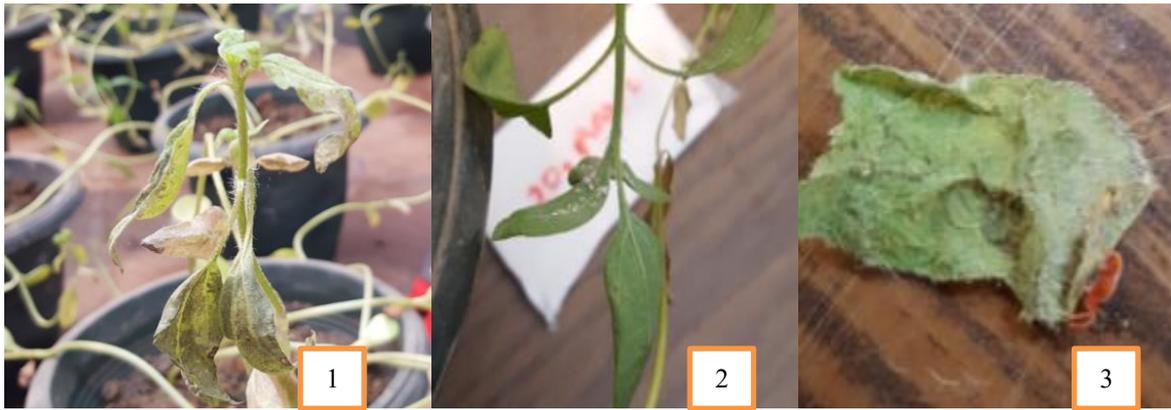


Figure 4.8. Insect and lice problems on the sunflowers (*Helianthus annuus*).

#### 4.4. Root and Shoot Weights

The sunflower (*Helianthus annuus*) plants were harvested at the end of 3-month growth period. The weights of dry root, dry shoot and fresh shoot were determined in the first run greenhouse pot experiment. However, in the second run greenhouse pot experiment, only the dry weights of shoot could be measured. The root weights of the plants couldn't be determined since the limited root samples were used in mycorrhizal colonization determinations. Nutrient deficiency, high heavy metal concentration, and especially the high bulk density of the Cr mine tailings restricted the root and plant growth of the sunflowers (*Helianthus annuus*). The bulk density of the mine tailings was too high for sunflower (*Helianthus annuus*) lateral roots to grow healthy. Supportively, McKenzie et al., (2004) reported that soils having a bulk density higher than  $1.6 \text{ g/cm}^3$  cause to restriction in plant and root growth by eliminating movement of air and water through the soil. The physical properties of the soil such as bulk density, compatibility, 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 (2000) observed differences in AMF association and plant biomass production for the substrates of similar particle size under similar growth conditions and host plant.

##### 4.4.1. The Sunflower Planted In the First Run of Greenhouse Experiments

The fresh shoots weights of the plant were presented in Figure 4.9 It has been observed that the artificial nutrient (MSR medium) application didn't make an important contribution to plant growth. However, the sludge application enhanced the growth of the plant in terms of fresh shoot weights, dry shoot weights and dry root weights compared to artificial nutrient application. The highest plant growth, measured in terms of plant fresh shoot weight, was obtained in the pots amended with 25% buffer soil, AMF inoculation and 20 g/kg sewage sludge addition. The fresh weights of these plants

were  $51.33 \pm 3.56$  gram. Important differences on plant biomass were found when sewage sludge was added to mine tailings. The fresh weights of plants grown on treated mine tailings with 25% buffered soil and 10 g/kg sewage sludge, inoculated with AMF were  $44.67 \pm 2.5$  grams. The weights of non-mycorrhizal plants grown on mine tailings amended with 10 g/kg and 20 g/kg sewage sludge were  $27 \pm 0.82$ ,  $32 \pm 3.97$  grams, respectively. The weights of the mycorrhizal plant grown in mine tailings without sewage sludge addition were  $17.34 \pm 0.47$  grams. These results indicated that the availability of plant nutrients and AMF inoculation increased the fresh weights of sunflowers by improving their growth. However; 30 g/kg sludge application decreased the fresh plant shoot biomass in comparison with 10 and 20 g/kg sludge applications. Dry shoots weights of the sunflowers were given in Figure 4.10.

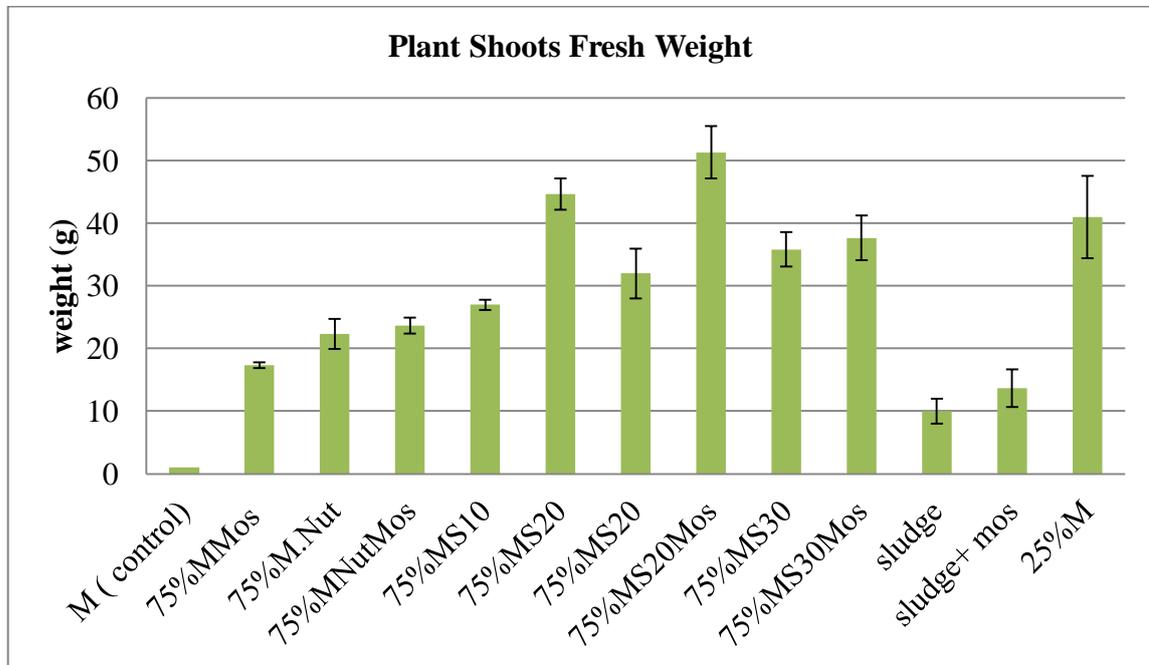


Figure 4.9. Fresh weights of plant shoots obtained in the first greenhouse experiment. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

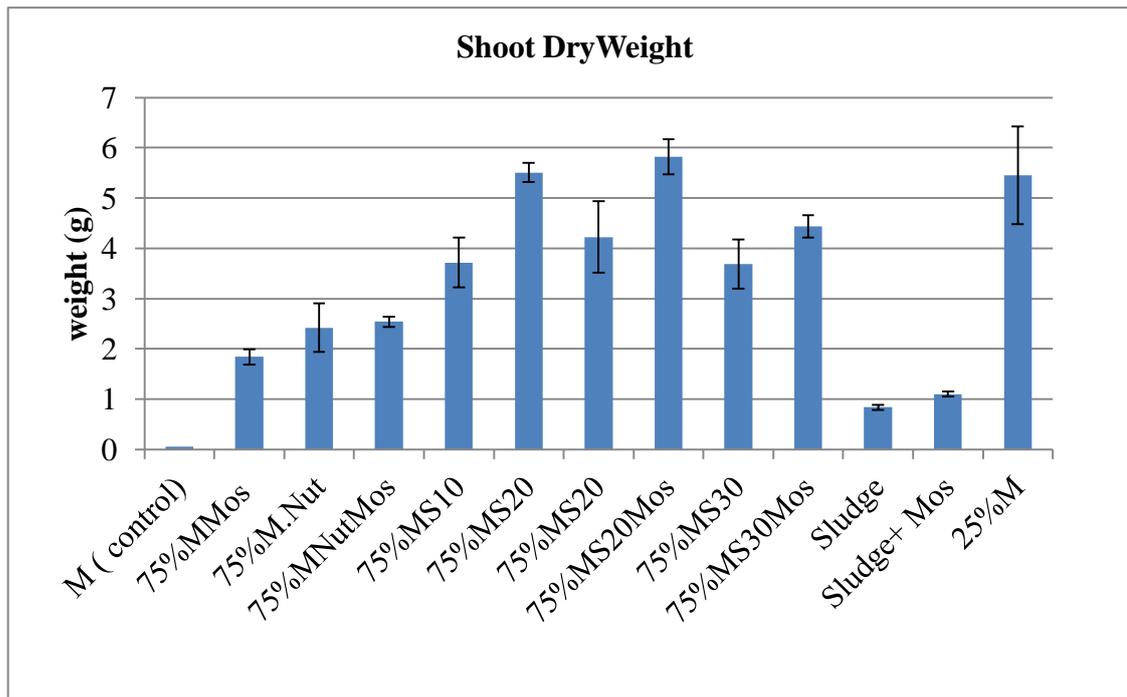


Figure 4.10. Dry weights of plant shoots obtained in the first greenhouse experiment. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution; Sludge: Plant grown in sewage sludge ).

The roots of the sunflower (*Helianthus annuus*) associated with AMF were heavier than non-inoculated plants. The plants developed in 75% buffer soils were also heavier than the others because the buffer soil was rich in nutrients and its texture was suitable for the growth of sunflower (*Helianthus annuus*) roots. The dry weights of plant roots obtained in the first run of the greenhouse experiments were given in Table 4.11. The roots of sunflowers (*Helianthus annuus*) grown in the sewage sludge were not developed compared to the other sunflowers (*Helianthus annuus*). The reduction in the root weights was determined to be 87% on average.

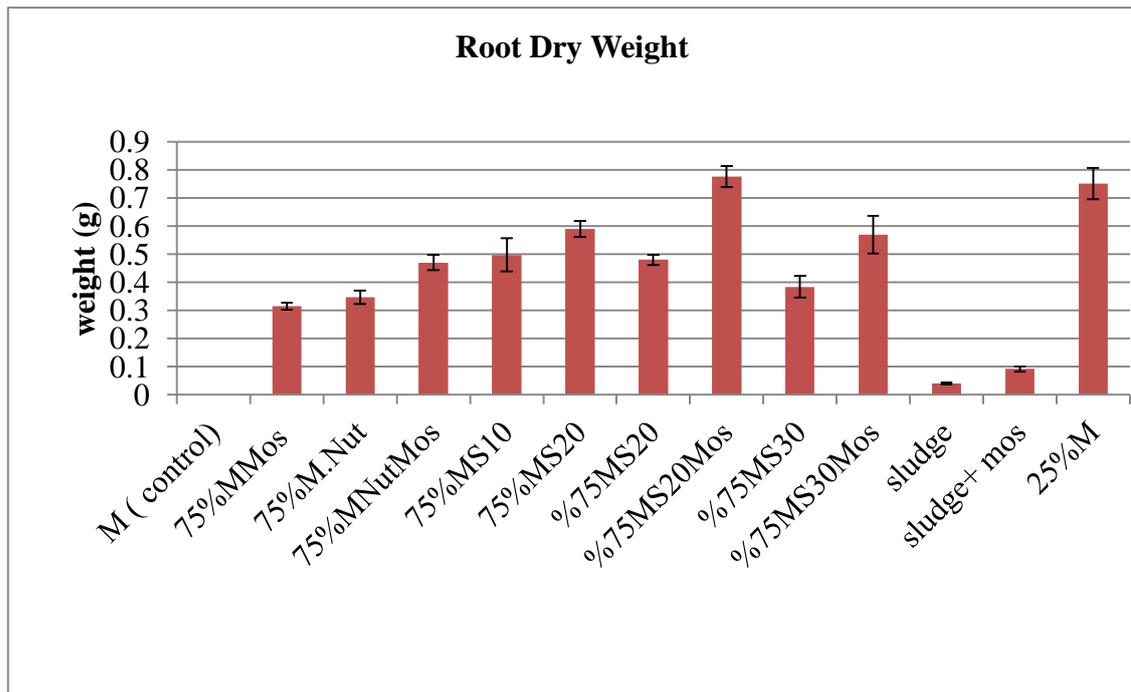


Figure 4.11. Dry weights of plant roots obtained in the first greenhouse experiment. (75%M: 75% Mine tailings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution; Sludge: Plant grown in sewage sludge).

Figure 4.12 shows the shoot dry weights in the second run plantation. AMF association and sludge application enhanced the plant growth. 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 tailings. The addition of 30 g/kg sludge may have toxic effect on the plant because the same result was obtained in the first series. Supportingly, Turk and co-workers (2006) observed that mycorrhizal symbiotic relationship suppressed the plant growth in the soils having optimum phosphorus concentration due to the carbon competition between the host plant and the mycorrhizal fungi. Furthermore, Lambert (1991) 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 nitrogen.

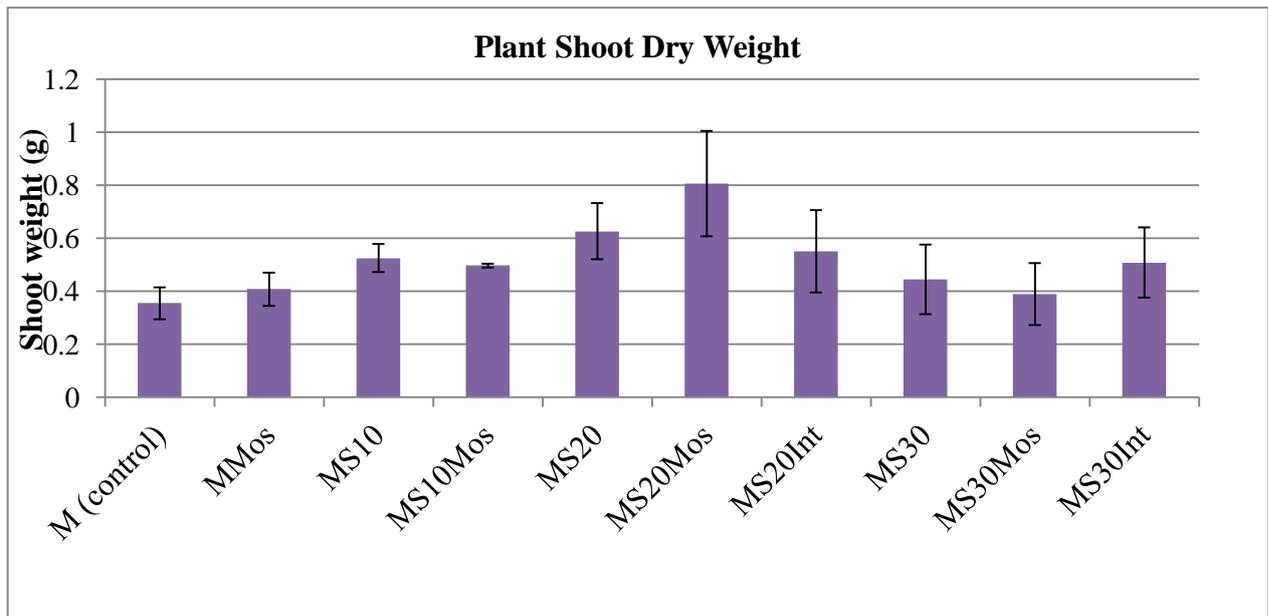


Figure 4.12. Dry weights of plant shoots obtained in the second run of greenhouse experiments. (M: Mine Tailings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

#### 4.5. Mycorrhization Rates

Mycorrhization rate is the indication of association level of AMF with the sunflower (*Helianthus annuus*) roots. Macroscopic observations helped to count AMF relationships with the plants. Mycorrhization rates were determined with the presence or absence of AMF on the visionary screen in the camera of microscope. Hyphae, arbuscules, vesicles and spores were counted according to these methods. Therefore, this creates an awareness of the proportion of occurrence of the root's AMF lengthways. This mycorrhization rate counting didn't provide the information about mycorrhizal strength.

Three months after the inoculation of *G.intraradices* and *G.mosseae*, the roots of sunflowers (*Helianthus annuus*) showed considerable differences that were established fungal intraradical structures. Mycorrhization rates were given in Figure 4.13. The highest mycorrhizal colonization rate of 66 % was observed in the pot MMos, containing *G.mosseae* inoculated plants. However, the second highest mycorrhizal colonization rate of 54.6% was obtained in the pot including 10 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 differences between control pots and the pots inoculated with *Glomus intraradices*. The application of 30 g/kg sewage sludge to the pots suppressed the mycorrhizal association. This can be attributed to the high nutrient, phosphorous and nitrogen, content of the sewage sludge.

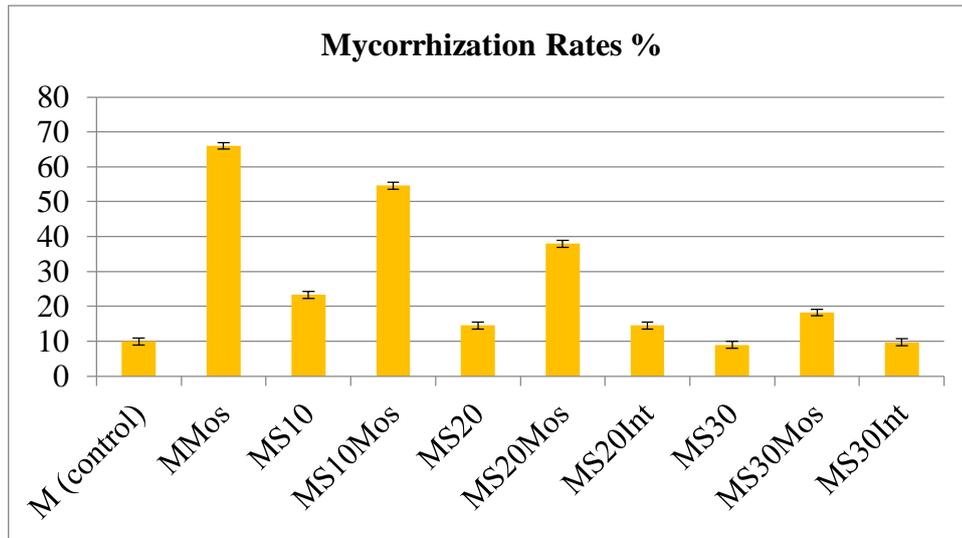


Figure 4.13. The average mycorrhization rates in the second run plantation (M: Mine Talings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

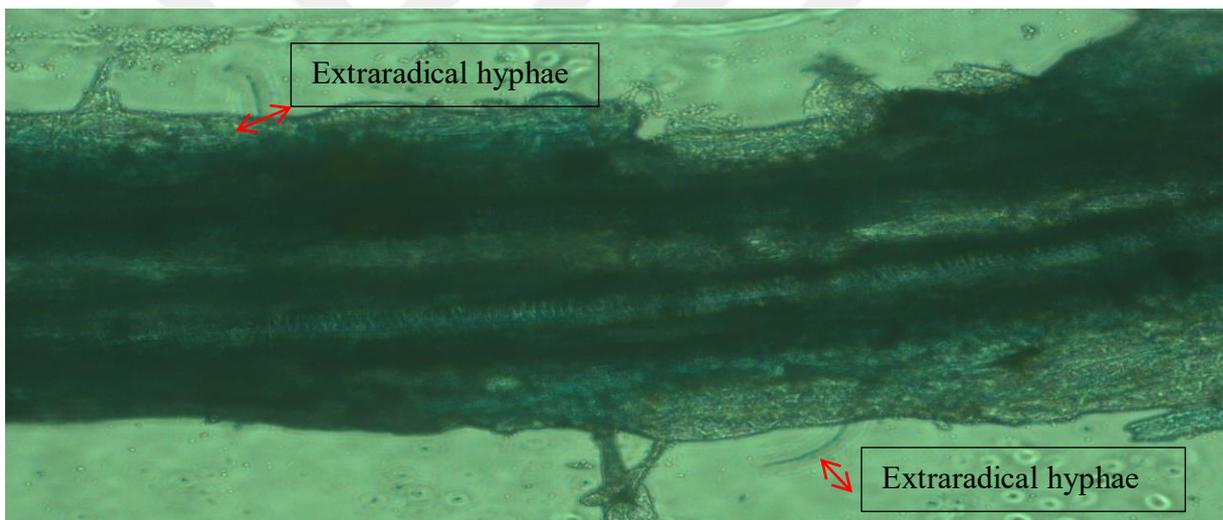


Figure 4.14. The roots of MMos plant have blue staining hyphae indicate AMF existence.

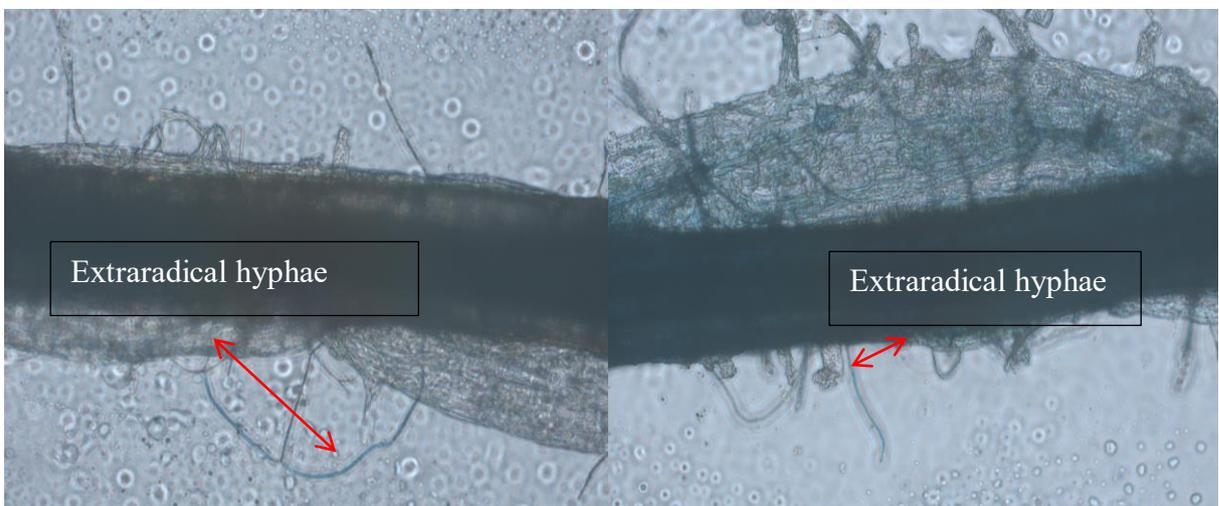


Figure 4.15. The roots of MS10Mos plant have blue staining hyphae indicate AMF existence.

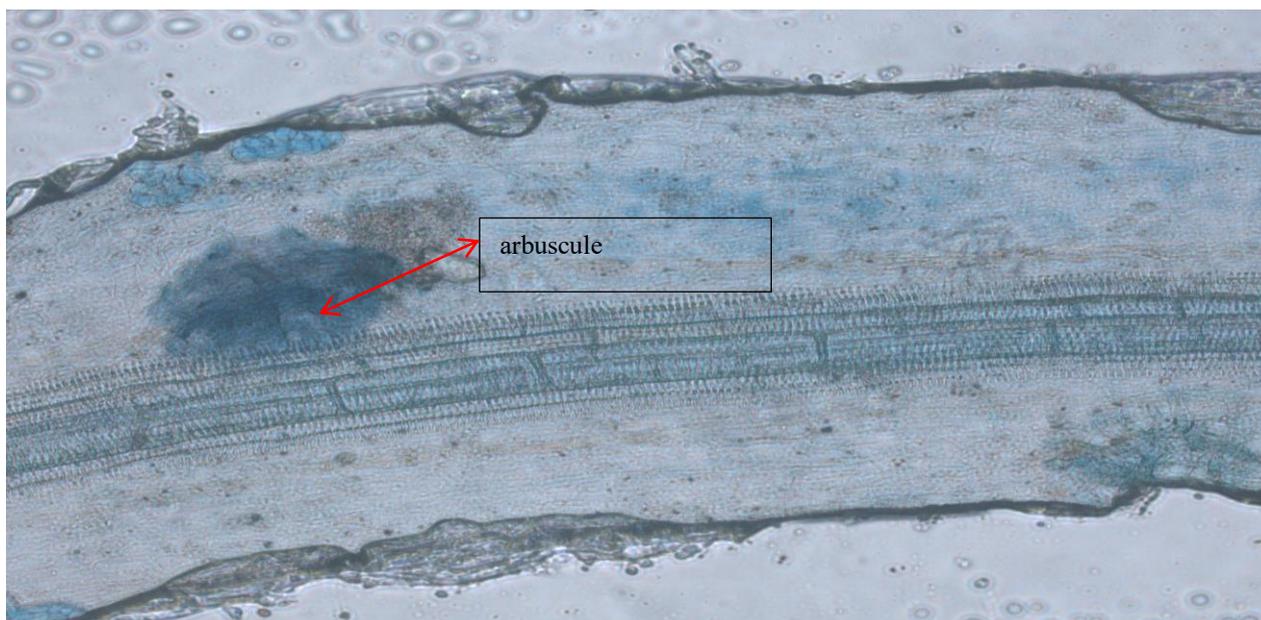


Figure 4.16. The roots of MS20Mos plant have blue staining hyphae and arbuscules indicate AMF existence.

#### 4.6. Glomalin Analysis

The glomalin calibration curve was prepared by using a solution with the known concentrations of glomalin proteins. This calibration curve indicated as a relevant calibration for the calculations to determine the glomalin concentrations accurately. The  $R^2$  value was 0.9928 which points out that the experiment was correct. Glomalin quantities of the extracts were calculated according to the glomalin calibration curve. The absorbance values and calibration curve of the standards were shown in Table 4.3 and Figure 4.17, respectively.

Table 4.3. Absorbance values of indicated BSA samples at 595 nm.

Concentration of protein standard (mg/L)	Absorbance at 595nm
0	0
0.2	0.118
0.4	0.217
0.6	0.399
0.8	0.531
1.0	0.662

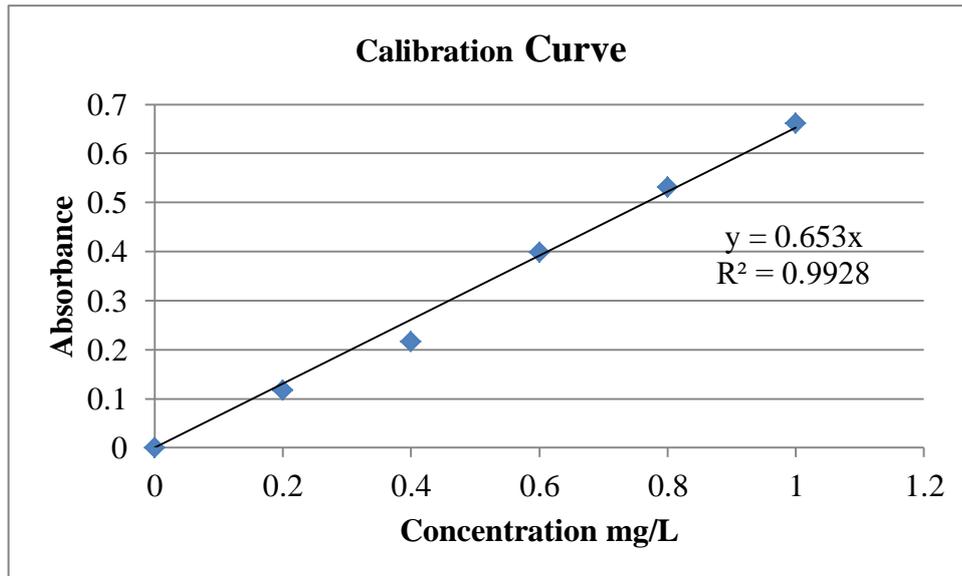


Figure 4.17. Calibration curve of BSA standards.

The results of glomalin protein determinations showed that mycorrhizal association increased the glomalin protein concentrations of the mine tailings in the pots. Supportively, the recent studies pointed out that mycorrhizal association increased the glomalin protein concentrations in the soil. Gadkar and Rillig (2006) reported that mycorrhizal colonization released the glomalin proteins. *G.mosseae* was found to be more effective than *G. intraradices*. Tunalı (2015) showed that *G.mosseae* was effective species for sunflower (*Helianthus annuus*) mycorrhizal colonization. Figure 4.18 showed the glomalin concentrations in the amended and un-amended mine tailings.

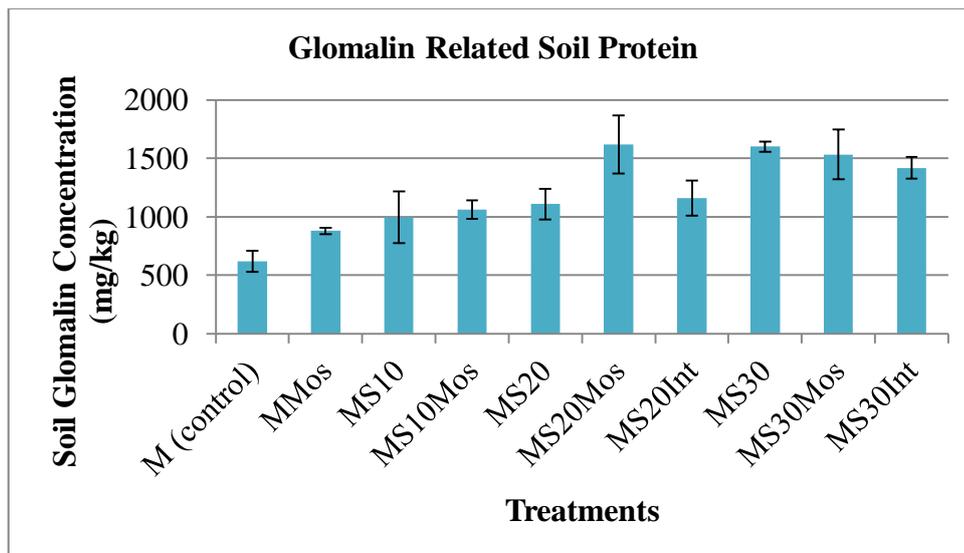


Figure 4.18. Glomalin concentrations in the amended and un-amended mine tailings. (M: Mine Tailings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

The results of the pot experiments showed that glomalin related soil protein was increased more with the application of sewage sludge. The highest glomalin protein was observed in plants grown in mine tailings amended with 20 g/kg sludge application and *Glomus mosseae* inoculation. Since a typical activated sewage sludge contains 224 – 462 mg protein g<sup>-1</sup> VSS (Ras et al., 2008), application of sewage sludge may increase the glomalin content of mine tailings.

## 4.7. Metal Analysis

### 4.7.1. First Run Plantation

Figures 4.19 - 4.26 show the heavy metal accumulations in the sunflowers (*Helianthus annuus*) planted in the mine tailings.

In the first run of the greenhouse experiments, the considerable differences were observed in heavy metal uptakes of the plant shoots. The plants grown in combined 25% mine tailings and 75% buffer soils accumulated the maximum chromium, nickel, aluminum, iron and silicon. The addition of buffer soil supported the plant root growth by supplying nutrient and improving the structure of mine tailings. Phytoremediation and their efficiency strongly related to root density. Heavy metal extractions in mycorrhizal plants were reduced during poor root growth. Beside this, when the roots are embedded in the entire soil area and can take up adequate nutrients, mycorrhization has no effect on heavy metal extraction. The root density was thought to be the crucial parameter in the heavy metal extraction and mycorrhizal plant efficiency.

Arbuscular mycorrhizal fungi associations enhanced the Cr accumulation in the sunflowers (*Helianthus annuus*) grown in 75% buffer soil and 25% mine tailings mixture. The Cr accumulations of the plants were represented in Figure 4.19. Combined 10 g/kg sludge application, 25% buffer soil, 75% mine tailings) addition and *Glomus mosseae* inoculation resulted with the highest chromium uptakes. The 10 and 30 g/kg except 20 g/kg sludge decreased the Cr uptake in the sunflower (*Helianthus annuus*) plants. The sewage sludge contains high concentration of phosphorus. The high P content decreases the Cr uptake because phosphate (H<sub>2</sub>PO<sub>4</sub><sup>-</sup>) stops CrO<sub>4</sub><sup>2-</sup> adsorption in the soil structures and P and Cr compete for surface sites (Davies et al., 2001).

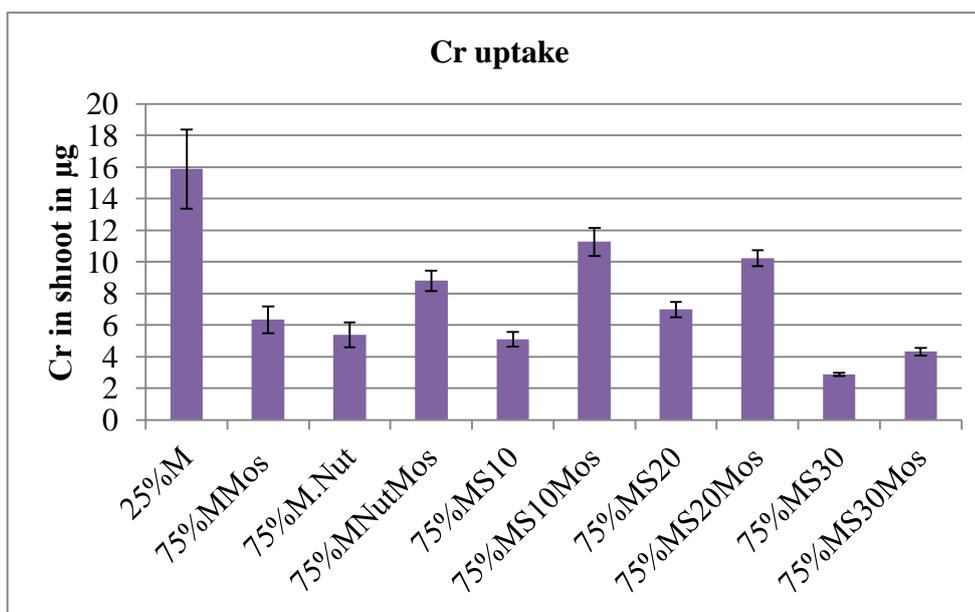


Figure 4.19. Chromium accumulation in the shoot of the first run plantation. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

Manganese accumulations were higher in the inoculated plants as shown in Figure 4.20. Especially the 75%MS20 plants had the greatest manganese accumulation in their shoots. Inoculated plants uptakes were higher than non-inoculated ones. This result was contradictory with the second run plantations, because it was observed that manganese extraction was lower in the mycorrhizal plants (Pacovsky, 1986). However, because of the low mycorrhizal association in the plants as a result of excessive buffer soil addition and P as well, Mn uptake was high in terms of plants biomass.

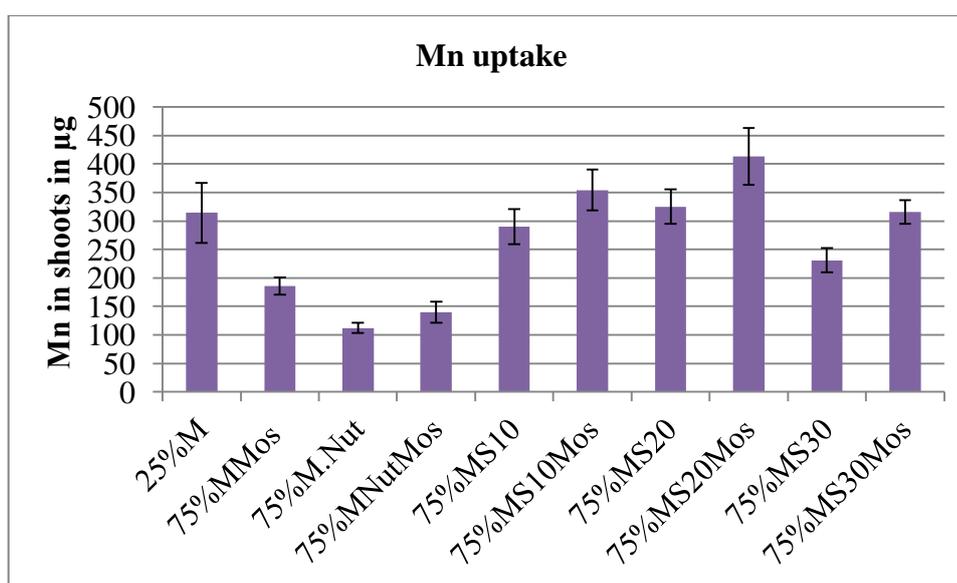


Figure 4.20. Manganese accumulation in the shoot of the first run plants. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

Figure 4.21 shows Ni accumulation in the shoot of the first run plants. Sewage sludge addition increased the Ni uptake by improving plant growth. Plants grown in pots containing 75%MS20 had the higher biomass than plants grown in pots containing 75%MS20Mos, 75%MS30 and 75%MS30Mos. As a result, the highest Ni uptakes in plant shoots were obtained in these pots containing 75%MS20.

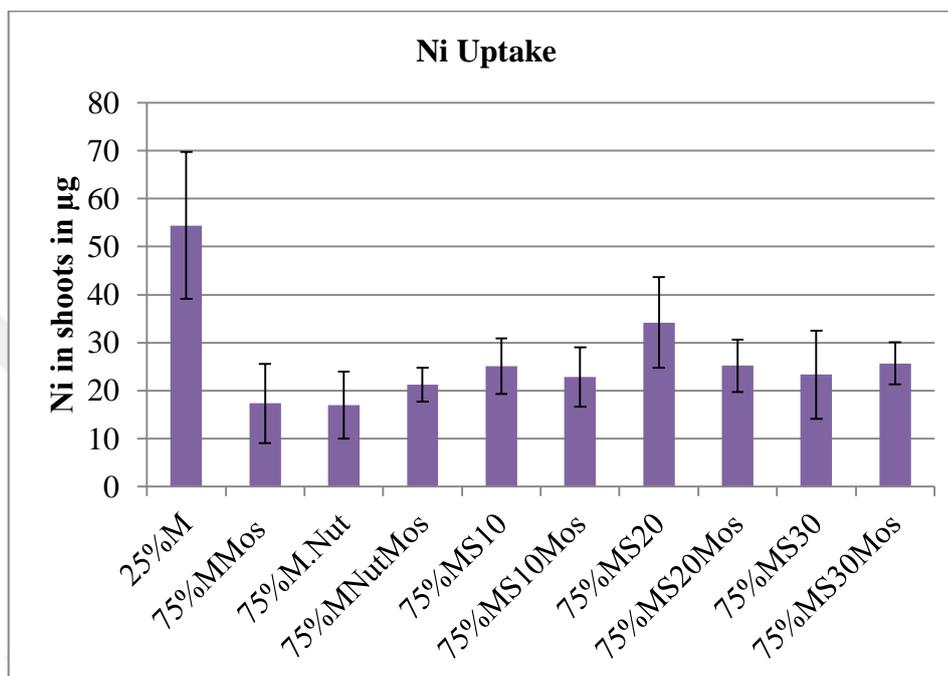


Figure 4.21. Nickel accumulation in the shoot of the first run plants. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

Figure 4.22 shows Cu accumulation in the shoot of the first run plants. The sewage sludge copper and zinc concentrations were 676 ppm and 1815 ppm respectively. Nevertheless, mine tailings Cu and Zn concentrations were 7.49 and 15.5 ppm which were much lower than the sludge, therefore sludge application were expected to increase the Cu and Zn accumulation. The results confirmed the expectation as Cu and Zn uptake increased with the sludge application. *G. mosseae* association enhanced the Cu and Zn accumulation in the sludge amended pots.

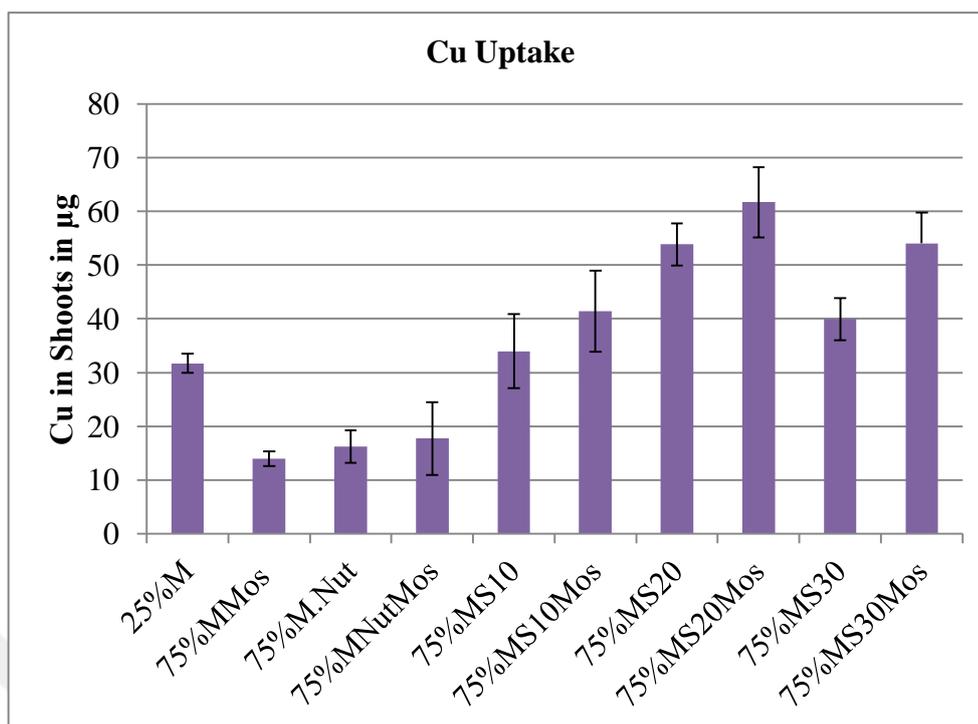


Figure 4.22. Copper accumulation in the shoot of the first run plants. (75%M: 75% Mine tailings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

The other studies supported the present study. Chen and co-workers (2007) worked with four different species of plants with the inoculation of *G. mosseae* to enhance the plants for Cu and Zn uptake in copper mine tailings. The results showed that the inoculated plants accumulated more Cu in their plant body. Furthermore, Wang and co-workers (2007) observed that shoot uptake of Cu and Zn increased in the mycorrhizal plants.

Marques and co-workers (2006) compared zinc accumulation in *Solanum nigrum* in the different Zn concentrations of the sand soil. The AMF species of *G. colaroideum*, *G. mosseae* and *G. intraradices* were inoculated with plants to enhance the zinc accumulation. The data of the study showed that Zn accumulation was higher in the mycorrhizal plant body in all concentrations of Zn but AMF didn't affect the translocation factor.

Buffer soil addition increased the iron, silicon and aluminum concentration in the plants shoots. *G. mosseae* inoculation increased the Fe, Si and Al uptake compared to non-inoculated plants. Zn, Al, Fe and Si accumulations in the shoot of the first run plants were given in Figure 4.23, Figure 4.24, Figure 4.25, and Figure 4.26, respectively.

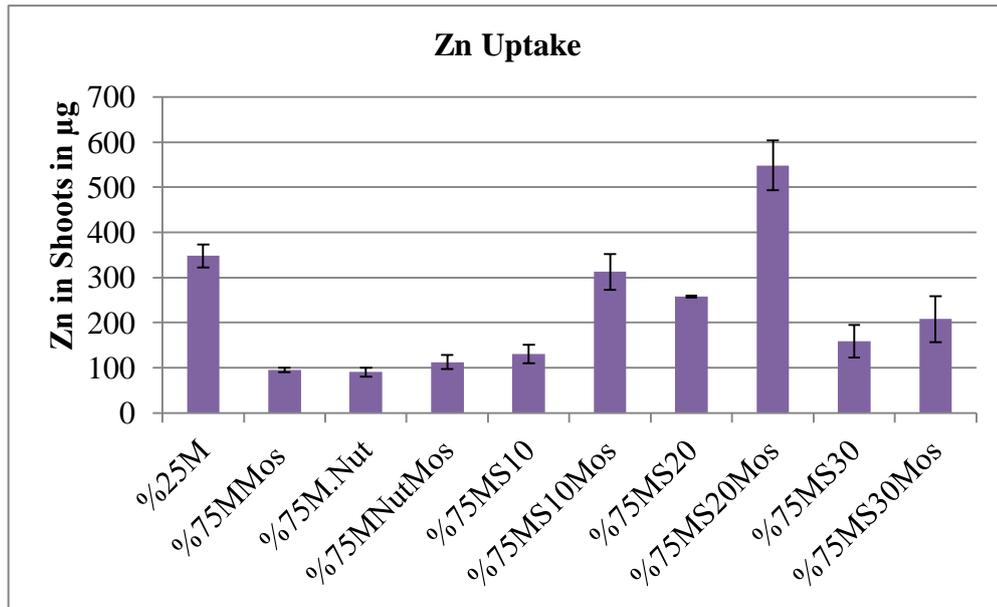


Figure 4.23. Zinc accumulation in the shoot of the first run plants. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

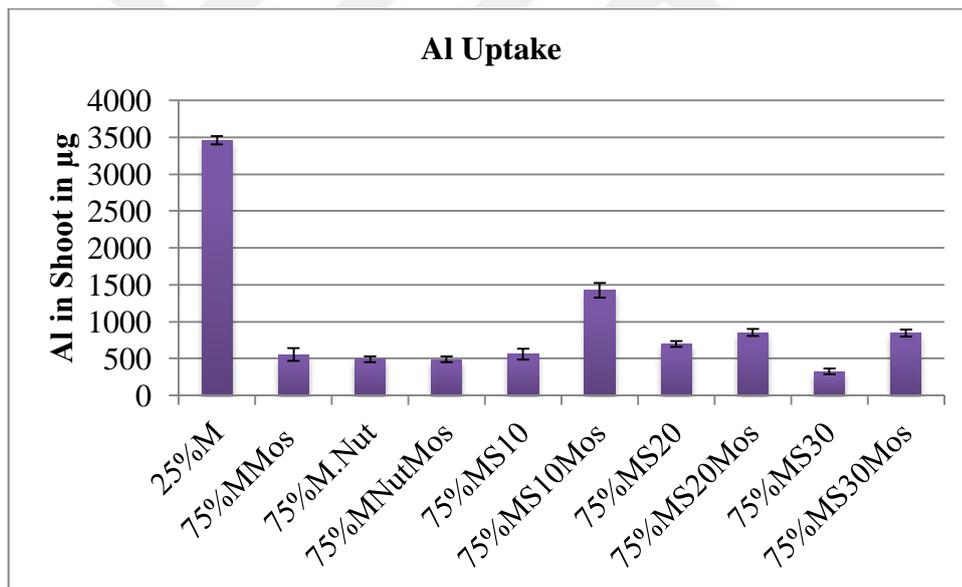


Figure 4.24. Aluminum accumulation in the shoot of the first run plants. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

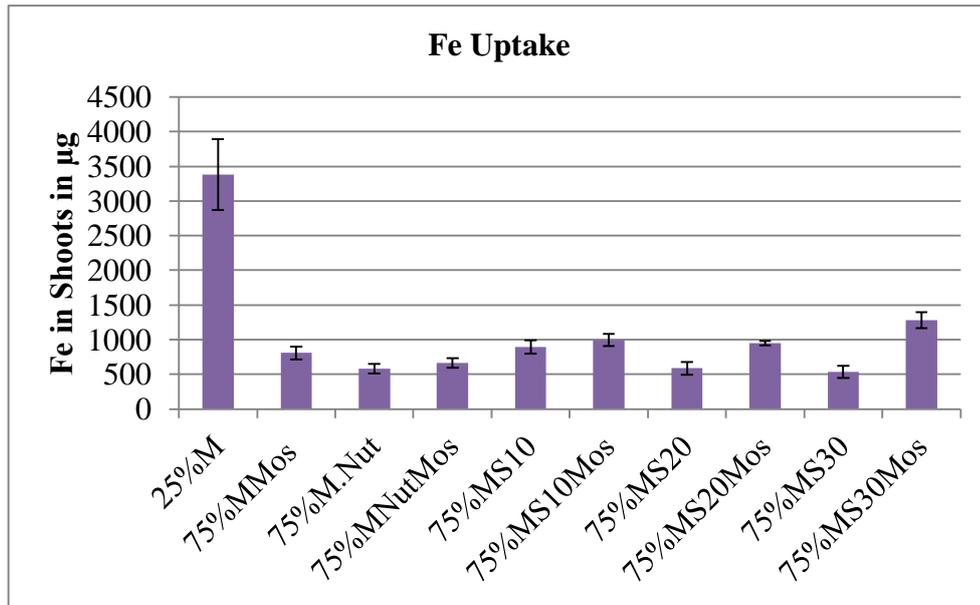


Figure 4.25. Iron accumulation in the shoot of the first run plants. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

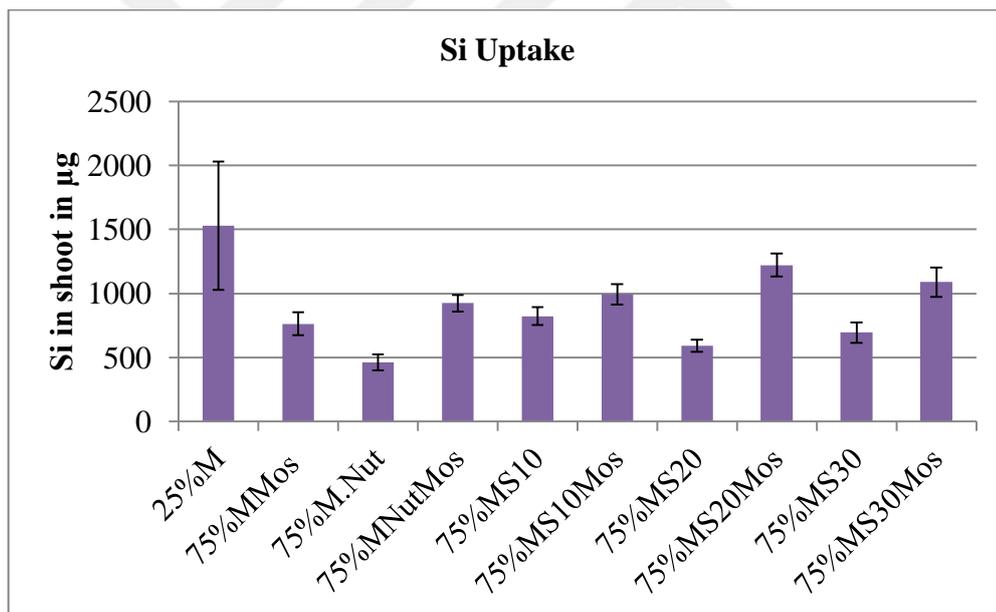


Figure 4.26. Silicon accumulation in the shoot of the first run plants. (75%M: 75% Mine talings; Mos: *G. mosseae*; S10: 10 g/kg Sewage sludge addition; Nut: Nutrient solution).

In the first run plantation, the six pots were filled with the sewage sludge, and then sunflower (*Helianthus annuus*) seeds were planted in them. The heavy metal accumulation in the roots and shoots of the plants were evaluated. Figures 4.27 and 4.28 showed the heavy metal accumulations in the shoots. Cr, Cd, lead, cobalt, and molybdenum uptakes were shown in a figure as their accumulation was lower than the other metals. Accumulations in the mycorrhizal plant shoots were

higher except Cr accumulations. The differences of Cu, Pb, Co and Mo shoot uptake were not important in inoculated and non-inoculated plants.

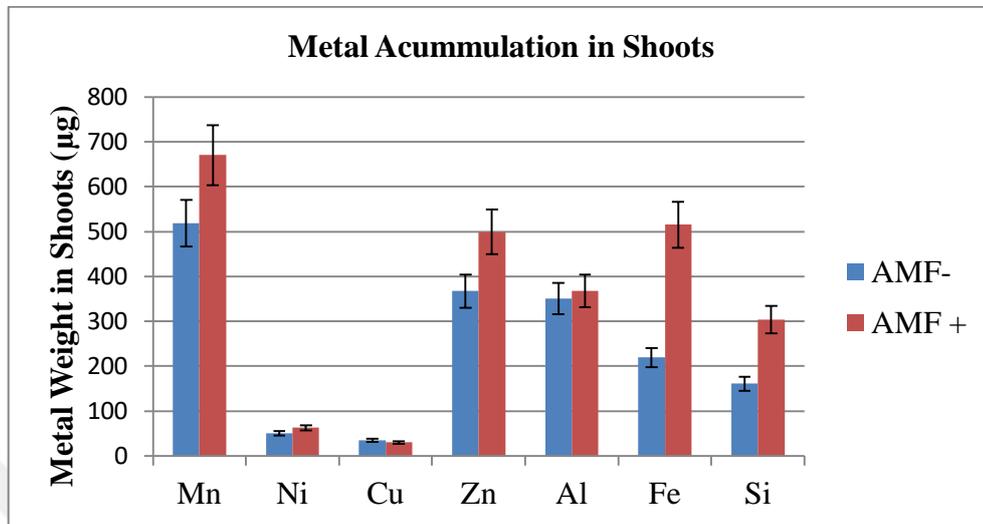


Figure 4.27. Metal accumulation in plant shoots grown in sewage sludge.

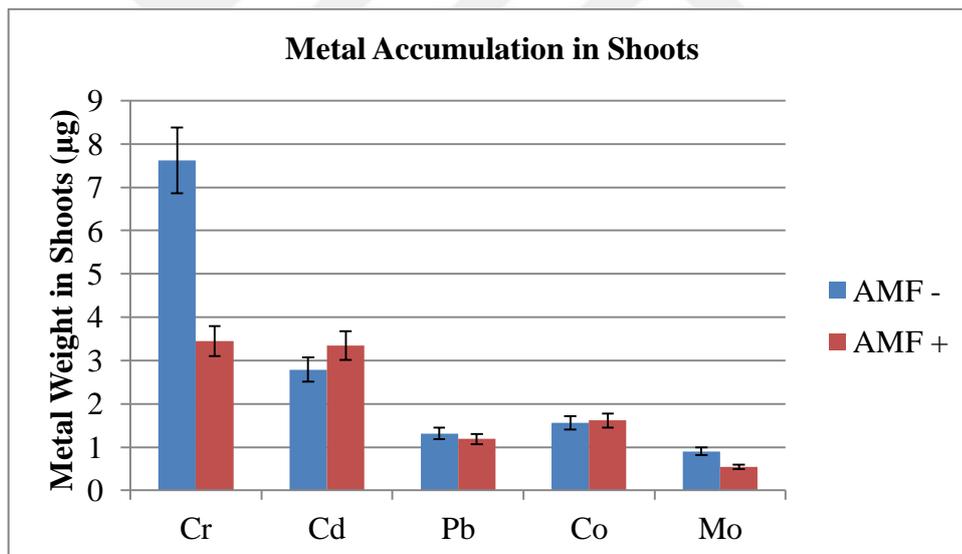


Figure 4.28. Metal accumulation in plant shoots grown in sewage sludge.

Figures 4.29 and 4.30 show the metal accumulations in the plant roots of *G. moseae* inoculated plants were higher except iron accumulation.

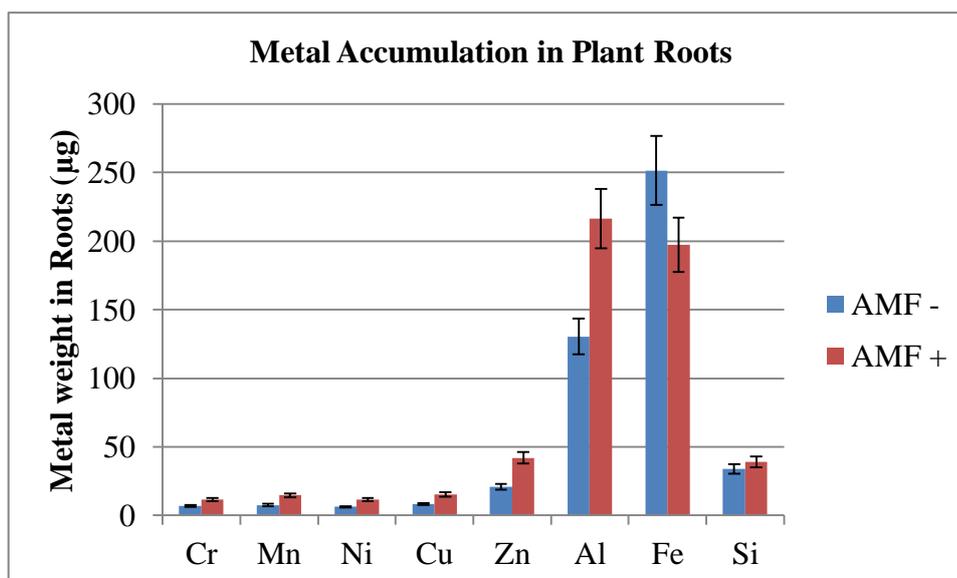


Figure 4.29. Metal accumulation in plant roots grown in sewage sludge.

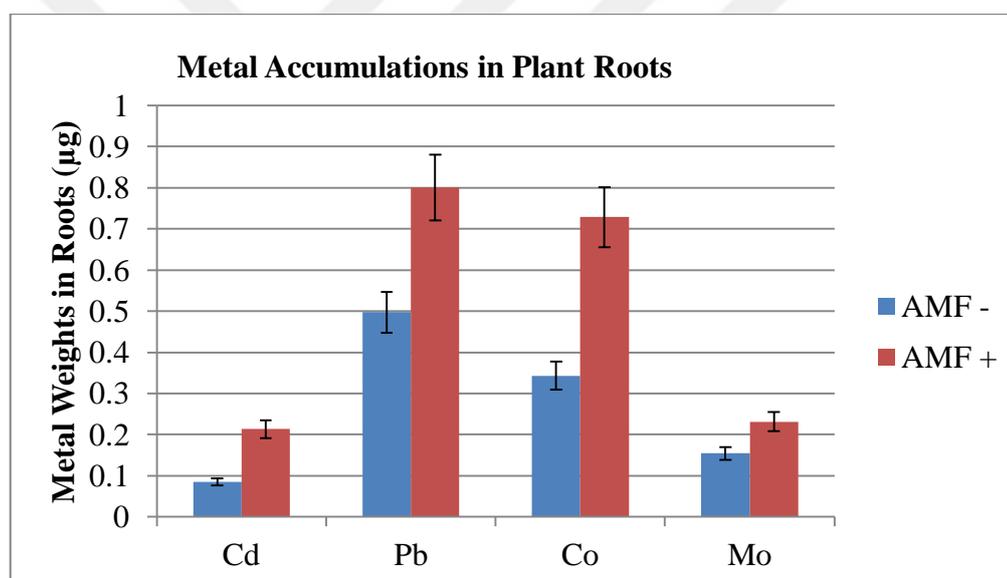


Figure 4.30. Metal accumulation in plant roots grown in sewage sludge.

#### 4.7.2. Second Run Plantation

In the second run plantation, there were considerable differences in the shoot heavy metal uptakes between non-inoculated plants and *G. mosseae* inoculated plants except 10 and 30 g/kg sludge applied plants. Especially, *G. mosseae* association without sewage sludge application increased the total metal uptakes in plant shoots. The 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 and co-workers (2006) 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.

Cr extractions by sunflower (*Helianthus annuus*) shoots were given in Figure 4.31. Cr accumulation increased by 100% with the mycorrhizal association (in pots MMos), 50% with sludge addition (in pots MS10) and 34% with sludge addition (in pots MS20) respectively. When inoculated plants were grown in mine tailings amended with 20g/kg sludge addition, the plant shoot uptake increased by 225%. The results of the study were in agreement with the study of Anna, AMF increases the Cr uptake in sunflower (*Helianthus annuus*).

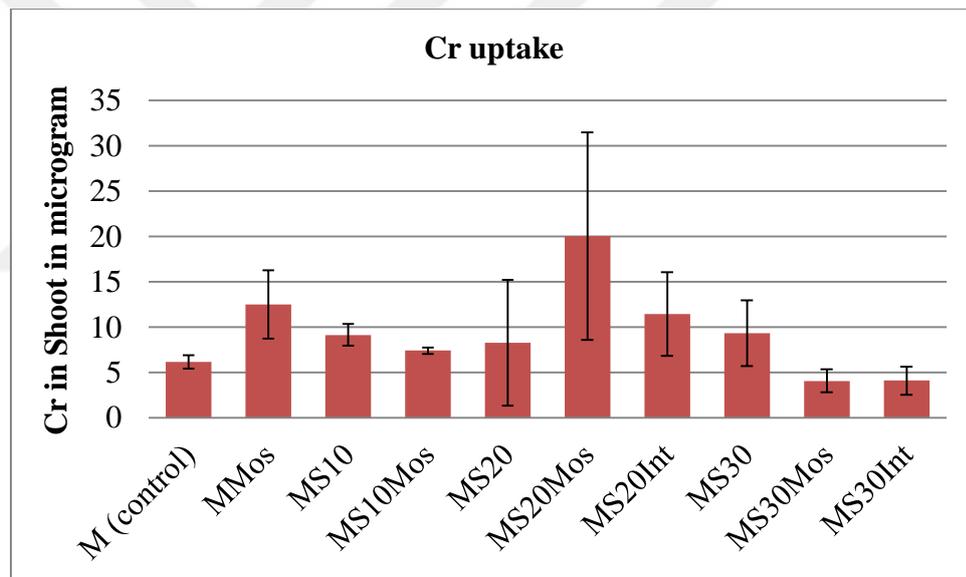


Figure 4.31. Chromium accumulation in the shoot of the second run (M: Mine Tailings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

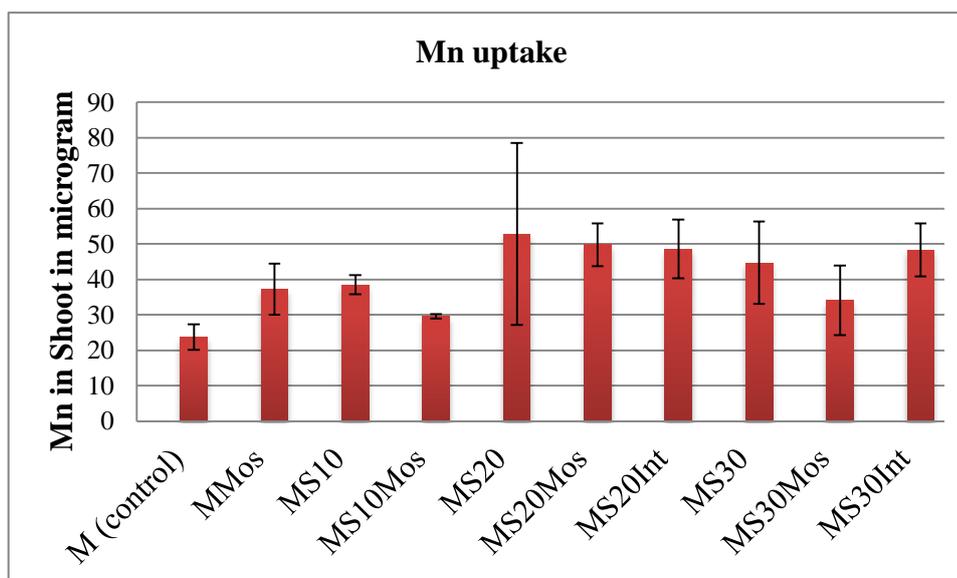


Figure 4.32. Manganese accumulation in the shoot of the second run (M: Mine Tailings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

Mn extractions by sunflower (*Helianthus annuus*) shoots were given in Figure 4.32. Mn uptakes in plant shoots were improved with both AMF association (in pots MMos) and 10, 20 and 30 g/kg sludge addition (in pots MS10, MS20 and MS30) by 57%, 54%, 122%, and 87% 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 tailings, without mycorrhizal association, increased the Mn uptakes in sunflower (*Helianthus annuus*) shoots. High phosphorus content of sludge was thought to increase the solubility of manganese. Shuman (1988) reported that Mn uptake increased with phosphorus level of the soil in non-inoculated plants. Mycorrhizal association caused a slight decrease in Mn accumulation in the plants grown in sludge amended pots (in pots MS20Mos). Liu and co-workers (2000) observed that Mn accumulation was lower in the inoculated plants than non-inoculated in the presence of high micronutrient concentrations. Furthermore, Arines and co-workers (1992) 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. In this study, both sludge addition and *G. mosseae* inoculation may have increased the Mn-oxidising bacteria population.

If the symbiosis between fungus and plant is effective in terms of protection against excess Mn, then total Mn uptake should be lower in plants with mycorrhizae (Pacovsky, 1986).

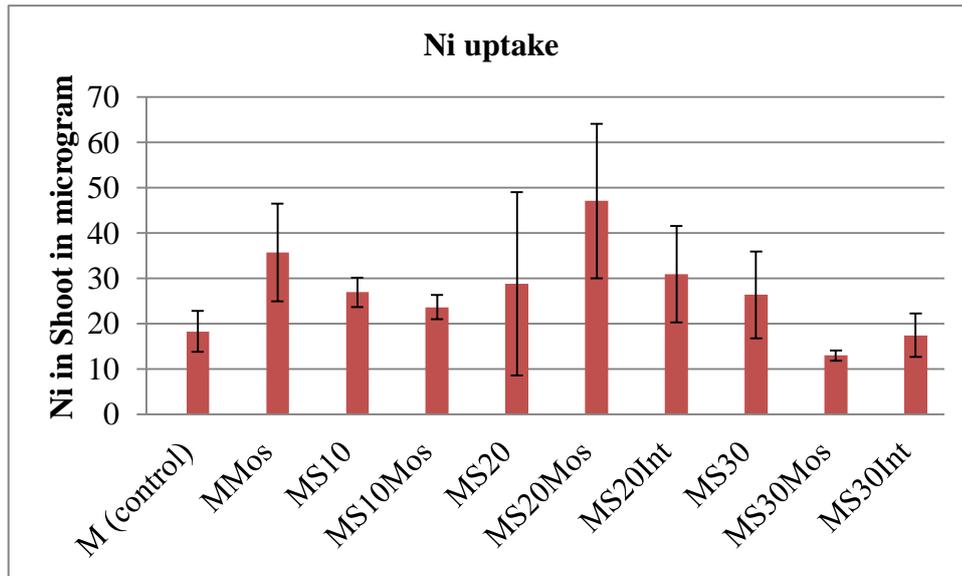


Figure 4.33. Nickel accumulation in the shoot of the second run (M: Mine Talings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

Ni extractions by sunflower (*Helianthus annuus*) shoots were given in Figure 4.33. Nickel uptakes of sunflowers (*Helianthus annuus*) were higher in pots MS20Mos and MMos. Mycorrhizal association increased the Ni accumulation in the plant shoots. Citterio and co-workers (2005) also observed that *Glomus mosseae* colonization increased the Nickel uptake in *Cannabis sativa* stem and leaf of inoculated plants grown in contaminated soil.

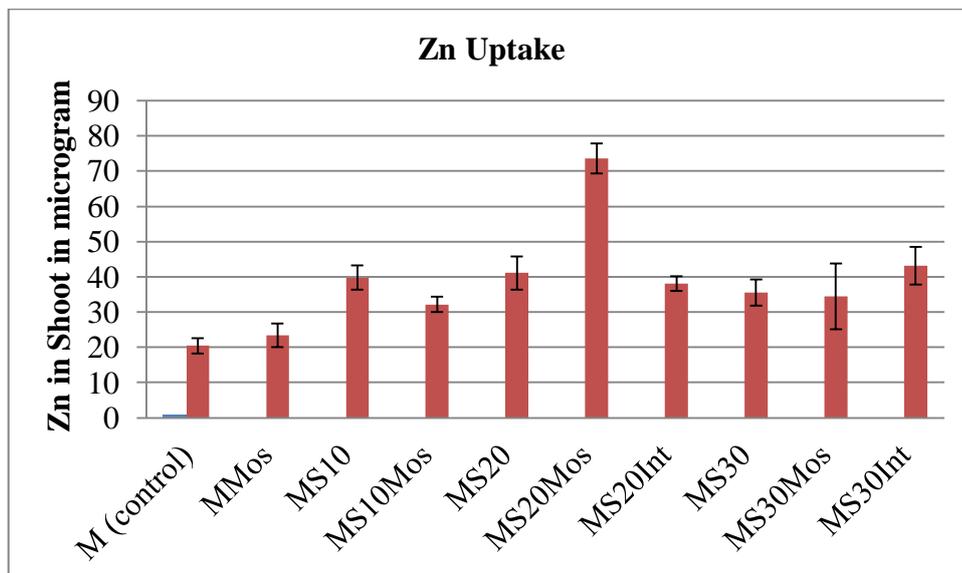


Figure 4.34. Zinc accumulation in the shoot of the second run (M: Mine Talings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

Zn extractions by sunflower (*Helianthus annuus*) shoots were given in Figure 4.34. 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 (2006) pointed that zinc plant content was higher in plants inoculated with *G. mosseae* or *G. intraradices*. Moreover, zinc and phosphorous are antagonistic metals because their hyphal uptake and translocation mechanisms are similar (Cooper and Tinker, 1978). Zn uptake increases with the sludge addition like the first run plantation due to the sewage sludge addition containing high Zn and P. Therefore, mycorrhiza didn't need an uptake of phosphorous by mycorrhizal hyphae. The plant in this scenario would take enough amount of P by roots. AMF enhanced plant Zn uptake with the mycorrhizal hyphae.

Christie and Chen (2004) showed that mycorrhizal association was improved the root Cu acceptance, nevertheless in highly contaminated soils Zn uptake were reduced by the mycorrhizal plants shoot to tolerate the toxic effect of zinc.

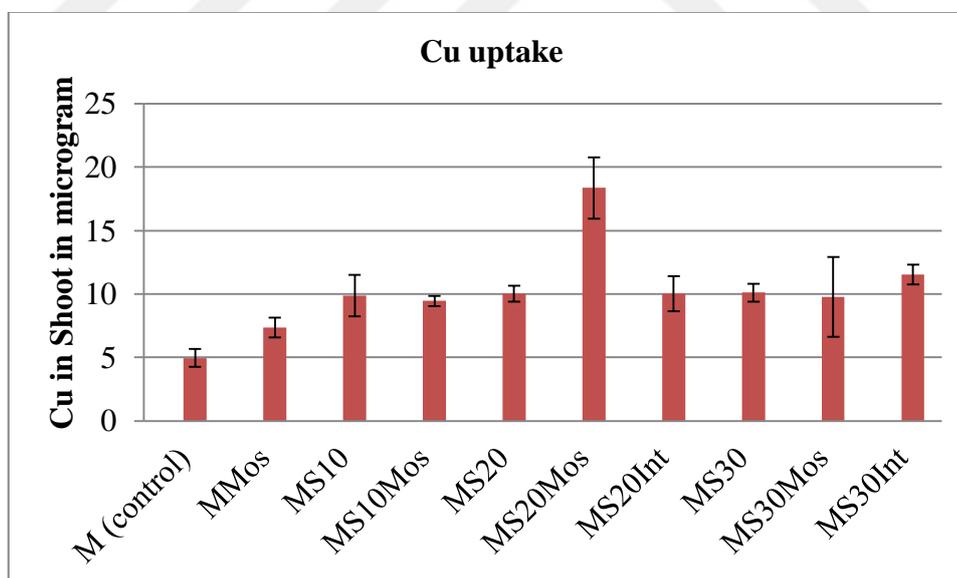


Figure 4.35. Copper accumulation in the shoot of the second run (M: Mine Tailings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

In the same way, plant shoot Cu uptakes were found to be high in AMF inoculated plants. It was shown in Figure 4.35. 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 (Tarafdar and Maraschner, 1995). Contrarily, Joner and co-workers (2001) found that Cu uptakes were 50% low in the inoculated clover shoots.

Iron, aluminum and silicon uptakes were showed in Figure 4.36, 4.37 and 4.38. The accumulations in the host plant shoots were high in the 20 Mos and the Mos treatment. Clark and Zeto (2000) confirmed that Si accumulation was higher in the *Glomus mosseae* inoculation in maize plants.

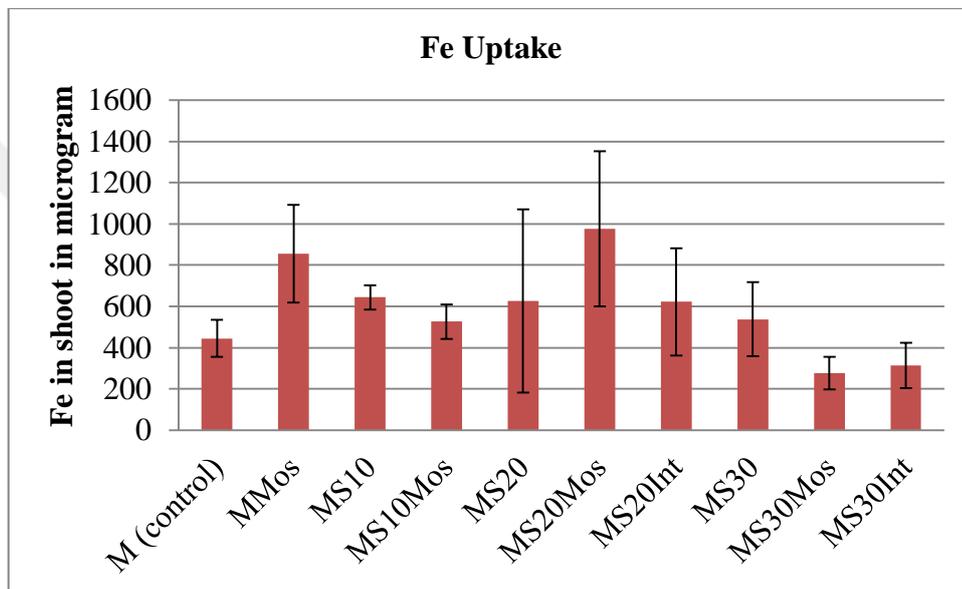


Figure 4.36. Iron accumulation in the shoot of the second run (M: Mine Tailings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

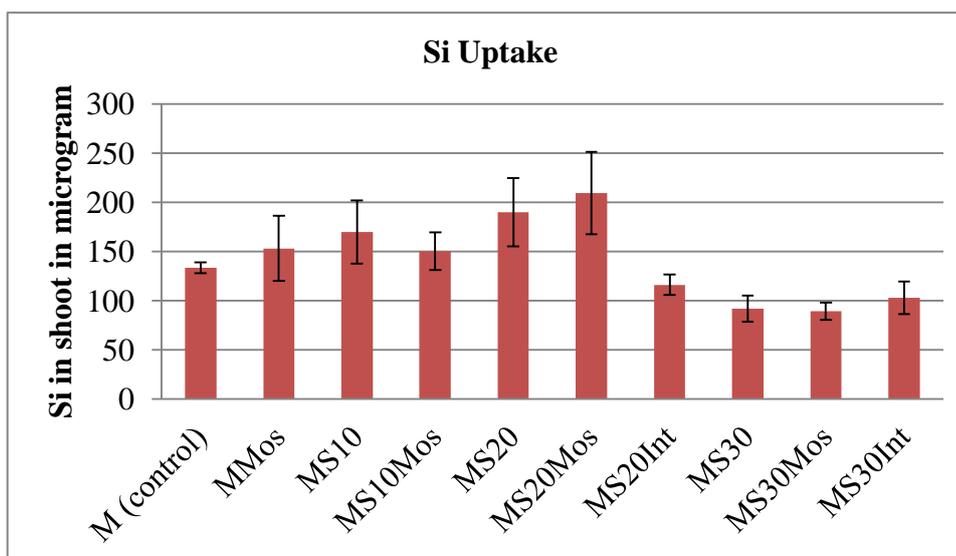


Figure 4.37. Silicon accumulation in the shoot of the second run (M: Mine Talings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

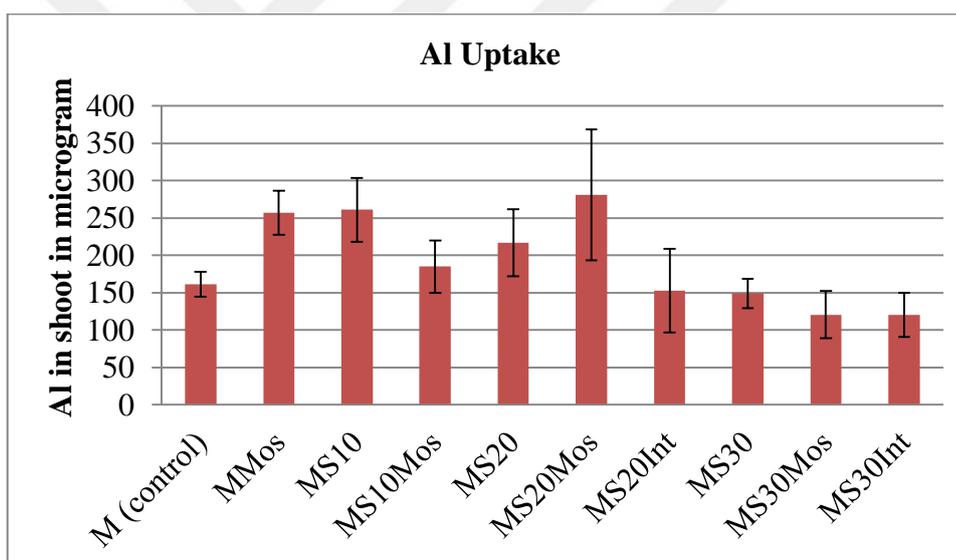


Figure 4.38. Aluminum accumulation in the shoot of the second run (M: Mine Talings; Mos: *G. mosseae*; Int: *G. intraradices*; S10: 10 g/kg Sewage sludge addition).

While Mn, Ni uptake exhibited different behaviours among the First and Second runs of greenhouse experiments, Cr, Cu, Zn, Al, Fe, and Si exhibited similar behaviours.

The variations in the metal uptakes of the plants can be attributed to the non-homogeneity of the samples taken from pots.

### 4.7.3 Cluster Analysis of Second Run Plantation

Cluster analysis was applied in this study to observe the differences of metal uptake by sunflower. The dendrogram obtained from hierarchical cluster analysis is shown in Figure 4.39. The case and group numbers appearing in Figure 4.39 were given in Table 4.4. The group numbers of 6, 7 and 8 represented Fe Al and Si, respectively. Cluster analysis revealed that the uptake of Fe (group number 6) was significantly different from the other metals regarding the rescaled distance cluster combine as 20. Since the uptake of Fe indicated a strong separation due to its high concentration, except case number 59, the part of the dendrogram grouping Fe was redrawn and given in Figure 4.40. The case number 59 refers to the combination of *G. mosseae* inoculation and 30 g sludge addition. This condition resulted with low Fe uptake. It confirms that the symbiotic relationship of mycorrhiza turned into parasite.

Si and Al were grouped together. It is likely that Fe, Si and Al are abundant in the earth's crust and the plant is accustomed to these metals, therefore, the uptake of these metals are expected to be higher than the other metals.

The metal uptake of Cr, Ni, Cu, Mn and Zn were grouped together (Figure 4.39). There is an exception for Zn in case number 46 which refers to the addition of 20 g of sludge and *G. mosseae* inoculation. In this condition, Zn uptake in plant shoots grouped together with the Fe, Al and Si because *G. mosseae* and sludge increased the Zn uptake by the shoots of plant.

The cluster analysis was performed separately to see in detail the mycorrhiza and sludge effect of Cr and Ni accumulations in plants because these metals were toxic for the environment. Figure 4.41 showed that 30 g sludge addition with the *G. mosseae* and *G. intraradices* grouped together and AMF inoculation decreased the accumulation of Cr in plant shoots. The Cr uptake by the shoots of plant, 10 g/kg, 20 g/kg and 30 g/kg sludge grouped together. Also, Figure 4.41 indicated that control and 10 g/kg sewage sludge addition with *G. mosseae* inoculation grouped together implying that this condition has no effect on the Cr uptake. The reason for this couldn't be explained with the information we gathered from this part of the study. It would be better to repeat and understand the reason behind this outcome. On the other hand, the addition of 20 g/kg sludge with *G. mosseae* inoculation increased the uptake of Cr.

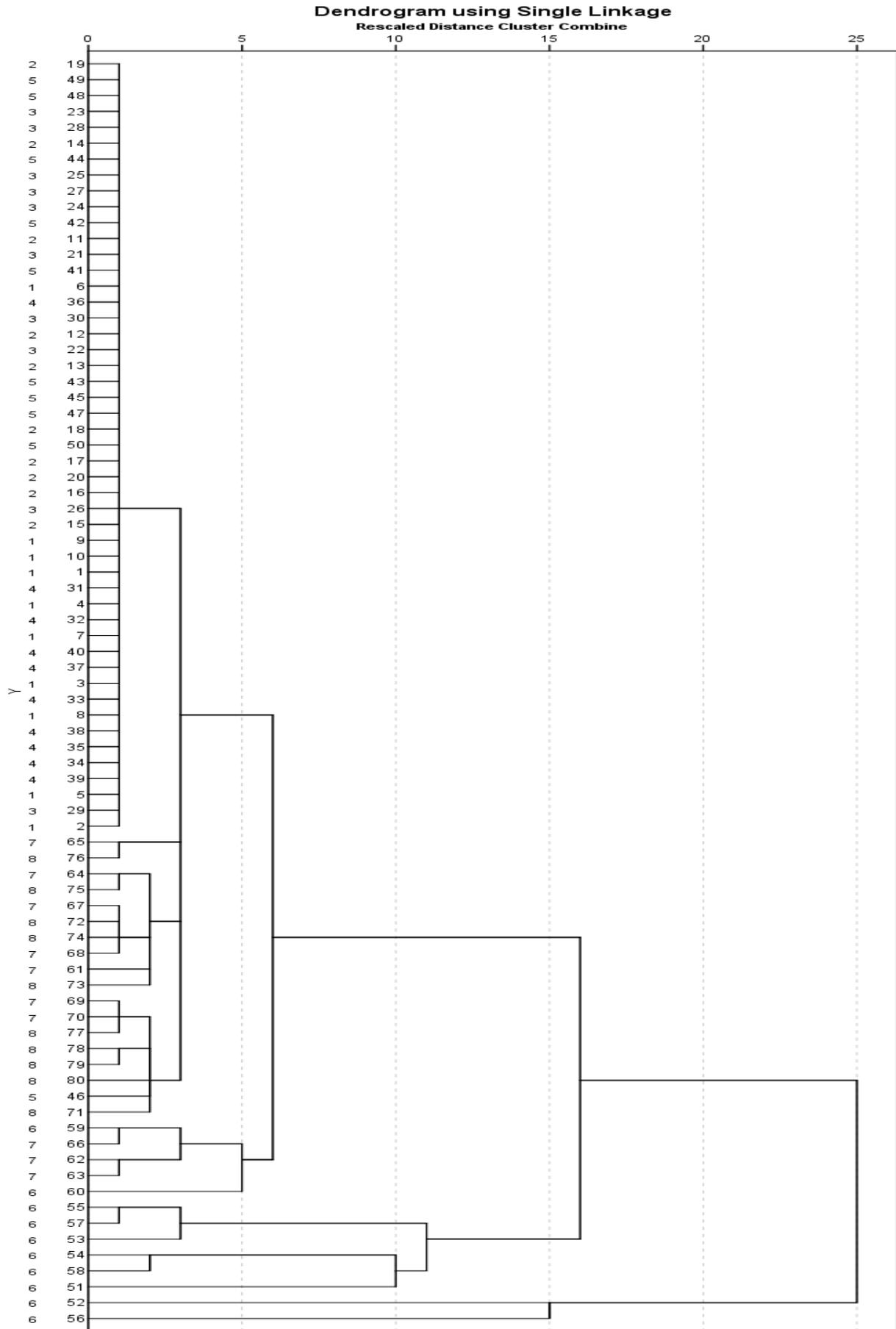


Figure 4.39. Hierarchical dendrograms for clustering of metals with respect to their uptake by sunflower.

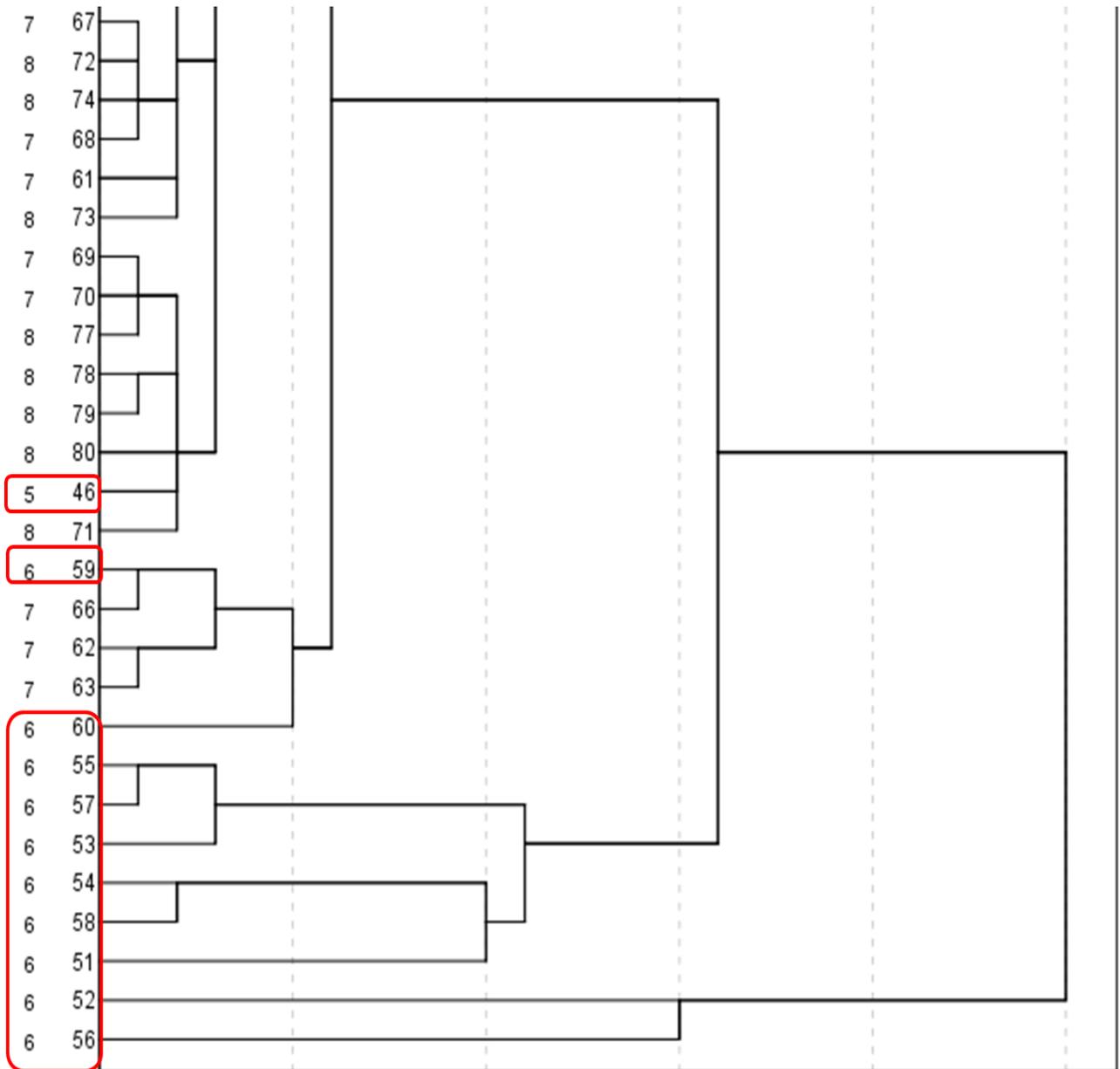


Figure 4.40. Hierarchical dendrograms for clustering of metals with respect to their uptake (Redrawing of Figure 4.40 focusing on the grouping of only Fe)

Table 4.4. Meaning of terms used in cluster analysis.

Case No	Metal Name	Metal No	Mycorrhizae	Mycorrhizae No	Sludge Addition	Sludge No	Metal Uptakes ( $\mu\text{g}$ )
1	Cr	1	No	1	-	1	6.15
2	Cr	1	<i>G. mosseae</i>	2	-	1	12.47
3	Cr	1	No	1	10 g/kg	2	9.12
4	Cr	1	<i>G. mosseae</i>	2	10 g/kg	2	7.38
5	Cr	1	No	1	20 g/kg	3	8.24
6	Cr	1	<i>G. mosseae</i>	2	20 g/kg	3	20
7	Cr	1	<i>G. intraradices</i>	3	20 g/kg	3	11.42
8	Cr	1	No	1	30 g/kg	4	9.28
9	Cr	1	<i>G. mosseae</i>	2	30 g/kg	4	4.06
10	Cr	1	<i>G. intraradices</i>	3	30 g/kg	4	4.07
11	Mn	2	No	1	-	1	23.74
12	Mn	2	<i>G. mosseae</i>	2	-	1	37.27
13	Mn	2	No	1	10 g/kg	2	38.45
14	Mn	2	<i>G. mosseae</i>	2	10 g/kg	2	29.62
15	Mn	2	No	1	20 g/kg	3	52.87
16	Mn	2	<i>G. mosseae</i>	2	20 g/kg	3	49.84
17	Mn	2	<i>G. intraradices</i>	3	20 g/kg	3	48.62
18	Mn	2	No	1	30 g/kg	4	44.75
19	Mn	2	<i>G. mosseae</i>	2	30 g/kg	4	34.17
20	Mn	2	<i>G. intraradices</i>	3	30 g/kg	4	48.36
21	Ni	3	No	1	-	1	18.26
22	Ni	3	<i>G. mosseae</i>	2	-	1	35.71
23	Ni	3	No	1	10 g/kg	2	26.92
24	Ni	3	<i>G. mosseae</i>	2	10 g/kg	2	23.62
25	Ni	3	No	1	20 g/kg	3	28.78
26	Ni	3	<i>G. mosseae</i>	2	20 g/kg	3	47.05
27	Ni	3	<i>G. intraradices</i>	3	20 g/kg	3	30.88
28	Ni	3	No	1	30 g/kg	4	26.34
29	Ni	3	<i>G. mosseae</i>	2	30 g/kg	4	12.93
30	Ni	3	<i>G. intraradices</i>	3	30 g/kg	4	17.41
31	Cu	4	No	1	-	1	4.96
32	Cu	4	<i>G. mosseae</i>	2	-	1	7.35
33	Cu	4	No	1	10 g/kg	2	9.88
34	Cu	4	<i>G. mosseae</i>	2	10 g/kg	2	9.45
35	Cu	4	No	1	20 g/kg	3	10.01
36	Cu	4	<i>G. mosseae</i>	2	20 g/kg	3	18.37
37	Cu	4	<i>G. intraradices</i>	3	20 g/kg	3	10.03
38	Cu	4	No	1	30 g/kg	4	10.1
39	Cu	4	<i>G. mosseae</i>	2	30 g/kg	4	9.77

Case No	Metal Name	Metal No	Mycorrhizae	Mycorrhizae No	Sludge Addition	Sludge No	Metal Uptakes (µg)
40	Cu	4	<i>G. intraradices</i>	3	30 g/kg	4	11.51
41	Zn	5	No	1	-	1	20.44
42	Zn	5	<i>G. mosseae</i>	2	-	1	23.37
43	Zn	5	No	1	10 g/kg	2	39.76
44	Zn	5	<i>G. mosseae</i>	2	10 g/kg	2	32.16
45	Zn	5	No	1	20 g/kg	3	41.07
46	Zn	5	<i>G. mosseae</i>	2	20 g/kg	3	73.6
47	Zn	5	<i>G. intraradices</i>	3	20 g/kg	3	38.07
48	Zn	5	No	1	30 g/kg	4	35.46
49	Zn	5	<i>G. mosseae</i>	2	30 g/kg	4	34.44
50	Zn	5	<i>G. intraradices</i>	3	30 g/kg	4	43.18
51	Fe	6	No	1	-	1	444.45
52	Fe	6	<i>G. mosseae</i>	2	-	1	855
53	Fe	6	No	1	10 g/kg	2	643.51
54	Fe	6	<i>G. mosseae</i>	2	10 g/kg	2	525.75
55	Fe	6	No	1	20 g/kg	3	625.81
56	Fe	6	<i>G. mosseae</i>	2	20 g/kg	3	976.05
57	Fe	6	<i>G. intraradices</i>	3	20 g/kg	3	622.79
58	Fe	6	No	1	30 g/kg	4	537.39
59	Fe	6	<i>G. mosseae</i>	2	30 g/kg	4	276.43
60	Fe	6	<i>G. intraradices</i>	3	30 g/kg	4	314.25
61	Al	7	No	1	-	1	160.98
62	Al	7	<i>G. mosseae</i>	2	-	1	256.88
63	Al	7	No	1	10 g/kg	2	260.94
64	Al	7	<i>G. mosseae</i>	2	10 g/kg	2	185.09
65	Al	7	No	1	20 g/kg	3	216.72
66	Al	7	<i>G. mosseae</i>	2	20 g/kg	3	281.13
67	Al	7	<i>G. intraradices</i>	3	20 g/kg	3	153.08
68	Al	7	No	1	30 g/kg	4	149.07
69	Al	7	<i>G. mosseae</i>	2	30 g/kg	4	120.66
70	Al	7	<i>G. intraradices</i>	3	30 g/kg	4	120.16
71	Si	8	No	1	-	1	133.55
72	Si	8	<i>G. mosseae</i>	2	-	1	153.12
73	Si	8	No	1	10 g/kg	2	169.9
74	Si	8	<i>G. mosseae</i>	2	10 g/kg	2	150.47
75	Si	8	No	1	20 g/kg	3	190.22
76	Si	8	<i>G. mosseae</i>	2	20 g/kg	3	209.48
77	Si	8	<i>G. intraradices</i>	3	20 g/kg	3	116.18
78	Si	8	No	1	30 g/kg	4	91.75
79	Si	8	<i>G. mosseae</i>	2	30 g/kg	4	89.14
80	Si	8	<i>G. intraradices</i>	3	30 g/kg	4	103.09

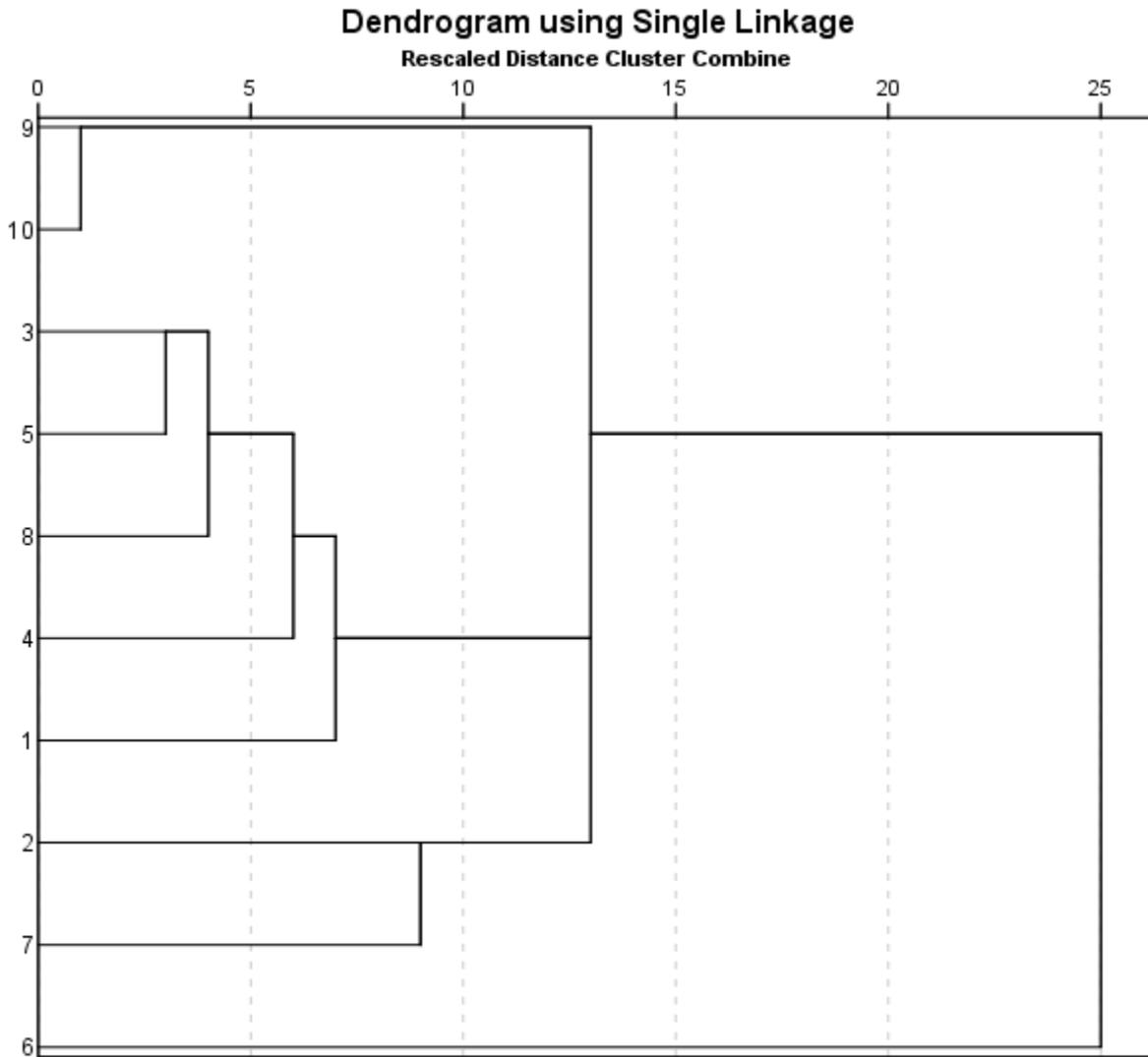


Figure 4.41. The evaluation of plant Cr uptake by Hierarchical cluster analysis on dendrogram.

Cluster analysis applied only on the uptake of Ni by the shoots of plant in different conditions and dendrogram are shown in Figure 4.42. The sludge addition didn't affect the uptake of Ni as the uptake of Cr. The addition of 10, 20, 30 g sludge referring 23, 25 and 28, respectively grouped together. The hierarchical cluster analysis indicated that 20 g/kg sludge addition with *G. mosseae* inoculation increased the uptake of Ni (Case No: 26) because it grouped separately from the others.

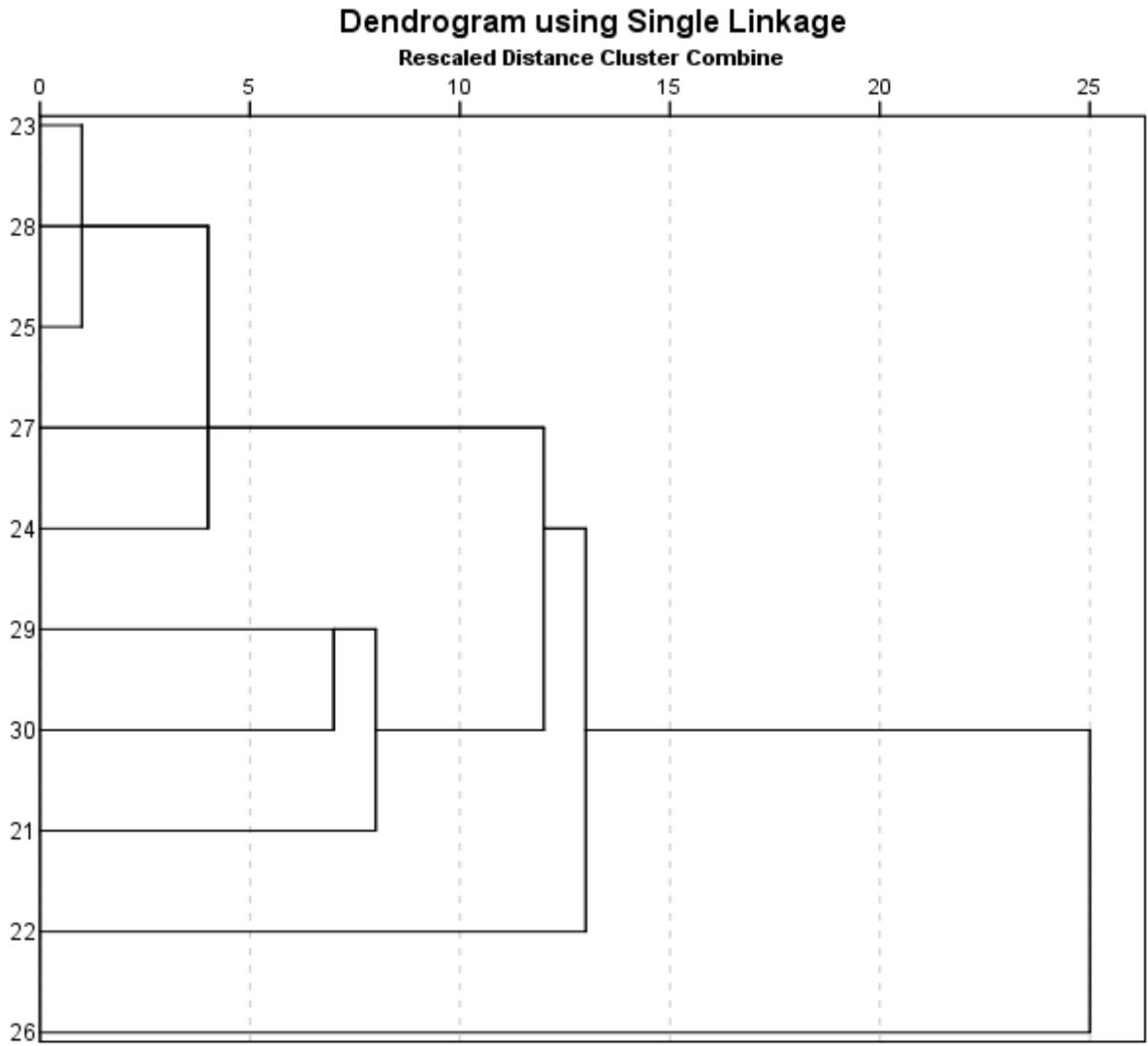


Figure 4.42. The evaluation of plant Ni uptake by Hierarchical cluster analysis on dendrogram.

## 5. CONCLUSION

The symbiotic relationship of AMF to sunflower (*Helianthus annuus*) roots, sewage sludge applications and addition of buffer soil were observed to increase the phyto-extraction of Cr mine tailings.

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 (*Helianthus annuus*) mycorrhizal colonization. However, *G. intraradices* were more tolerable to plant growth in presence of high P levels.

The combined AMF and 20 g/kg sludge amendments resulted with the highest plant heavy metal uptakes and phytoremediation efficiency. Therefore, sewage sludge increased the body mass of the sunflowers (*Helianthus annuus*) and phytoextraction efficiency of mine tailings. Mycorrhizal plants increased the surface area of the plant roots enabling them to reach more metal and nutrients. 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* suppressed the plant growth in soils having optimum phosphorus concentration due to the carbon competition between the host plant and the mycorrhizal fungi.

The statistical analysis (Cluster analysis) was applied in this study and it also showed that the combined AMF and 20 g/kg sludge amendments resulted with the highest plant heavy metal uptakes and phytoremediation efficiency.

Soil bulk density may hold crucial significance for phytoextraction proficiency with AMF interactions and plant body mass.

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