PLANT AND MICROBE INTERACTIONS IN THE PHYTOREMEDIATION OF HEAVY METAL CONTAMINATED SOILS

by

MEHMET MERİÇ TUNALI

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PLANT AND MICROBE INTERACTIONS IN THE PHYTOREMEDIATION OF HEAVY METAL CONTAMINATED SOILS

In this study, phytoremediation; the amount of heavy metal (Cu) uptake by plant species, host plants' symbiosis potential with two different species of arbuscular mycorrhizal fungi (AMF), interaction between AMF and host plants for sequestration of Cu available in the soil, as well as generation of glomalin-related soil protein (GRSP). Heavy metal concentrations were 100, 500 and 1000 ppm. For phytoremediation, two commercial crops (sorghum and sunflowers) which are widely cultivated in majority of agricultural fields in Turkey were used as host plants.

The results showed a strong variability in sunflower and sorghum plants colonized with both AMF species in terms of GRSP (0.6–11.2 mg g^{-1} and 0.2-10.75 mg g^{-1}), respectively. Cu content (29.34–249.86 mg kg^{-1} for the total Cu in sunflower and 12.06–73.97 mg kg^{-1} for sorghum rhizosphere colonized by *G. mosseae* (up to 60%) were observed.

This study provides evidence on the role of the plant microbe interactions in Cu sequestration as well as Glomalin generation related to heavy metal stimulation. Findings suggest highly efficient mechanism for AMF to mitigate stress leading to stabilization of polluted soils. Results also provide a new suggestion on the contribution of Glomalin in copper sequestration in polluted soils.

AĞIR METAL İLE KONTAMİNE OLMUŞ TOPRAKLARIN FİTOREMEDİASYONUNDA BİTKİ VE MİKROORGANİZMA ETKİLEŞİMLERİ

Bu çalışmada, fitoremediasyon; bitki türleri tarafından kontamine topraktan ağır metalin (Cu) uzaklaştırılması, konak bitkilerin iki farklı tür arbusküler mikoriza mantarı (AMF) ile simbiyoz potansiyeli, mikoriza ile bitki etkileşiminin toprakta mevcut olan kirletici bakırın stabilizasyonu ve topraktan ayrılması, yanı sıra arbüsküler mikoriza mantarı tarafından üretilen, glikoprotein olan toprak protein Glomalin (GRSP). Topraktaki ağır metal konsantrasyonları 100, 500, ve 1000 ppm'dir. Fitoremediasyon çalışması için, Türkiye'deki tarım alanlarında yaygın olarak üretilen iki tür ticari mahsül olan sorgum ve ayçiçeği bitkileri konak bitki olarak kullanılmıştır.

Sonuçlar; arbüsküler mikoriza türleriyle koloni oluşturmuş olan ayçiçeği ve sorgum bitkilerinin glomalin (GRSP) seviyelerinde ciddi artış göstermektedir (sırası ile 0.6 – 11.2 mg g^{-1} ve 0.2-10.75 mg g^{-1}). Ağır metal – bakır muhtevası *G. mosseae* ile mikorizasyonun %60'a kadar ulaştığı ayçiçeği bitkisinde toplam bakır miktarı 29.34 – 249.86 mg kg^{-1}, sorghum bitkisinde ise 12.06 – 73.97mg kg^{-1} olarak gözlemlenmiştir.

Bu çalışmada elde edilen sonuçlar, bitki ve mikoriza etkileşiminin topraktan kirletici (Cu) uzaklaştırılmasında ve bitki kökünde oluşan mikorizasyon tarafından ağır metal uyarımı sonucu üretilen Glomalin miktarları üzerindeki etkisini kanıtlar niteliktedir. Bulgular, yüksek miktarda kontaminasyona uğramış toprakların oluşturduğu baskının mikoriza aktivitesi ile hafifletilerek toprak stabilizasyonuna katkıda bulunduğunu desteklemektedir. Ayrıca, Glomalin üretiminin topraktan kirletici giderilmesi mekanizması üzerindeki etkisi konusunda yeni bir yaklaşım sunmaktadır.

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Abbreviation	Explanation
HM	Heavy Metal
HMCS	Heavy Metal Contaminated Soils
AMF	Arbuscular Mycorrhizal Fungi
GRSP	Glomalin-Related Soil Protein
PGPR	Plant Growth Promoting Rhizosphere
GST	Glutatthione S-Transferares
EKRT	Elektro-Kinetic Remediation Technology

1. INTRODUCTION

Soil structure and mechanisms inside soil are important environmental factors because of their role as natural buffers; hence, controlling the transport of nutrients and contaminants to the atmosphere, hydrosphere and biosphere [1]. Therefore due to these interactions, soil contamination has been increasing over the years by natural processes and anthropogenic activities.

European Environment Agency (EEA) reported a dramatic estimation of total contaminated areas across Europe, which covers 2.5 million sites. 14% of these presumably polluted areas (340,000 sites) need an immediate remediation planning [2]. Consequently, to build a suitable environment that can support organism activities in the ecosystem by reducing the contamination in soil, planning should involve appropriate implemental remediation methods which covers; economic feasibility, efficiency, and applicability to the concerned area [3].

Through the soil pollution, heavy metals are common compounds which are metallic chemical elements found naturally on earth. Industrial development triggered their accumulation in the nature and soil; therefore, heavy metal pollution emerges as a main concern. Two essential resources of heavy metals can be listed as: (i) natural background: parent rock is a main source for this kind of heavy metal concentration and (ii) anthropogenic activities: including urbanization, agro-chemical, organic amendments, chemical fertilizers, sewage sludge and industrial waste.

Human activities and its input exceeded the natural input of several heavy metals into the soil on regional and global scales [4,5]. It is well known that heavy metals in higher doses have toxic effects for ecological life and human health. Therefore, it is crucial to develop reasonable policies for controlling heavy metal pollution as well as implementing proper soil remediation techniques in order to manage contaminated areas in this respect.

Remediation of heavy metal contaminated soils (HMCS) can be carried out via various techniques and applications. These methods can be classified as, (i) physical methods; such as thermal desorption & destruction and soil replacement method, (ii) chemical methods; including electrokinetic remediation, chemical leaching, chemical fixation [6], chemical extraction, and nanoremediation (iii) biological methods; phytoremediation, biological remediation.

Biological methods are getting more attention recently by legal and governmental authorities based on their economical reliability and environmental aspects. Among the biological methods for soil remediation; phytoremediation, explained as the use of plants for the reclamation of air, soil and water contaminated by organic and inorganic pollutants, had increased its importance during the last decade.

In soil pollution, phytoremediation is becoming the pioneer and developing technology for degradation and removal of pollutants in developed countries because of its advantages such as; cost effectiveness, beneficial environmental impact, and public acceptance. However, because of long term operation to perform cleaning of contaminated sites this technique has also some drawbacks, when compared to traditional engineering technologies. These technologies are much faster than biological remediation means but also extremely expensive for the municipalities and may disturb the ecological life within the area on environmental aspect [7].

Soils, as a host environment have tremendous number of biological and non-biological elements and species. Biological ecosystem in soil is the mixture of thousands of species of fungus, bacteria, protozoa (microorganisms) nematodes, worms and other living things which all together contribute as first level to the food chain in biosphere. Among soil biological elements arbuscular mycorrhizal fungi (AMF) are the most common soil microorganisms in natural and agricultural soils. Main organs of plants growth, roots, can form symbiotic associations meaning beneficial relationship with AMF to develop and promote growth. In this respect plant plays as host organism, while AMF support plants' life through this symbioses relationship. In nature, flora and fauna are generally able to adapt and survive when encountered with crisis such as drought, salinity, water flood and toxic substances like heavy metals via adjustment and adaptation abilities.

Soil-plant interaction studies have demonstrated that AMF can help their host plants to endure heavy metal stress, and can increase metal uptake and translocation to plant bodies. Corresponding the host plants provide AMF with photosynthesis outcome like carbon compounds, and in return, AMF obtain nutrients (*i.e.* phosphorus and nitrogen) which are not available for plant roots or too low for their hosts. In the specific and dramatically circumstance this relationship lead the AMF to produce metal chelation of glomalin, fungal polyphosphates and metallothioneins that have high binding capacities for heavy metal [8].

Mycorrhizal (AMF) interaction with plants produces the glycoprotein (Glomalin) [9,10]. In various soils glomalin is detected in large amounts as glomalin-related soil protein (GRSP) [11]. Glomalin is a mean of carbon storage in the soil and has been studied from so many angles and objectives. Glomalin is the product of sequestration of potentially toxic element as heavy metals (HM), and plays an important role in soil stability. It is established that, some well-known heavy metals such as Cu, Cd, Pb and Zn can bind with GRSP and sequestration occurs [12,13].

In this study, heavy metals (Cu) contaminated soil and its phytoremediation potential was simulated for evaluating the role of different interactions between different mycorrhiza (AMF) species such as *Glomus intraradices* and *Glomus mosseae*; and plant growth promoting rhizosphere (PGPR) in scope of soil phytoremediation.

Specific objectives of this study are as follows;

(1) Evaluate the role of soil microbial (AMF) and host plants interactions in the formation of appropriate phytoremediation technique to improve soil health, stabilization and sustainability by the uptake rate of heavy metals from the surface soil.

(2) Assess the formation of soil proteins under exposure of heavy metals.

(3) Understand functional role of mycorrhizal fungi diversity in formation of specific phytoremediation networks.

This study aimed to observe the absorption, translocation, efflux, and metabolites of selected heavy metals as inorganic pollutants in the plant-root-rhizosphere system. Heavy metals influence on two different species of AMF (*Glomus mosseae* and *Glomus intraradices*), and effect of this interaction on plant growth and heavy metal uptake performance. In order to understand the functions of the plant-microbe-soil interactions in rhizosphere, ecology of mycorrhizosphere network and the diversity of plant-microbe-soil combinations, particularly the tight co-operation between different species of AMF soil bacteria and plant root in the rhizosphere were monitored. Therefore, the aim of this study was to understand the basic processes in the root zone and correlating them with the microorganism symbioses effect on plant growth and heavy metal remediation capacity in contaminated soils.

2. LITERATURE REVIEW

2.1. Heavy Metals Pollution

In the recent centuries due to industrial progression, anthropogenic activities triggered the extensive release of industrial wastes, a complex mixture of materials and components. These enormous quantities of industrial pollutants, containing several hundreds of different substances have been released into the environment over years and many of them are toxic and create serious pollution in agricultural ecosystems upon disposal.

Due to the nature of heavy metal pollution sources, solid waste management is becoming more complicated by characteristic differences among industrial waste, particularly heavy metal involvement is challenging in this aspect. Inadequate practice and handling waste management by the industries resulted in continuous addition of organic and inorganic wastes in ecosystems. Safe and nature sense of urban and arable land prohibited by being encountered with heavy metals released and leached from neighborhood landfills and contaminates water sources to with toxic metals [14].

Heavy metals are thus commonly defined as those having a specific density of more than 5 g/cm^3. The main threats to human health from heavy metals are associated with exposure to lead, cadmium, mercury and arsenic (arsenic is a metalloid, but is usually classified as a heavy metal) [15].

The doses and concentration of heavy metals as well as their physical and chemical forms make them mobile or bioavailable. Heavy metals release into the environment can occur via a wide range of processes and pathways, including to the air (e.g. during combustion, extraction and processing), to surface waters (via runoff and releases from storage and transport) and to the soil (and hence into ground waters and crops) [16]. Heavy metal (Cu) concentrations on different contaminated sites are given in Table 2.1.

Bioaccumulation of heavy metals in flora and fauna proved to have toxic effects. Several studies showed that toxicity of the heavy metal - Cu applies to, microbial processes in soils [17], earthworms [18,19], plants [20,21], microalgae [22], aqueous plants [23], and fish species [24,25]. Also on environmental health hazard point of view, Cu has influence on various diseases observed in humans as well. Namely; hepatocerebral and neurodegenerative diseases such as; Alzheimer's [26], Parkinson's disease [26], Wilson's disease [26,27], Menkes disease [27,28], Skogholt's disease [29], and liver diseases [30]. Hence, it is crucial to implement remediation measures to areas contaminated with heavy metals.

Table 2.1. Cu concentration in soils on different heavy metal contaminated sites.

Location of the Site	Cu (ppm)	Reference
Yangtze Delta, China	32.4	[5]
Alaba, Nigeria	4308	[31]
Guiyu, China	683.8	[31]
Sarcheshmeh mine, Iran	110 - 1330	[32]
Darezaar mine, Iran	30 - 450	[32]
Sereydoon mine, Iran	150 - 210	[32]
Karachi, Pakistan	26.79	[33]
Eskişehir, Turkey	39.33	[34]
Novi Sad, Serbia	21.9	[35]
Vineyard – France	201-689	[36]
Vineyard – Italy	194-448	[36]
Palermo, Italy	77	[37]
Lus Tunas, Cuba	94	[38]

2.2. Remediation of Heavy Metal Polluted Soils

2.2.1. Physical Methods

Physical remediation of heavy metal contaminated soils can be classified with two main categories; (1) Soil replacement method, and (2) Thermal treatment methods.

(1) Application of soil replacement method is based on removing the polluted soil and importing fresh soil. This method provides fast remediation of the site; however, it is only applicable within a small area because of the required labor and high cost.

Among the engineering techniques soil replacement method can also be applied without removing the specified soil, by importing high amounts of fresh soil into the system. This method is based on physically diluting the contamination and decreasing the toxic effects caused by heavy metals [6]. This approach is also not suitable for large areas for the same reasons explained above.

(2) The thermal treatment (<1000 °C) and smouldering (600–1100 °C) remediation methods relies on heating the associated soils to high temperatures via different techniques and apply filtering to the volatilized contaminants. These two physical/engineering based applications, has been implemented and verified to remove significant amounts of organic pollutants from soil with low footsteps on environmental impacts.

Yet, remediation of the heavy metal contaminated sites by thermal means is more complex, since in-situ devices used for heating is expensive and desorption time for HM is rather long, also high temperatures affects the biological and chemical properties of the soil as well, after application of thermal means, restoring the soil introduces further expense, making the process more challenging [39,40]. Illustration of thermal remediation is presented in Figure 2.1.

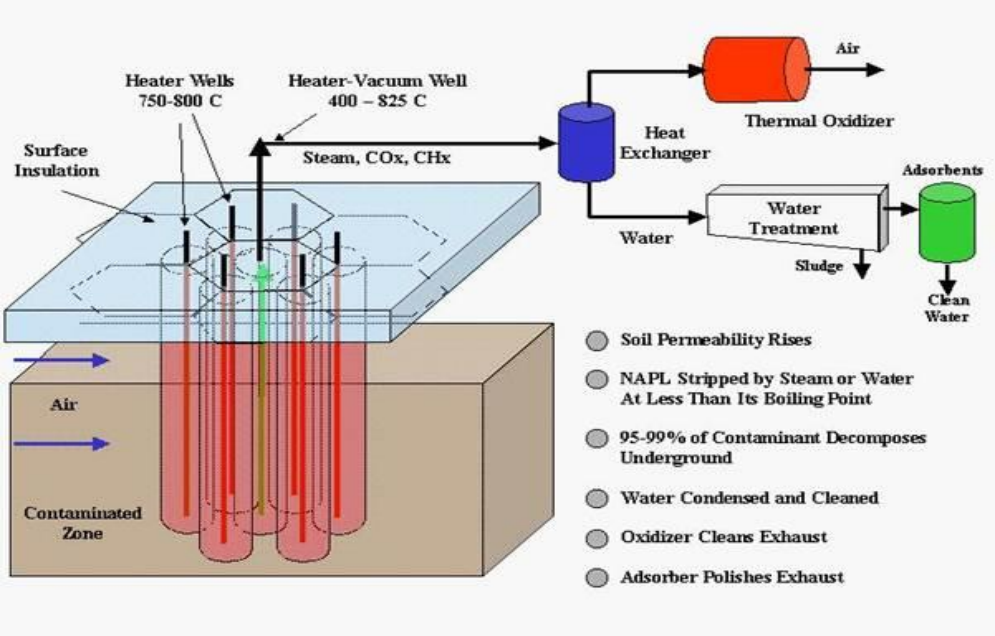

Figure 2.1. Schematic representation of thermal remediation [41].

2.2.2. Chemical Methods

Chemical methods used for reclamation of heavy metal polluted soils are; (1) Electro-kinetic remediation technology (EKRT),(2) Chemical fixation, (3) Chemical leaching, and (4) Nanoremediation.

(1) Electro-kinetic remediation technology (EKRT); which is an emerging method to treat both organic and inorganic pollutants. Working principle of the EKRT is using low level direct current between anodes and cathodes in soils to mobilize and recover the contaminants [42]. EKRT is suitable for low permeable soils and easy to operate with a low cost [43]. Recent studies on HM remediation using modified versions of EKRT resulted in 75% Cu removal [44], another study achieved 60% Hg removal [45], also 54% of Cu and 30% of As removal from contaminated soil observed [46].

(2) Chemical fixation; aims to immobilize the pollutants in the soil by introducing reagents and forming relatively low-toxic compounds and reduce their bioavailability and potential transport of pollutants to water bodies and other plants. Fe(hydr)oxides proved to be effective reagents used for heavy metal fixation (Cd, Cu, Ni, Pb and Zn). Results from a related research by Contin et al., on chemical fixation of heavy metals showed that, extractable HM compounds such as Cd, Cu and Zn decreased significantly in the arable

soil by 88%, 93%, 36% and grassland soil by 95%, 98%, 65% [47]. Another study carried out by using Mn(oxides) and Fe(oxides) for arsenic fixation from soil resulted in 56% and 67% reductions of leachable arsenic compared to untreated soil in two different sites [48].

(3) Chemical leaching; can be applied by using means adsorption, ions exchange, chelation, and precipitation with fresh water or reagents in liquid or gaseous forms to leach the pollutants such as heavy metals and recovering them from leachate; thus, remediating the contaminated soils. Plant-derived biodegradable compound – saponin can be used as biosurfactant on heavy metal contaminated soils. In a study carried out by Hong et al., using saponin utilization achieved 90-100% Cd, 85-98% Zn extraction from related soil sites [49].

(4) Nanoremediation; is the utilization of nanoparticles to treat contaminated bodies, and applicable to soil, groundwater, and surface waters. Successful applications of macroscale metallic substances for reclamation, there is an increasing interest on nanoscale materials for in situ remediation application [50]. Main drawback for using nanoscale materials is that widely used form for environmental remediation contains iron compounds, and due to lack of knowledge on the fate and transport of these nanoscale materials in the environment, ongoing researches pertaining to the potential toxicological effects of nanoscale materials [51].

2.2.2. Biological Methods

Due to their nature, heavy metals cannot be degraded or disintegrated by biological means; hence, biological methods for HM remediation solely relies on their bioaccumulation, and migration potential. Introducing biological processes for treatment of contaminated soils can be done via two different methods; (1) In-situ applications, such as phytoremediation, bioaugmentation, bioventing, and biosparging. (2) Ex-situ applications, including composting, land farming, and bioreactors.

Bioremediation methods are based on the activities of soil microorganisms', therefore; in situ applications are easily applicable and feasible. Soil microorganisms essentially need an energy source, and nutrients in a habitable environment (pH,

temperature, moisture, type of soil, oxygen level, and electron acceptor) with the absence of toxic constituents.

2.3. Phytoremediation

Phytotechnologies can be defined as the utilization of plants to remediate, treat, stabilize or control the contamination in soils, and phytoremediation is one of these, dedicated to the removal or the destruction of pollutants. The application of phytoremediation technologies, as an alternative to high cost chemical and physical methods, represents a great inexpensive opportunity to reclaim contaminated soils, maintaining or even improving their biological features. In fact, the main concern for biological studies of soil rehabilitation is to improve their efficiency. One of the disadvantages in phytoextraction technology regards the plants employed for metal extraction from soil, which are annuals and show a seed-to-seed life cycle occurring over a few months, usually in spring and summer [52]. Advantages and plausible drawbacks of phytoremediation technologies are detailed in Table 2.2, and some applications of these technologies in Europe region are presented in Table 2.3.

Table 2.2. Advantages and disadvantages of phytoremediation [53].

Advantages	Disadvantages / Limitations
Suitable for variety of organic and inorganic compounds.	Restricted to sites with shallow contamination within rooting zone of hyperaccumulators.
Remediation can be practiced both In Situ and Ex Situ.	Slow process, may take up to several years for remediation.
In Situ applications decrease the amount of soil disturbance compared to conventional methods.	Restricted to sites with low contaminant concentrations.
Can reduce the amount of waste up to 95%.	Harvested plant biomass from phytoextraction may be classified as a hazardous waste, hence, should be properly disposed.
In Situ applications decrease spread of pollutant via air and water.	Climatic conditions are a limiting factor for plant growth.
Does not require expensive equipment or highly specialized personnel.	Introduction of non-native species may affect biodiversity.
In large scale applications the biomass can be utilized to generate energy.	Consumption/utilization of contaminated plant biomass should be monitored.

Selective uptake of certain plant root systems are unique capabilities which phytoremediation method relies on. This combined with the metals translocation, bioaccumulation, and contaminant degradation abilities of the whole plant upper and under grand parts [54]. Studies showed that, various plants species have been successful in accumulating heavy metals such as lead, cadmium, chromium, and arsenic. This method also utilizes heavy metals that are essential for plant growth (Fe, Mn, Zn, Cu, Mg, Mo, and Ni). Some metals with unknown biological function (Co, Ag, Se, Hg) can also be accumulated [55].

Phytoremediation can be applied in various forms depending on contaminants and soil characteristics. For organic pollutants; phytodegradation, phytovolatilization, rhizofiltration, or rhizodegredation mechanisms can be implemented. For inorganic compounds such as heavy metals; suitable mechanisms of phytoremediation are; phytoextraction, phytostabilization, rhizofiltration, and phytovolatilization. Schematic explanation of pytoremediaton mechanisms are portrayed in Figure 2.2.

Phytoextraction, described as using plants to extract heavy metals from contaminated soil. Accumulated heavy metals in the shoots of grown plants and can be removed from soil by harvesting. Latest phytoextraction researches aims to unveil those species showing high biomass production that are native to the area requiring remediation and, are easily cropped [56].

Phytostabilization typically involves using suitable perennial plants to the contaminated area to immobilize the pollutants and reduce their bioavailability in soil bodies. The method depends on roots ability to cope with concerned contaminants' level in the soil. Phytostabilization can occur via sorption, precipitation, complexation, or metal valence reduction. The plantation's primary purpose is to reduce the amount of water percolating through the soil; thus, decreasing the risk of formation for hazardous leachate and prevent soil erosions [57].

Rhizofiltration, is similar to phytoextraction process but instead of plant shoots it focuses on the part of the plant below surface. Roots' ability to absorb, concentrate, and precipitate the contaminants from polluted areas determines the effectiveness of the

application. Rhizofiltration can partially treat industrial discharge, and can be used for various heavy metals' remediation such as lead, cadmium, copper, nickel, zinc and chromium, which are primarily retained within the roots [58].

During phytovolatilization, while organic and inorganic contaminants uptake from the soil, plant's biological processes can transform them into a different volatile form. Later on the final form of the pollutant components are transpired from plant shoots to the atmosphere. Phytovolatilization has been primarily used for the removal of mercury from soil, the mercuric ion is transformed into less toxic elemental mercury; yet, volatilized form of mercury released into the atmosphere is very likely to be recycled by precipitation and then redeposit back into ecosystem [59].

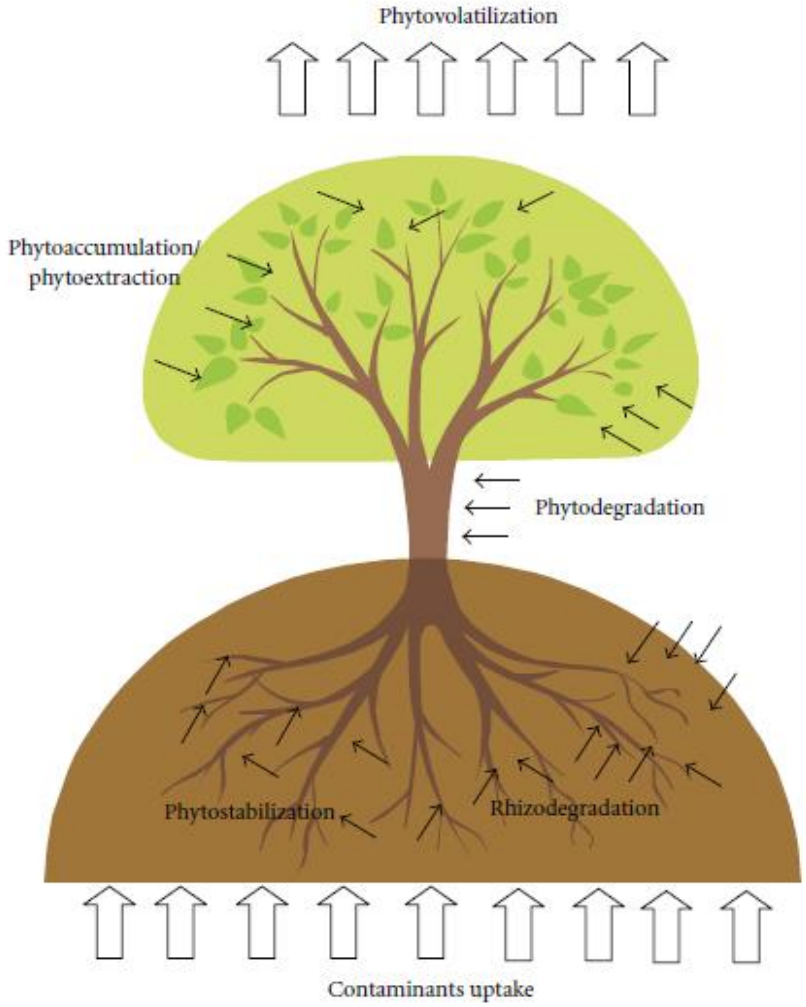

Figure 2.2. Schematic representation of phytoremediation mechanisms [55].

Phytodegradation, organic compounds which are present in the soil can be degraded by plant metabolism and reduce the amounts of related organic contaminants by revenue of transformation or break down. The breakdown of organics taken up by the plant to smaller molecules requires the presence of proteins and enzymes produced by the plant or by soil organisms such as bacteria or fungi. Phytodegradation can be successful as a result of the symbiotic relationship between plants and soil microbes. Symbiosis mechanism: plants provide nutrients necessary for the microbes to thrive, while microbes provide a healthier soil environment for the plant [60].

Table 2.3. Heavy metal phytoremediation field applications in Europe [61,62].

Site Location	Plant Species	Heavy Metals
Katovice, Poland	*Brassica juncea*	Pb, Cd
Switzerland, (Former Landfill)	*Salix viminalis (willow)*	Zn, Cd
Ronneburg, Germany	*Triticale, H. Annuus Brassica juncea*	Cd, Cu, Fe, Mn, Ni, Zn, Pb
United Kingdom, (Sewage Disposal Site)	*Salix species (willow)*	Ni, Cu, Zn, Cd
Hlemyzdi, Chech Republic	*H. annuus, C. sativa, Z. mays, C. Halleri*	Zn
Balen, Belgium	*Brassica napus*	Zn, Cd, Pb
Dornach, Switzerland	improved tobacco plants	Cu, Cd, Zn

Based on the phytoremediation method, plants' involvement for degradation or stabilization of concerned pollutants from soil depends on their growth activity. Optimum seasonal growth period enables plants to establish and develop their root area and expand in the rhizosphere as strong as possible. Duration of the growth period affects plants' performance to spread through rhizosphere wider, thus, stabilizing more heavy metals available in the soil. In addition, shoots of the plants continue to grow and produce more biomass as long as climate conditions are favourable. Mediterranean climate; which has hot and long term summer with longer sunshine duration is suitable for C_4 plants growth like Sorghum and Sunflower. Therefore, phytoremediation application in Turkey is suitable and it can be effective and feasible for remediation of heavy metal contaminated soils.

2.4. Plant Morphology

2.4.1. Hyperaccumulators

Hyperaccumulator plants are able to absorb remarkably high amounts of heavy metals, either as single elements or complex compounds from the soil when compared to other plant species [63]. Furthermore, the heavy metals are not just stabilized in the roots but also translocated to the shoot and accumulated in above ground organs, especially leaves. The accumulation concentrations in hyperaccumulator plants may exceed between 100–1000 fold higher than those found in non-hyperaccumulating species. Hyperaccumulators show no symptoms of phytotoxicity [64,65]. Despite having different feature, hyperaccumulation also be dependent on hypertolerance, an essential key property allowing plants to avoid heavy metal poisoning, to which hyperaccumulator plants are as sensitive as non-hyperaccumulators [66].

About 450 angiosperm species have been identified so far as heavy metal (As, Cd, Co, Cu, Mn, Ni, Pb, Sb, Se, Tl, Zn,) hyperaccumulators, accounting for less than 0.2% of all known species. However, recent reports reveals that with late identification of hyperaccumulator plants, the number of hyperaccumulators continue to increase [67,68]. It is very plausible that unidentified hyperaccumulators may function in nature. On the other hand, species classified as hyperaccumulators due to their analysis from field samples might be removed from the hyperaccumulator plants list, if this feature could not be confirmed by experimentation under controlled conditions [69]. For instance, in researches regarding leaf surface contamination by field samples triggered a critical re-examination of the Cu and Co hyperaccumulators [70,71].

The hyperaccumulator species are spread through worldwide. This feature can be a result of adaptation of the plant in heavy metal polluted soils. Function mechanism of hyperaccumulating plants are still under study and discussion. However, series of suggestions to explain this mechanism have been discussed. These objectives will be discussed in the following section.

2.4.2. Mechanism of Heavy Metal Hyperaccumulation

The degree of hyperaccumulation of one or more heavy metals can differ and may be significant in different species or also in populations and ecotypes of the same species [72,73]. However, hyperaccumulation relies on three essential indicators that distinguish hyperaccumulators from related non-hyperaccumulator species. Common traits of hyperaccumulators are; (1) Much greater capability of taking up heavy metals from the soil; (2) Faster and effective root-to-shoot translocation of metals; (3) Capacity to depollute and sequester high amounts of heavy metals into the leaves. The investigation and understanding of hyperaccumulation function in those plants to uptake heavy metals has been deeply developing. Several studies performed to understand the mechanism. These studies focused on different approaches such as physiological, genomic, and proteomic features of hyperaccumulators and related non-hyperaccumulator plants. *T. caerulescens* and *A. halleri* are two plants that have been used in many studies and researches, thus, they became model plants for other applications [74,75]. Verbruggen et al., have revealed an interesting feature of hyperaccumulators, that they do not rely on novel genes, but depend on genes common to hyperaccumulators and non-hyperaccumulators [76].

2.4.3. Root-to-Shoot Translocation

Hyperaccumulators, different from non-hyperaccumulator plants, does not retain the heavy metals in the root cells, and does not detoxify them by chelation or store them into vacuoles. Shoot parts of hyperaccumulators are the places which elements can be translocated rapidly and efficiently. This entails the heavy metal availability for xylem loading, originating from low sequestration into a ready efflux out of the vacuoles, possibly due to specific features of root cell [77]. Amount of specific heavy metal - Zn sequestered into cell root vacuoles is 2–3-fold lower and the Zn efflux out of vacuoles almost twice as fast in the hyperaccumulators *T. caerulescens* [77] and *S. alfredii* [78] than in non-hyperaccumulating species. A lower amount of metals separately accumulates into root vacuoles as translocation in hyperaccumulator compared with non-hyperaccumulator species of Pteris [79].

Another feature of hyperaccumulators is the presence of small organic molecules in the roots, which makes metals binding. However, what kind of involvement of different chelators in hyperaccumulation has not been well-known yet. Some organic acids, such as malate and citrate has a role of ligands in the root cells, has shown low association with metals that makes complexation negligible, making this function is particularly arguable[80]. Meanwhile, some free amino acids, such as histidine and nicotinamine play key role in heavy metal hyperaccumulation, which form stable complexes with bivalent cations [81].

Various hypotheses have been proposed to explain the role of high elemental concentrations in leaves of the plants [82], namely; metal tolerance/disposal, drought resistance, interference with neighboring plants, and defense mechanism against natural enemies. According to the tolerance/disposal hypothesis, the unusual hyperaccumulation form would allow plants to translocate heavy metals away from the roots to tolerant leaf tissues. This eliminates them from the plant body by shedding the high-metal upper-grand organ. Another explanation is that large amounts of heavy metals might increase plant drought resistance via water's role in cell walls or its possible respondance like osmolytes inside the cells. These hypotheses, however, are hardly supported by experimental confirmation, so that their validity requires further investigation.

Hyperaccumulators have been believed to have limited potential of phytoremediation because most of the hyperaccumulators are metal selective, and does not accumulates for all elements, also they can only be used in their native habitats, above all, they have relatively small biomass, shallow root systems and slow growth rates, which limits the speed of heavy metal removal [83,84].

Several pot and field studies have showed that the hyperaccumulator *T. caerulescens* grown as a crop can accumulate as high as 5 tons ha^{-1} by breeding to increase the combination of yield and shoot metal concentration [85]. Moreover, the recycling of shoot metals may provide another value to the ash originated from metal hyperaccumulators; hence, there is no need for further expense to dispose the plants. Various species of *Thlaspi* are known for hyperaccumulating more than one heavy metal. Mostly, *Thlaspi* planted on Ni contaminated sites and accumulates about 3% of its dry matter as metal but

T. caerulescens can accumulate Cd, Ni, Zn, and Pb. As a hyperaccumulator of Cd and Zn, it could remove as much as 60 kg Zn ha^{-1} and 8.4 Kg Cd ha^{-1} [86]; *T. goesingese* and *T. ochroleucum* hyperaccumulate Ni and Zn while *T. rotundifolium* hyperaccumulates Ni, Pb, and Zn [87]. The brake fern *P. vittata*, produces a large biomass under favorable climate conditions, and can accumulate (from relatively low As concentration in the soil) 22 g As kg^{-1} in dry weight, with26% removal rate for the soil's initial As concentration [88,89]. These results indicate that phytoremediation of moderate levels of As contamination in sites is feasible. On the other hand, Pb compounds in soil mostly immobile and its extraction is limited by its solubility and diffusion rate to the root surface, and common buckwheat (*Fagopyrum esculentum*, *Polygonaceae*), were the first known Pb hyperaccumulator species with high biomass, can accumulate up to 4.2mg g^{-1} dry weight of Pb in the shoots [90].

2.5. Arbuscular Mycorrhizal Fungi

Universal and global symbiotic microorganisms such as azotobacters and arbuscular mycorrhizal fungi (AMF); belonging to *Glomerales order*, form symbiotic relationships with roots of 80~90% land plants in natural and agricultural ecosystems [91], including halophytes, hydrophytes and xerophytes [92]. Mycorrhizal fungi are known to benefit plant nutrition, growth and survival, due to their greater exploitation of soil for nutrients [93]. These associations represent a key factor in the below ground networks which influence diversity and plant community structure [94]. The degree of benefit to each partner in any AMF-plant host interaction depends not only on the particular plant and AMF species involved but also on the rhizobacteria and soil abiotic factors. Soil microorganisms are known to play role in the mobilization and stabilize of metal cations, thereby changing their availability to plants [95].

AMF are among the most common soil microorganisms and constitute an important functional component of the soil plant system occurring in almost all environments and climates, including disturbed soils [96]. More specifically, it has been shown that AMF can be affected by heavy metal toxicity, but in many cases mycotrophic plants growing in soils contaminated with heavy metals are colonized by AMF [8]. Many reports concerning this

have quantified spores and estimated root colonization. Others have gone further and described metal tolerant AMF in heavy metal polluted soils [97].

In recent years, research interest has focused on the diversity and tolerance of AMF in heavy metal contaminated soils trying to understand the basis underlying adaptation and tolerance of AMF to heavy metals in soils, since this could facilitate the management of these soil microorganisms, for phytoremediation/bioremediation programs. Few analytical studies have focused on AM in polluted soils or soils that are under constant heavy metals application.

While some workers observed that the external mycelium of AMF was the main site for trace element localization [98], others reported selective exclusion of toxic and non-toxic elements by adsorption onto chitinous cell walls [99], extra-cellular glycoprotein, glomalin, or intra-cellular precipitation. All these mechanisms have implications in reducing a plant's exposure to potentially toxic elements, i.e. mycorrhizo-remediation technology. Gonzalez, studied the form and localization of heavy metal accumulation in the extra-radical mycelium of three AM fungi isolated from the same polluted soil contaminated with Cu and As, and found that AMF-related soil protein; Glomalin, plays a vital part in sorption and sequestration of potentially toxic elements, and reducing their bioavailability [12].

Differential capacity of AMF to absorb and accumulate Cu as determined by scanning and transmission electron microscopy (SEM and TEM) is reported [100]; yet, the nature of accumulation and mechanisms involved require further studies in order to better understand the participation of AMF in plant tolerance and its ecological significance in polluted soils.

AMF can be screened for their ability to produce maximum levels of extra-radical mycelium in polluted soils, and to utilize adapted AMF to help accumulate heavy metal both within the plant roots (phytoaccumulation) and the extracellular fungal mycelium. During fungal degradation of lignin, carbohydrates are generated, but toxic phenols are also concomitantly released. Fungi generally biotransform pesticides and other organic xenobiotic by inducing minor structural changes to the pesticide, rendering in nontoxic.

The metabolic fate of soil pollutants such as organic xenobiotics is dependent on abiotic environmental conditions (temperature, moisture, soil pH, etc.), microbial community or plant species (or both), pesticide characteristics (hydrophilicity, p$K_{a/b}$, K_{ow}) and biological and chemical reactions.

Abiotic degradation is due to chemical and physical transformations of the pesticide by processes such as photolysis, hydrolysis, oxidation, reduction, and rearrangements. However, enzymatic transformation, which is mainly the result of biotic processes mediated by plants and microorganisms, is by far the major route of detoxification [100]. A major difficulty in translating the results of research into practical recommendations is the interaction between factors affecting the AMF symbiosis and the separation of cause and effect.

2.5.1. Interactions between Arbuscular Mycorrhiza and Plant Rhizosphere

In nature, most of the actively absorbing rootlets form symbiotic association with mycorrhizal fungi which are ubiquitous soil inhabitants. The changes in root exudates affect the microbial communities around the roots, leading to formation the "mycorrhizosphere". The mycorrhizosphere is the zone of soil exposed with pollutions where the physical, chemical, and microbiological processes are influenced by plant roots and their associated mycorrhizal fungi [93]. In laboratory studies with axenic plants, rhizosphere processes were not considered, e.g. role of mycorrhiza for soil phytoremediation.

Numerous organic xenobiotics and xenobiotic organic pollutants are detoxified in plants to glutathione conjugates. Following these reactions, xenobiotic GS-conjugates are compartmentalized in the vacuole of plant cell. These xenobiotic may interact deleteriously with an organism, causing toxic and in animal sometimes carcinogenic effects. Nevertheless, plants are able to detoxify organic pollutants by conjugation reactions, e.g. mediated by glutathione S-transferases (GST). Some GSTs are constitutively expressed in certain tissues, but GST regulation can be modified by agrochemicals, including herbicide safeners (antidotes) and synergists. It is hypothesized that plant GST gene promotes have multiple regulatory elements that respond differently to specific or more general stress-

related singles [100]. The role of GSTs in plants may encompass several major functions. A second function may be the regulation and transport of both endogenous and exogenous compounds which are often GS-X tagged for compartmentalization in the vacuole or cell wall [101].

It is generally accepted that xenobiotic glutathione conjugates are sequestered in the vacuole of leaves. However, recent literature documents that vascular storage might be either an alternative or temporary stage in the fate of xenobiotic, as evidence accumulates for plasmalemma transporters for xenobiotic and extracellular degradation enzymes.

Recently, evidences were presented for long range transport of conjugates in barley plants. From these data, it becomes clear, that a significant fraction of the resulting metabolites reaches the rhizosphere, where they may impact other plant`s roots and microorganisms in the root zone [100].

2.6. Glomalin Related Soil Protein (GRSP)

Glomalin is a glycoprotein produced by AMF [10]. Operationally defined by extraction and detection conditions [102-106], it is detected in large amounts in diverse soils as glomalin-related soil protein [11]. As such, is widely studied for its implications in the carbon storage, sequestration of potentially toxic element as HM, and its role in soil stability. GRSP could represent a significant fraction of the pool of soil proteins due to its persistence [107]. While the identity of the protein proper has been revealed to be a putative hsp60 homolog [10], the biochemical nature of the substance extracted from soil is still not fully revealed. GRSP appears to be a component of the hyphae and spore wall of AMF, likely released into the soil by mycelium turnover [108], where it subsequently contributes to linking soil particles and stabilizing aggregates [109]. Moreover, recent studies indicate that GRSP can bind and sequester some heavy metals such as Cu, Cd, Pb and Zn [12,13,110]. Based on these data, in this study it was hypothesized that the release and accumulation of GRSP in soils can be a very important mechanism for the stabilization of soils degraded by mining activities, and that this substance may also contribute to sequestration of significant quantities of heavy metal characteristic of this kind of environmental pollution.

3. MATERIALS AND METHODS

In order to determine the interaction between AMF and rhizosphere of the selected plant species under heavy metal contamination, following tasks were carried out in this study;

i. Preliminary germination tests – Different species of selected plants were germinated in petri dishes to determine the suitable plant varieties for greenhouse experiment.

ii. AMF species' propagules (production) – two adapted species of AMF; *Glomus mosseae* and *Glomus intraradices* inoculums were supplied from Ege University and cultivated under greenhouse conditions to use for inoculation source in this study.

iii. Greenhouse experimental unit – Set-up the main experiment with cultivation of selected plants' seeds (sunflower & sorghum) in stream sand and inoculating with two Arbuscular Mycorrhizal Fungi (AMF) species, *Glomus mosseae* and *Glomus intraradices*.

iv. Harvest, sampling, and analysis –measurements of heavy metal uptake by AAS method, and evaluation of mycorrhization in the roots via microscopic analyses as well as determination of glomalin (GRSP) content via Bradford method.

3.1. Germination Test

Preliminary germination tests were conducted in order to determine the germination capacity of the sorghum (*Sorghum bicolor*) and sunflower (*Helianthus annuus*) seeds in the presence of Arbuscular Mycorrhizal Fungi (*Glomus mosseae*) and heavy metal (*Cu*); design of the test setup consists of; control groups with 100 ppm, 500 ppm, and 1000 ppm of *Cu* concentrations for both sorghum and sunflower seeds without AMF, and 3 parallels of petri dishes for each concentration with the presence of AMF inoculation (Figure 3.1). AMF species (*Glomus mosseae*& *Glomus intraradices*) were adapted local climate and

weather conditions over the past few years and supplied from Ege University. 24 petri dishes were sterilized and prepared to conduct the germination test.

Sorghum and sunflower seeds were supplied from Turkish commercial farming business located in Izmir and Edirne, respectively. Seeds were treated and washed with distilled water and ethanol repeatedly. Contact times for distilled water and ethanol were 5 minutes and 2 minutes, respectively. This cycle was repeated 3 times and in the following step seeds were rinsed 5 times with distilled water. Planted numbers of seeds in petri dishes were 10 for sunflower, and 30 for sorghum.

Introduction of the Arbuscular Mycorrhizal Fungi; *Glomus mosseae,* into the germination system, was carried out as following procedure; 30.002 g of Glucose Hydrate ($C_6H_{14}O_7$) was dissolved in 100 ml distilled water. Afterwards, 1.8 g of AMF spores was added to the prepared solution. 5 ml of this mixture was added to each petri dishes –except controls-, to observe the effect of *Glomus mosseae* on germination. The initial concentration of the AMF spores was 100 mg mycorrhizal fragments including spores and hyphae per petri dish [91]. Presence of glucose may cause absesic acid formation, which raises typical stress response, germination may be delayed or inhibition of seedling development may occur.

Artificial heavy metal (Cu) pollution was provided by prepared $CuSO_4$ solution; where 0.5003 g of $CuSO_4$ was solved in 500 ml distilled water in order to maintain 1000 ppm copper concentration. Prepared solution was diluted accordingly to 500 ppm, and 100 ppm. 3 ml of the prepared $CuSO_4$ solutions were added to petri dishes accordingly. Seeds' germinations and seedling growth features were monitored for 8 days.

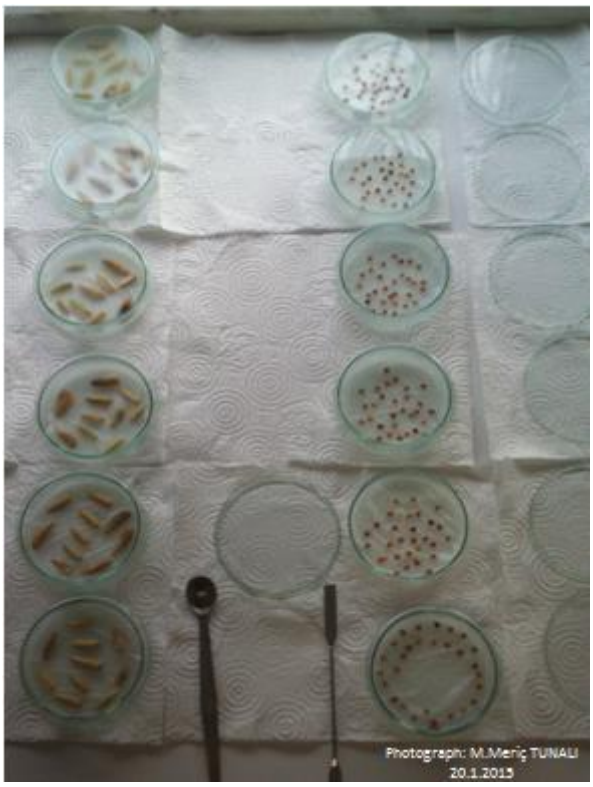

Figure 3.1. Germination set-up in petri dishes.

3.2. Propagules of Arbuscular Mycorrhizal Fungi (AMF)

Glomus mosseae and *Glomus intraradices s*pecies had been adapted to the Turkey's soils and climate during past several years in Ege University. Provided cultures of AMF were cultivated for mass production for phytoremediation study, using dark-green plastic pots with volume of 0.6 l and 40 gr tare weight (Figure 3.2). 15 pots for each species were initially set up with 500 g of sanitized stream sand, sorghum seeds and 100 ml of distilled water. Temperature of the greenhouse environment was controlled via electrical heaters between 18-22ºC. For watering the pots, 20 ml water irrigation was carried out every day to maintain soil humidity at 20%.

Figure 3.2. Application of *Glomus intraradices* (Left) and *Glomus mosseae* (Right).

One week after the cultivation setup, the average number of plants per pot was 54 for *Glomus mosseae,* and 34 for *Glomus intraradices.* In order to provide enough nutrients to plants and maintain healthy environment for the sorghum seeds and AMF spores, elimination of inadequate plants was carried out via trimming; thus, number of plants for each pot were halved during the process.

All of the plants were trimmed after 21 days (Figure 3.3), and all pots covered with black plastic bags to accelerate the decomposition of plants and initiating the production of AMF spores.

Figure 3.3. Plants with *Glomus intraradices* (Left) and *Glomus mosseae* (Right) after 21 days.

3.3. Preliminary Greenhouse Experiments

Sorghum and sunflower seeds in petri dishes with 3 different copper concentrations (100 ppm, 500 ppm, and 1000 ppm) including controls and AMF introduced seeds were transferred to pots after 7 days of germination. Dark-Green plastic pots with volume of 0.6 l and 40 g tare weight were used. Stream sand was sanitized in oven under 151°C for 20 hours prior to the transfer of the seedlings. Pots and stream sand were supplied from a local botanic shop in Istanbul.

500 g of stream sand utilized for each pot along with seeds and 15 mg of *Glomus mosseae* spores. Afterwards, 100 ml of distilled water was added to each pot for star up, and 20 ml added daily in order to maintain humidity at 20%. 10 ml of Ammonium Ferric Sulfate ($NH_4Fe(SO_4)_2$) solution with a concentration of 0.3 g/l also added to pots every 2 weeks to provide nutrients for the plants.

Initial heavy metal (Cu) dosage for Sorghum planted pots were 3 ml of each concentration, after 5 weeks second dosage of heavy metal (Cu) was carried out with 15 ml for each concentration in all pots. Meanwhile, the initial heavy metal (Cu) dosage for Sunflower planted pots were 25 ml of each concentration. After 2 weeks from the transfer, second dosage of heavy metal (Cu) applied to all pots with 15 ml for each concentration.

3.4. Phytoremediation Set

Dark-Green colored plastic pots with volume of 3.7 liters and 325 gr tare weight were used for phytoremediation of heavy metals (Cu). Sorghum and sunflower seeds' sterilization was carried out with following procedure; seeds treated with ethanol solution (70%), with a contact time of 1 minute and afterwards seeds were rinsed with distilled water for 3 minutes. This process repeated for 3 times prior to plantation.

Stream sand was sanitized in oven under 151°C for 20 hours, after cooling period; all pots were filled with 2 kg of stream sand. Sunflower and sorghum seeds were placed on the top of the sand, afterwards inoculated with previously propagulated *Glomus mosseae* &

Glomus intraradices cultures added to the system along with mentioned organisms' spores. After that, seeds and AMFs covered with 1 kg of stream sand.

With initial 5% moisture of the sanitized stream sand, and 600 ml of distilled water used for irrigation; moisture inside the pots were adjusted to 23~25 %, and regularly 50 ml distilled water was added every 2 days in order to maintain humidity at intended levels(20-23% field capacity).

In order to provide enough nutrients for plant growth, nutrient solution based on Modified Strullu-Romand medium (Table 3.1) was prepared. 20 ml of the nutrient solution was added to each pot every 2 weeks.

Table 3.1. Composition of MSR medium [111].

Elements	Concentration, µM
$N(NO_3^-)$	3800
$N(NH_4^+)$	180
P	30
K	1650
Ca	1520
Mg	3000
S	3013
Cl	870
Na	20
Fe	20
Mn	11
Zn	1
B	30
Mo	0.22
Cu	0.96

Heavy metal (Cu) dosage over 6 week period for potted sunflower and sorghum plants were 30 ml, 50 ml, 50 ml, 20 ml, 25 ml, and 25 ml for each concentration. Heavy metal additions done once a week, and the total dosage of heavy metal per pot was 200 ml for all concentrations. Figure 3.4 and Figure 3.5 display the growth of the sunflower plants over time in the experimental setup.

Figure 3.4. Sunflower (Hornet) plants' growth over time (3rd & 4th Week).

Figure 3.5. Sunflower (Hornet) plants' growth over time (5th & 6th Week)

For every combination of plant – AMF in three different heavy metal concentrations, 3 parallel pots were maintained. Breakdown of the total number of the pots in greenhouse was; Sunflower – *G. mosseae:* 3 parallels for 3 heavy metal concentrations; 9 pots, Sunflower – *G. intraradices:* 3 parallels for 3 heavy metal concentrations; 9 pots, Sorghum – *G. mosseae:* 3 parallels for 3 heavy metal concentrations; 9 pots, Sorghum – *G. intraradices:* 3 parallels for 3 heavy metal concentrations; 9 pots.

In total; 36 pots were maintained for this phytoremediation experiment. Number of plants were; for sunflower planted pots: 5 plants/pot, for sorghum planted pots: 10 plants/pot.

3.5. Harvesting and Sampling

8 weeks after plantation of the seeds, sunflower and sorghum plants were harvested. Whereas, roots and shoots were sampled and collected separately.

During harvesting, samples from roots of each pot were taken for AMF symbiosis observation via microscope and stored in 70% ethanol solution at 4°C for long time conservation. Sand samples taken from each pot for Glomalin (GRSP) determination and were packaged separately at -20°C. Harvested shoots and roots of the sorghum and sunflower plants (fresh biomass) were subjected to drying process at 60°C for 4 days in the oven, and then transferred to desiccator. Dried samples from shoots and roots were grinded in stand blender until formation of powder state and packed accordingly.

3.6. Digestion Method

To determine the capacity of heavy metal uptake by examined plants, 0.25 g of powdered sample was taken and digested with nitric acid –(HNO_3) (65%), hydrogen peroxide –(H_2O_2) (35%), and distilled water. Ratio of the digestion media was fixed at, HNO_3: H_2O_2: dwater (9:1:1). Digestion procedure was adapted from USEPA, Method 3052 [112].

The procedure specifications for digestion was; Pressure: 800 psi, Power: 1600 watt, Temperature: 180°C, Ramp Time: 5.25 minutes, and Hold Time: 11 minutes, Machinery: MARS 6 Microwave Accelerated Reaction System Instrument (CEM), USA. Measurement of Cu in digestion was carried out with Atomic Absorption Spectroscopy (AAS) method, (Standard Methods 3111B) with Perkin Elmer AAnalyst 300.

3.7 Determination of Mycorrhization

In order to determine the level of mycorrhization in root samples preserved in 70% ethanol solution at 4°C prior to root staining. Root staining process carried out in following order; each root sample carefully packed in tulle and kept in 10% KOH solution (w/v) for 4 hours in water bath at 60°C. This procedure used for cleaning of the roots and extracts tannins from root samples and dissolved in KOH solution. Afterwards, roots were contacted with 1% HCl solution for 3 minutes and rinsed carefully. 0.05% (w/v) trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) for 4 days [113]. After staining process is completed all root samples handled carefully and aligned on glass slides horizontally for microscope examination (Figure 3.6).

For this analysis; Microscope Axio Observer.Z1, with EC Plan-Neofluar 10x/0.3 objective, and AxioCam MR5 camera was used.

Figure 3.6. Prepared slides for determination of mycorrhization

3.8 Glomalin Related Soil Protein (GRSP) Measurement

GRSP levels of the soil samples were evaluated by Bradford method. Extraction of reactive soil protein was determined by Rillig et al., [11], and final measurement method was carried out according to the Wright and Upadhyaya [103]. For this combined method, 1 gram of soil sample (below roots) from each set was treated with 50 mM citric acid, and autoclaved at 121°C for 60 minutes. Then, centrifugation of samples was performed at 8000 rpm for 30 minutes. Afterwards, all samples were filtered through Whatman No.4 filter paper.

Calibration curve for the analysis of GRSP, Bovine Serum Albumin (A4503 SIGMA**),** with concentrations of 50, 150, 200, 250 mg with citric acid, and Bradford Reagent (B6916 SIGMA**),** with distilled water (Ratio: 1:4) was prepared and filtered through Whatman No.4 filter paper. 20 µl of prepared Bradford Reagent was added to each Bovine Serum Albumin [11].

All samples, including calibration solutions were analyzed at 595 nm with UV-160A Spectrophotometer, Shimadzu.

4. RESULTS

4.1. Germination Tests

To determine seeds' germination potential and related features via screening of the suitable seeds, five different species of sunflower were tested in petri dish units. According to germination rates; Hornet has shown quite positive germination characters (Table 4.1). Hence, further examination carried out for understanding the behaviour of Hornet species with presence of AMF under the influence of heavy metals. Evaluation of the results concludes that, among all species of sunflower, Hornet variety showed the best performance for germination and seedling development characteristics, therefore, it was the most suitable variety of sunflower for further phytoremediation experiment.

Table 4.1. Germination ratio of different sunflower varieties

Sunflower Type	Germinated Seeds / Total Seeds				
	1d	2d	3d	4d	5d
Tunca -1	4/18	15/18	17/18	17/18	17/18
Tunca -2	5/20	19/20	20/20	20/20	20/20
09 TRÇ/004 -1	0/15	3/16	5/16	7/16	8/16
09 TRÇ/004 -2	0/15	3/15	3/15	4/15	6/15
Hornet – 1	**9/16**	**16/16**	**16/16**	**16/16**	**16/16**
Hornet – 2	**9/16**	**16/16**	**16/16**	**16/16**	**16/16**
Bosfora - 1	1/15	11/15	11/15	12/15	14/15
Bosfora - 2	2/15	6/15	9/15	11/15	11/15
10 TRÇ/027 - 1	0/15	0/15	1/15	3/15	6/15
10 TRÇ/027 - 2	1/16	4/16	4/16	7/16	10/16

After the selection of the suitable variety among selected species, plants' seeds were cultivated under heavy metal stress, and the contamination levels of Cu for experimental procedure were selected as 100ppm, 500ppm, and 1000ppm. In order to observe the interactive effects of AMF and heavy metal, seeds were inoculated with two different arbuscular mycorrhizal fungi (AMF) species: *Glomus mosseae* and *Glomus intraradices.*

Monitored germination characteristics include: germination ratio, radicle length, and hypocotyl lengths of the seedlings plants. These parameters were measured daily for 7 days and gathered data is given in Table 4.2.

According to the results, Sunflower – Hornet showed a strong and significant ability in seed germination up to 100% under heavy metal concentrations (100, 500 and 1000ppm $CuSO_4$) within three days. Meanwhile, sorghum seeds showed relatively low germination rate, with a total 91.1% after same period of time. Data exhibited an elicitation of a heavy metal resistance in the both seed species without showing toxic effects. In terms of phytoremediation, successful germination and seedlings' development performance are essentially fundamental parameters for the selection of the plant species for application and re-vegetation of contaminated soils.

Radicle growth, as a major germination characteristic for root development among other growth factors for seed was measured during this experiment. Sorghum plants were significantly reacted to stress caused by two different sources; (1) Heavy metals in the soil inhibited the growth rate, while (2) activity of arbuscular mycorrhizal fungi brought another stress to the plants. After 7 days from plantation of the seeds, average radicle lengths of the control group; 100, 500, 1000ppm $CuSO_4$ were 25.75 mm, 2.25mm, and 1.75 mm, respectively. Increasing heavy metal concentrations inarguably had a negative effect on root development. Seeds inoculated with *Glomus mosseae* for the same Cu concentrations had average radicle lengths of 6 mm for 100 ppm Cu, 3.92 mm for 500 ppm, and for 1000 ppm radicle lengths were less than 1.5 mm after 7 days, (Figure 4.1). As for hypocotyls of the sorghum plants, observed heavy metal and AMF effects were parallel to radicle growth (Figure 4.2). It can be deducted that, prior to forming a symbiosis with AMF, plants' defence mechanism was active and it limited the intensity of root system and the effects can be clearly observed in root development.

Table 4.2. Results of germination and seedlings characters for selected varieties under the application of different Cu concentrations.

Plants-Treatment-Concentrations	Rate of Germinated Seeds (%)						Radicle Length (Avg.) mm.						Hypocotyl Length (Avg.) mm.					
	1d	2d	3d	4d	6d	7d	1d	2d	3d	4d	6d	7d	1d	2d	3d	4d	6d	7d
Sorg. Cont. 100 ppm	66.7	73.3	90	90	90	96.7	-	-	22.8	28.5	25.75	25.25	-	-	2.5	10.75	45.25	52.5
Sorg. Cont. 500 ppm	60	76.7	90	96.7	96.7	96.7	-	-	9	4	4.25	2.25	-	-	8	16.5	25.5	31
Sorg. Cont. 1000 ppm	80	86.7	86.7	90	93.3	93.3	-	-	2	3	2.25	1.75	-	-	12.3	26.75	16.5	18.25
Sorg. Glo. 100 ppm -1	30	86.7	93.3	96.7	96.7	96.7	-	-	3	4.75	4.75	5.75	-	-	3.25	8	11.25	10.75
Sorg. Glo. 100 ppm -2	13.3	50.0	86.7	86.7	86.7	86.7	-	-	2.5	7	6.25	6.75	-	-	3.75	9.25	14.5	15.25
Sorg. Glo. 100 ppm -3	33.3	43.3	90	90	93.3	96.7	-	-	3	6.5	6	5.5	-	-	3.75	7.5	11.75	11
Sorg. Glo. 500 ppm -1	10	73.3	90	90	90	90	-	-	1.5<	4	1.5<	3.75	-	-	1.5<	5.25	1.5<	6.5
Sorg. Glo. 500 ppm -2	10	66.7	86.7	86.7	86.7	90	-	-	1.5<	3.75	3	3.75	-	-	1.5<	7.5	9.25	9
Sorg. Glo. 500 ppm -3	3.3	30.0	96.7	96.7	96.7	100	-	-	1.5<	4	3.75	4.25	-	-	1.5<	5	13.25	12.75
Sorg. Glo. 1000 ppm -1	10	53.3	96.7	100	100	100	-	-	1.5<	1.5<	1.5<	1.5<	-	-	1.5<	1.5<	1.5<	1.5<
Sorg. Glo. 1000 ppm - 2	10	46.7	96.7	96.7	96.7	100	-	-	1.5<	1.5<	1.5<	1.5<	-	-	1.5<	1.5<	1.5<	1.5<
Sorg. Glo. 1000 ppm -3	17	40	90	93.3	96.7	96.7	-	-	1.5<	1.5<	1.5<	1.5<	-	-	1.5<	1.5<	1.5<	1.5<
Hornet Cont. 100 ppm	70	100	100	100	100	100	-	5.75	10	23.25	54.75	57	-	-	12.5	22.75	47.75	57.25
Hornet Cont. 500 ppm	80	100	100	100	100	100	-	9	10.75	18.5	20.25	21.25	-	-	16.25	28.25	53.5	56
Hornet Cont. 1000 ppm	80	100	100	100	100	100	-	4.5	8.75	12.75	18.75	21	-	-	16.5	20.75	37.5	44.5
Hornet Glo. 100 ppm	80	90	100	100	100	100	-	6.75	15.5	33.5	85.25	103.75	-	-	19.25	26.5	95.75	113.75
Hornet Glo. 500 ppm	60	100	100	100	100	100	-	5	10.25	12.5	21.25	22.5	-	-	15.75	23.5	63.5	67.75
Hornet Glo. 1000 ppm	50	90	100	100	100	100	-	5.75	7.75	9.5	10.5	10.75	-	-	11.75	15.5	25.75	37.5

*Sorg. – Sorghum, Cont. – Control, Glo. – Glomus mosseae

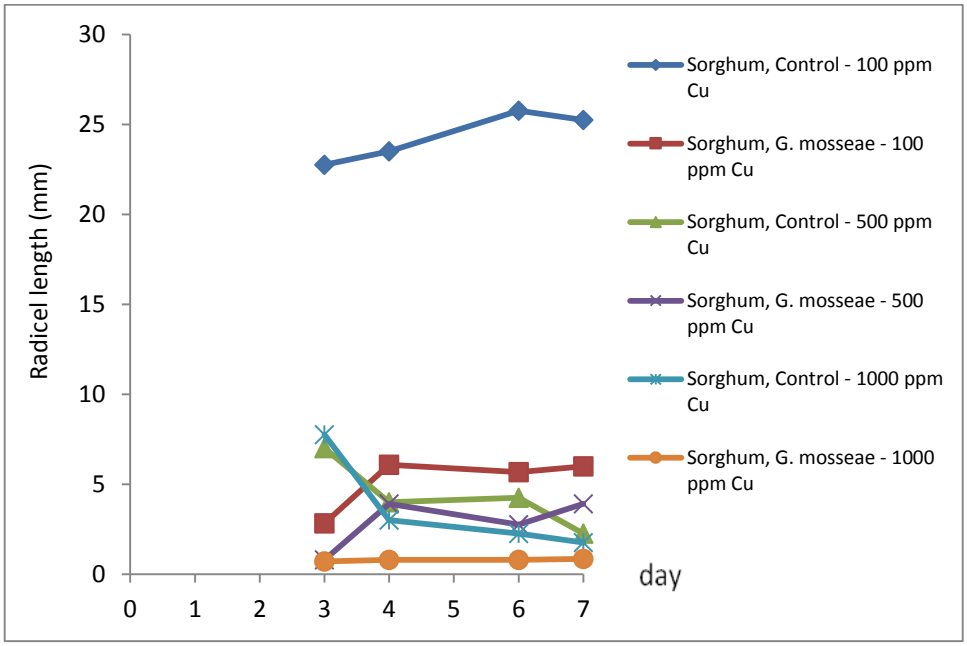

Figure 4.1. Comparison of radicle length (mm) of sorghum plants.

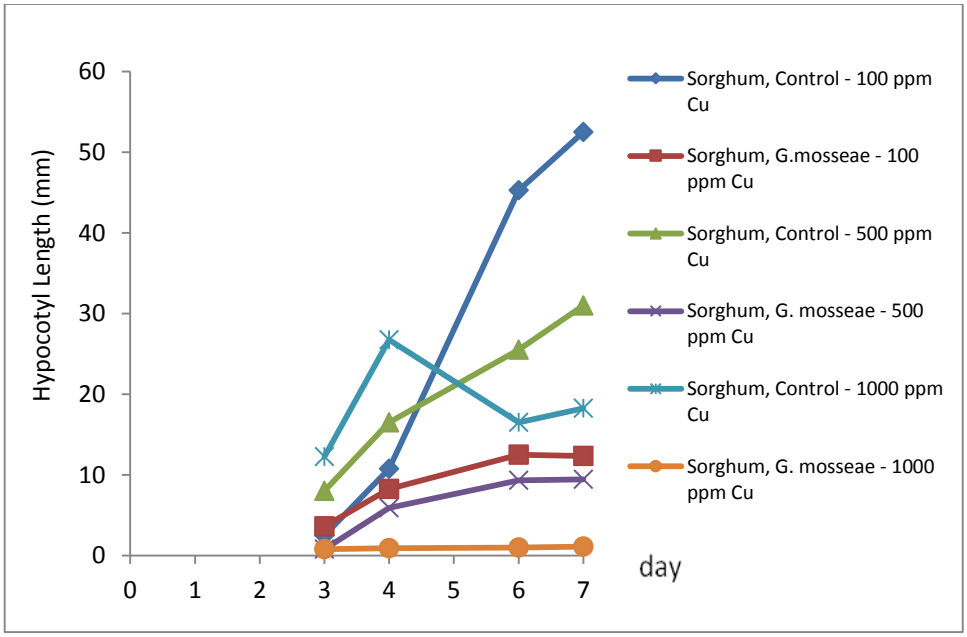

Figure 4.2. Comparison of hypocotyl length (mm) of sorghum plants.

Sunflower plants' ability to germinate and grow under heavy metal was considerably better than it was for sorghum plants. However, heavy metal levels in the soil undoubtedly showed negative effect on the growth rate for both plants; despite sunflower plants demonstrated adequately succeed. The activity of arbuscular mycorrhizal fungi, was also different in sunflower than sorghum plants, this may be due to plants' ability to cope with heavy metal stress, leading to accelerated symbiosis formation. Sunflower - Hornet plants' radicle lengths under 100 ppm Cu contamination were promoted 103.75 mm long in the seeds inoculated with *Glomus mosseae* while this was just 57 mm for control seeds after 7 days of growth period. Correspondingly, the average hypocotyl lengths were 113.75 mm and 57.25 mm. Therefore, at 100 ppm Cu the presence of AMF promotes almost doubled growth value in the radicle and hypocotyl lengths of sunflower plants (Figure 4.3 and Figure 4.4).

On the other hand, increased levels of Cu concentrations had adverse effect on both sunflower plants and activity of the AMF – *Glomus mosseae*. Experiments for cultivation with both 500 ppm, and 1000 ppm of Cu concentration showed that, *Glomus mosseae* can still operate and support sunflower plants at 500 ppm Cu concentration in terms of plant growth when compared to the control group; however, at 1000 ppm Cu concentration;non-mycorrhizal control group had 50% longer radicles and 18% longer hypocotyls than AMF inoculated seed group. This result suggests that, as explained above, when heavy metal stress overlaps with another organisms' (AMF) activity, it decelerates the growth speed of the plant. Although, at 1000 ppm, plants did not show effects of toxicity, thus, development of the plant may need longer duration before reaching an anticipated size.

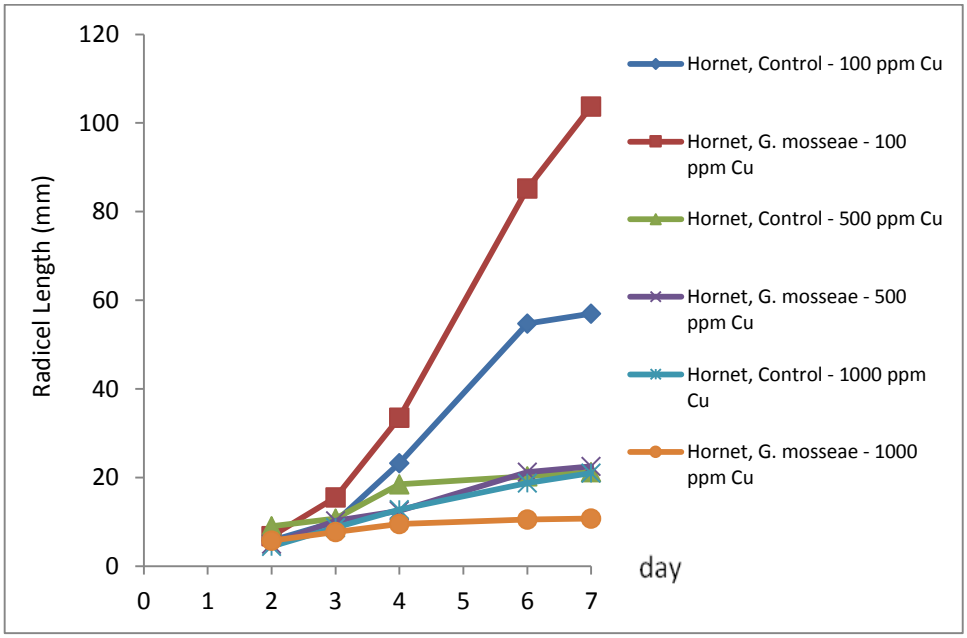

Figure 4.3. Comparison of radicle length (mm) of sunflower plants.

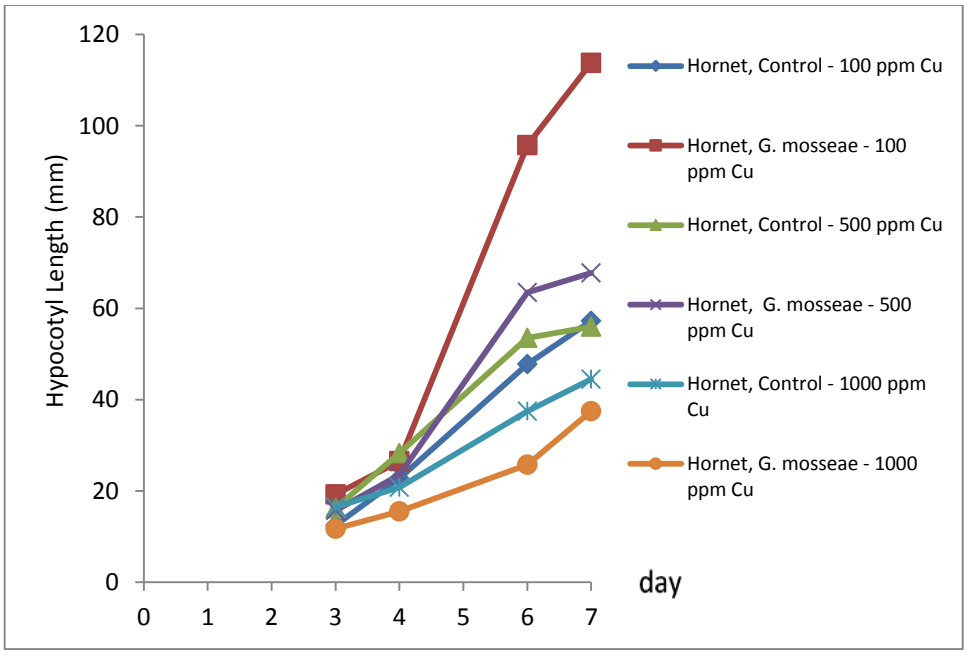

Figure 4.4. Comparison of hypocotyl length (mm) of sunflower plants.

In order to test the suggested hypothesis, *Sorghum bicolor*, grown on three different levels of Cu were monitored for a month. Plants were grown and morphological measurement applied at the end of every week; monitoring parameters included total number of plants per pot, number of leaves, and average plant height. Obtained data indicated that, there was an adaptation period of 2 weeks for sorghum plants with arbuscular mycorrhizal fungi. During this adaptation period, all plants' development seemed to be slowed down but it was continuous for both control and AMF inoculated plants. After 3rd week, plants associated with *Glomus mosseae,* had started to grow more rapidly in terms of leaves (number and size), and increasing plant height, along with higher number of healthy plants. Extensive data of the monitoring is presented in Table 4.3.

Table 4.3. Potted sorghum plants' growth over time.

Pot	Number of Plants	Number of leaves				Average Plant Height (cm)			
		1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Sorg. Cont. 100 ppm	6	5 x 1 Leaf	4 x 2 Leaf 2 x 1 Leaf	5 x 2 Leaf 1 x 1 Leaf	3 x 3 Leaf 3 x 2 Leaf	1.9	2.5	3.7	4.1
Sorg. Cont. 500 ppm	4	1 x 1 Leaf	1 x 2 Leaf 1 x 1 Leaf	2 x 2 Leaf 1 x 1 Leaf	1 x 3 Leaf 1 x 2 Leaf	2.2	3.7	5.3	6.5
Sorg. Cont. 1000 ppm	3	-	-	2 x 1 Leaf 1 x 1 Leaf	2 x 2 Leaf 1 x 1 Leaf	1<	1.4	1.6	1.8
Sorg. Glo. 100 ppm	7	1 x 1 Leaf	3 x 2 Leaf 2 x 1 Leaf	2 x 2 Leaf 2 x 1 Leaf	3 x 3 Leaf 4 x 2 Leaf	2	3.3	6.5	11.8
Sorg. Glo. 500 ppm	8	-	2 x 2 Leaf 5 x 1 Leaf	5 x 2 Leaf 1 x 1 Leaf	3 x 3 Leaf 4 x 2 Leaf	1.7	3.0	5.7	7.5
Sorg. Glo. 1000 ppm	6	-	-	3 x 2 Leaf 1 x 1 Leaf	2 x 3 Leaf 1 x 1 Leaf	1<	1.1	6.1	10.5

4.2. Experimental Units for Phytoremediation Study

In section 4.1, seed germination and AMF interactions' effect during germination period were highlighted, therefore, in main phytoremediation study effect of continuous discharge of heavy metals – which is similar to discharge frequency from industrial processes – were investigated during 8 week period of phytoremediation.

Table 4.4. Plant height in Sunflower (Hornet) during phytoremediation study.

Plant - AMF	Cu Concentrations ppm	Plant Height, cm (Average)					
		3rd Week	4th Week	5th Week	6th Week	7th Week	8th Week
Hornet - Control	100	11.50	14.67	23.33	27.33	31.17	35.33
	500	10.33	14.33	23.00	29.67	35.50	40.33
	1000	6.83	9.83	15.67	23.67	30.67	34.00
Hornet - *G. mosseae*	100	8.17	13.00	23.33	25.67	30.17	34.00
	100	9.17	12.50	19.83	27.33	32.83	36.67
	100	10.17	13.17	22.67	25.67	30.17	33.00
	AVG - 100	9.17	12.89	21.94	26.22	31.06	34.56
Hornet - *G. mosseae*	500	8.17	10.83	21.17	23.33	27.50	31.00
	500	8.00	10.83	22.33	26.00	32.33	36.00
	500	8.17	10.50	19.67	28.33	35.00	37.00
	AVG - 500	8.11	10.72	21.06	25.89	31.61	34.67
Hornet - *G. mosseae*	1000	8.50	11.67	18.00	26.33	28.50	31.00
	1000	9.00	12.17	15.33	26.67	32.33	34.67
	1000	8.50	11.67	18.00	26.33	30.67	33.67
	AVG - 1000	8.67	11.83	17.11	26.44	30.50	33.11
Hornet - *G. intraradices*	100	10.17	14.67	20.00	25.67	30.67	34.00
	100	9.00	12.17	20.33	29.67	34.00	38.33
	100	9.83	13.83	18.50	31.67	33.83	38.67
	AVG - 100	9.67	13.56	19.61	29.00	32.83	37.00
Hornet - *G. intraradices*	500	10.17	13.67	18.00	28.33	29.50	35.67
	500	10.33	13.83	19.67	28.67	33.67	37.67
	500	10.00	12.67	17.00	29.00	34.67	39.00
	AVG - 500	10.17	13.39	18.22	28.67	32.61	37.44
Hornet - *G. intraradices*	1000	8.50	11.17	18.00	24.33	26.83	30.67
	1000	6.83	9.50	20.33	24.33	28.17	36.67
	1000	9.33	12.50	19.17	27.67	33.50	36.00
	AVG - 1000	8.22	11.06	19.17	25.44	29.50	34.44

Phytoremediation study on both sorghum and sunflowers plants were carried out for entire eight weeks. Plant growth trend showed a similar pattern in both sunflower and sorghum plants either in control or in symbioses with AMF species. Continuous heavy metal discharge into the systems' adverse effect on plant growth was obvious. Plant developments for the experimentation were presented in Table 4.4 and Table 4.5.

Table 4.5. Sorghum plant height changes over phytoremediation duration

Plant - AMF	Cu Concentrations ppm	Plant Height, cm (Average)					
		3rd Week	4th Week	5th Week	6th Week	7th Week	8th Week
Sorghum - Control	100	6.00	7.33	9.67	11.00	11.67	12.67
	500	5.67	7.00	8.00	9.00	10.00	11.33
	1000	4.67	5.33	7.00	8.00	9.00	10.33
Sorghum - *G. mosseae*	100	4.67	4.67	7.00	7.67	9.00	9.67
	100	5.33	5.33	7.33	7.83	9.00	10.67
	100	4.33	5.33	6.00	6.50	7.00	8.00
	AVG - 100	4.78	5.11	6.78	7.33	8.33	9.44
Sorghum - *G. mosseae*	500	5.00	5.67	4.67	5.83	6.00	7.33
	500	5.33	5.67	6.33	7.50	10.67	12.67
	500	5.33	5.00	7.00	7.67	8.67	11.00
	AVG - 500	5.22	5.44	6.00	7.00	8.44	10.33
Sorghum - *G. mosseae*	1000	5.00	5.67	7.00	7.50	9.33	10.00
	1000	5.00	5.67	6.67	7.50	7.67	8.67
	1000	4.67	6.33	7.33	8.33	9.00	10.00
	AVG - 1000	4.89	5.89	7.00	7.78	8.67	9.56
Sorghum - *G. intraradices*	100	5.67	6.00	6.33	9.00	10.67	11.33
	100	4.67	6.33	6.67	7.33	9.33	11.00
	100	5.67	6.00	6.00	6.50	9.67	11.00
	AVG - 100	5.33	6.11	6.33	7.61	9.89	11.11
Sorghum - *G. intraradices*	500	4.67	5.67	5.67	6.33	7.67	8.67
	500	5.00	6.00	6.00	6.67	9.00	11.33
	500	5.00	5.00	5.00	6.00	7.67	8.33
	AVG - 500	4.89	5.56	5.56	6.33	8.11	9.44
Sorghum - *G. intraradices*	1000	5.33	5.33	5.67	6.67	10.33	11.67
	1000	5.33	5.00	5.00	6.00	9.67	10.33
	1000	6.00	6.00	6.00	6.67	9.33	10.67
	AVG - 1000	5.56	5.44	5.56	6.44	9.78	10.89

By loading the system with heavy metals continuously, the stress on AMFs prevented their activity to enhance nutritional uptake of the plants, hence, keeping the symbiosis' primary objective to deal with the pollution rather than plain plant cultivation (non-mycorrhizal).

Plants' harvested fresh weights after 8 weeks showed that; for sorghum plants fresh weights did not fluctuated significantly at 100 ppm and 500 ppm, however, at 1000 ppm Cu concentration inoculated sorghum plants with *Glomus mosseae* and *Glomus intraradices* showed better growth performance. Sunflower plants, on the other hand, did not show any significant differences in terms of produced fresh weight under same conditions and duration. Fresh weights of the plants are presented in Figure 4.5 and Figure 4.6.

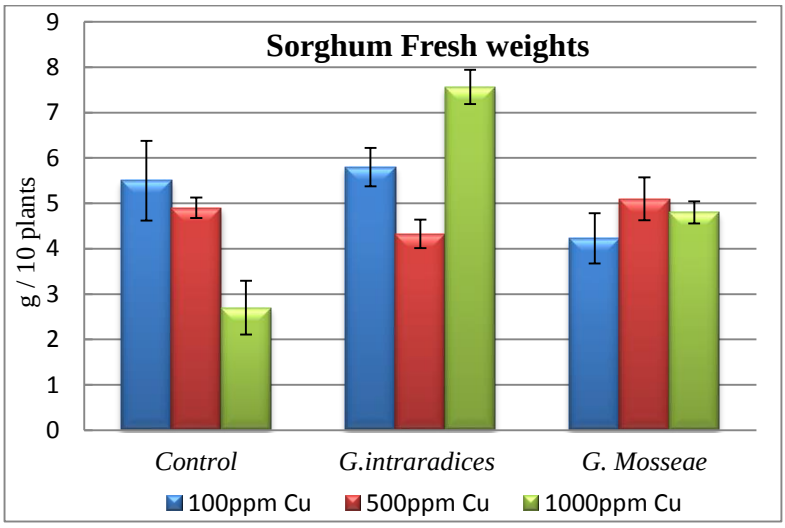

Figure 4.5. Fresh weights of the harvested sorghum plants.

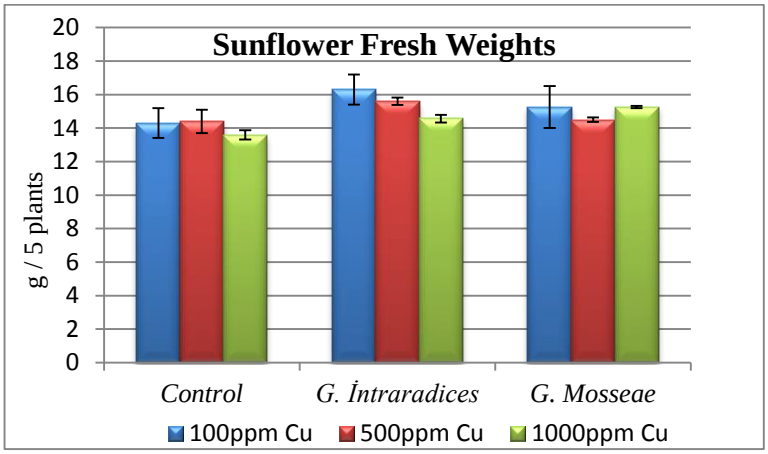

Figure 4.6. Fresh weights of the harvested sunflower plants.

4.2.1. Heavy Metal Uptake of the Plants

Uptake of heavy metals from soil was specifically higher on the roots than shoots of both plants. Determination of heavy metal concentration on the shoots of the sunflower plants suggested that the translocation of Cu to shoots was limited, although arbuscular mycorrhizal fungi seemed to increase this translocation. However, differences were not significant (Figure 4.7).

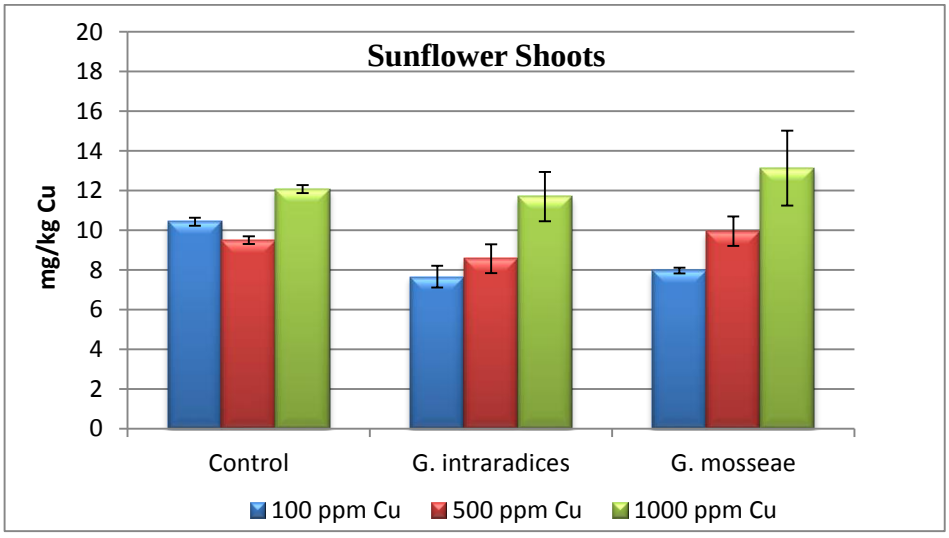

Figure 4.7. Cu translocation to sunflower shoots.

Indisputably, root mycorrhization significantly increased the Cu uptake on the plants exposed to different Cu concentrations (Figure 4.8). *Glomus mosseae* in symbioses with the roots of sunflower enhanced the uptake of Cu by 56.89% - 182.26% on plant roots. Whereas, *Glomus intraradices* had increased the root Cu concentrations by 44.97% - 96.14%. Fresh weight of shoots and roots as well as their Cu concentrations of sunflower plants are given in Table 4.6.

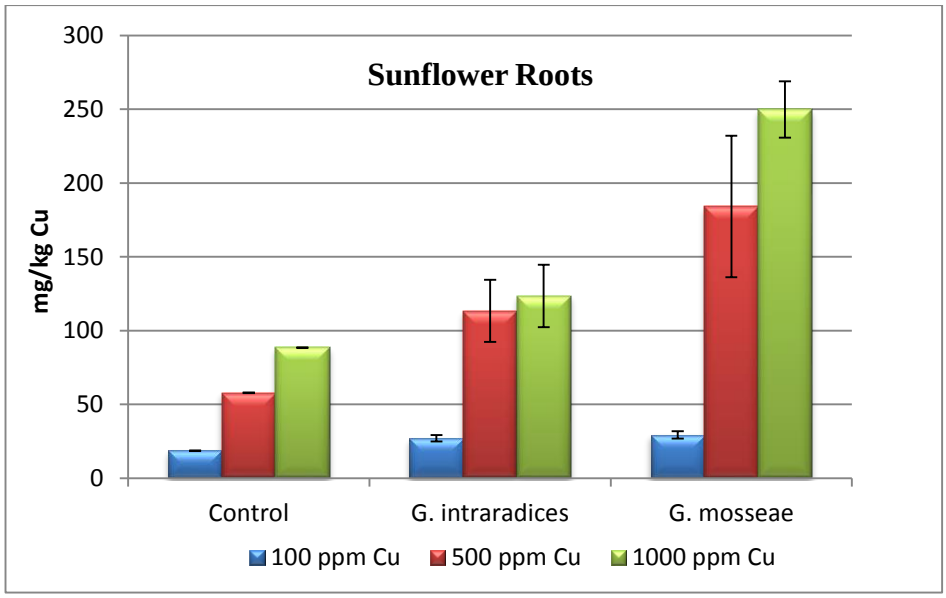

Figure 4.8. Cu stabilization on sunflower roots.

Table 4.6. Phytoremediation experiments data for sunflower plants.

Plant - AMF	Cu Concentrations (ppm)	Fresh Weight Root (g)	Root - Cu (mg/kg)	Fresh Weight - Shoot (g)	Shoot - Cu (mg/kg)
Hornet - Control	100	17.3	18.70	14.30	10.42
	500	17.5	57.81	16.40	9.50
	1000	21.9	88.52	13.60	12.07
Hornet *G. mosseae*	100	7.5	23.44	14.80	7.62
	100	15	34.93	17.00	8.03
	100	13.9	29.67	14.00	8.25
Hornet *G. mosseae*	500	6.7	180.74	12.50	8.06
	500	18.7	292.81	14.40	10.61
	500	17.1	78.51	16.60	11.17
Hornet *G. mosseae*	1000	10.6	224.84	15.20	12.89
	1000	18.8	299.37	15.00	9.02
	1000	17.8	225.36	13.60	17.48
Hornet *G. intraradices*	100	15.9	29.58	15.80	6.39
	100	12.3	30.33	17.00	8.82
	100	12.7	21.43	16.10	7.75
Hornet *G. intraradices*	500	6	119.95	14.60	9.13
	500	8.7	156.64	15.60	9.82
	500	23.9	63.58	15.00	6.74
Hornet *G. intraradices*	1000	13.8	81.83	14.70	11.79
	1000	7.5	174.68	14.60	14.42
	1000	13.1	113.84	12.40	8.87

As for sorghum plants' performance under the same conditions, were similar to sunflower plants on root stabilization, the most significant difference was *Glomus mosseae's* effect of increased translocation of heavy metal to sorghum shoots at 1000 ppm. The shoot concentrations of sorghum plants in control, *G. intraradices*, and *G. mosseae* group were; 5.8 mg/kg, 6.39 mg/kg, and 12.98 mg/kg, respectively. Therefore, at 1000 ppm *G. intraradices* increased the copper translocation to shoots by 10.17%; while *G. mosseae* raised the effectiveness by 123.79% (Figure 4.9).

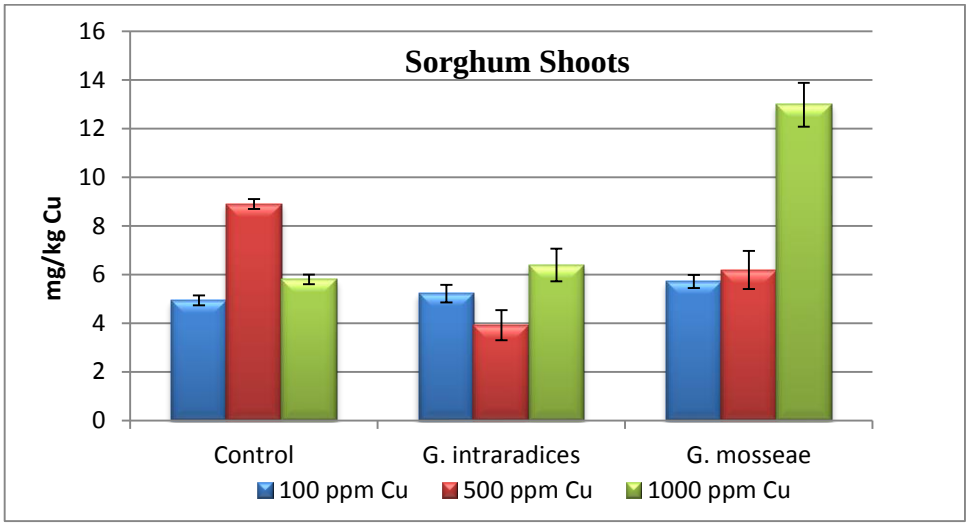

Figure 4.9. Cu translocation to sorghum shoots.

Copper stabilization on the roots of sorghum plants (Figure 4.10) were also influenced by AMF presence along with increasing concentration of heavy metals in the soil. At 1000 ppm, *G. mosseae* increased the Cu stabilization on the roots by 146.89%; while this value was 40.98% for *G. intraradices*, when compared with the control plants' roots (Table 4.7).

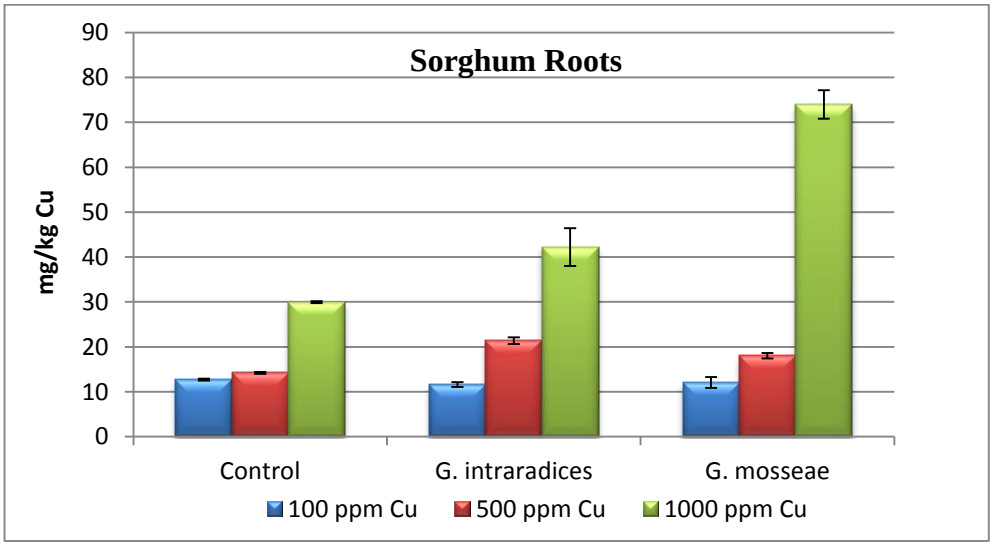

Figure 4.10. Cu stabilization on sorghum roots.

Table 4.7. Phytoremediation experiments data for sorghum plants.

Plant - AMF	Cu Concentrations (ppm)	Fresh Weight Root (g)	Root - Cu (mg/kg)	Fresh Weight - Shoot (g)	Shoot - Cu (mg/kg)
Sorghum - Control	100	38.5	12.71	6.00	4.94
	500	39.1	14.21	5.40	8.90
	1000	32	29.96	3.10	5.80
Sorghum *G. mosseae*	100	25.9	15.20	3.70	5.55
	100	45.3	10.08	4.30	5.20
	100	38.9	10.91	2.70	6.39
Sorghum *G. mosseae*	500	20.1	16.54	2.10	5.46
	500	41.9	18.33	5.80	4.92
	500	30.3	19.16	3.40	8.18
Sorghum *G. mosseae*	1000	43.7	75.84	6.80	15.13
	1000	11.2	66.16	3.80	11.16
	1000	19.8	79.90	3.80	12.64
Sorghum *G. intraradices*	100	26	12.90	6.30	4.35
	100	35.8	11.28	6.80	5.34
	100	22.2	10.62	4.30	5.96
Sorghum *G. intraradices*	500	18.7	23.14	3.80	4.21
	500	23.7	19.79	5.80	5.14
	500	37.4	21.27	3.40	2.42
Sorghum *G. intraradices*	1000	33.2	43.90	7.00	7.97
	1000	55.6	32.13	7.60	4.99
	1000	91.1	50.69	8.10	6.22

Table 4.8 indicates clear difference of Cu values in rhizosphere and non-rhizosphere measured after heavy metal application at the end of the experiment. Plant and microbes were able to absorb certain amount of Cu and partially immobilized fractions of soil Cu in the root area. As the young rhizosphere could not develop root area much after 8 weeks of growth. Initial expectation from these 8 week-plants in association with arbuscular mycorrhizal fungi was to achieve heavy metal immobilization along with plant uptake; and plant systems immobilized 70% of Cu in 100 ppm, 55% in 500 ppm, and 32% of total Cu available in 1000 ppm in the rhizosphere area.

Table 4.8. Comparison of Cu mass balance through rhizosphere and non-rhizosphere area and transition to the plant's compartments.

Plant - AMF	Treatment	Cu (mg) Stabilized on Roots	Cu (mg) Accumulated on Shoots	Total Cu (mg) Plant Uptake	Total Cu (mg) in Sand (Rhizosphere)	Total Cu (mg/pot)
Hornet - Control	100	0,32	0,15	0,47	3,28	3,75
Hornet - *G. mos.*	100	0,37	0,12	0,49	2,34	2,83
Hornet - *G. int.*	100	0,37	0,12	0,49	2,94	3,43
Sorghum - Control	100	0,49	0,03	0,52	2,05	2,57
Sorghum - *G. mos.*	100	0,42	0,02	0,44	3,99	4,43
Sorghum - *G. int.*	100	0,33	0,03	0,36	3,76	4,12
Hornet - Control	500	1,01	0,16	1,17	8,19	9,35
Hornet - *G. mos.*	500	2,67	0,15	2,82	12,81	15,63
Hornet - *G. int.*	500	1,20	0,13	1,33	12,17	13,50
Sorghum - Control	500	0,56	0,05	0,60	10,97	11,57
Sorghum - *G. mos.*	500	0,56	0,03	0,59	8,66	9,25
Sorghum - *G. int.*	500	0,57	0,02	0,59	8,66	9,24
Hornet - Control	1000	1,94	0,16	2,10	11,74	13,84
Hornet - *G. mos.*	1000	4,01	0,19	4,20	12,40	16,60
Hornet - *G. int.*	1000	1,31	0,16	1,47	15,77	17,24
Sorghum - Control	1000	0,96	0,02	0,98	13,07	14,05
Sorghum - *G. mos.*	1000	1,88	0,06	1,94	14,15	16,09
Sorghum - *G. int.*	1000	2,63	0,05	2,68	10,18	12,86

4.2.2. Mycorrhization on Roots

Determination of root mycorrhization was carried out under microscopic evaluation of root samples. Every plant's root colonization (mycorrhization) rate with both AMF species was examined by using 20 root samples and staining application. Highest mycorrhization rate was observed in Sunflower symbioses by *G. mosseae* species with 69.8% root colonization. Observation was also included the determination of average vesicle counts, total mycorrhiza count, and mycorrhizal colonization data was presented in Table 4.9. Arbuscular mycorrhizal fungi were spread though the roots and interaction between AMF and plant roots were visible (Figure 4.13 and Figure 4.14)

Table 4.9. Mycorrhization data of each plant - AMF combination.

Plant - AMF	Cu (ppm)	Vesicle Count	Total Mycorrhiza Count	Average Mycorrhization (%)
Sunf. - *G. mosseae*	100	6	34	53.45
Sunf. - *G. mosseae*	500	5	33	53.7
Sunf. - *G. mosseae*	1000	10	64	59.8
Sunf. *G. intraradices*	100	5	28	49
Sunf. *G. intraradices*	500	5	31	51.9
Sunf. *G. intraradices*	1000	6	35	56.65
Sorg. - *G. mosseae*	100	2	12	46.15
Sorg. - *G. mosseae*	500	2	13	52.75
Sorg. - *G. mosseae*	1000	2	16	56.2
Sorg. *G. intraradices*	100	2	12	49.3
Sorg. *G. intraradices*	500	1	11	46.75
Sorg. *G. intraradices*	1000	2	11	46.2

Root mycorrhizal symbiosis in sunflowers and sorghum through heavy metal – Cu – application showed a significant difference in terms of phytoremediation behaviour in sunflowers plants. Counted mycorrhiza number of *G. mosseae* and *G. intraradices* in sunflower roots was higher than sorghum plants (Table 4.9), hence, under the specified conditions sunflower plants symbiosis potential with AMF is favourable.

Correlation between mycorrhizal activity and root symbiosis was determined to be increasing parallel with escalating with heavy metal concentration available in soils. This is a promising defence mechanism, which was shown by plants under conditions of heavy metal stress for survival. Sunflower plants mycorrhization increased accordingly and best

rate achieved at 1000 ppm with *G. mosseae* (Figure 4.11). The standard error for root mycorrhization rate was approximately ±0.2%.

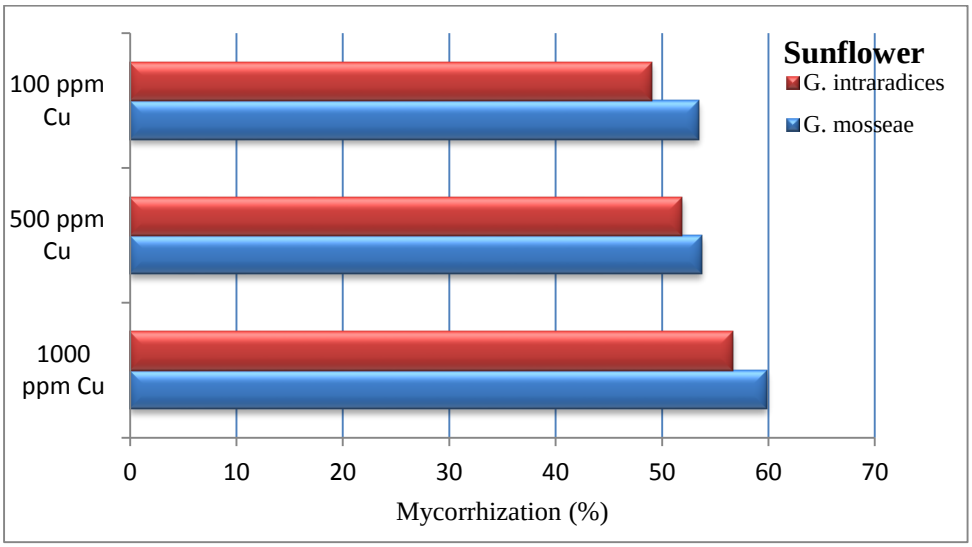

Figure 4.11. Comparison of mycorrhization rate of sunflower roots.

Sorghum; however, showed different mycorrhization levels with *G. mosseae* and *G. intraradices* under heavy metal stress. Increasing heavy metal concentrations resulted in better symbiosis rate for *G. mosseae,* and best ratio for sorghum was at 1000 ppm with *G. mosseae;* furthermore, root symbiosis with *G. intraradices* decreased when available heavy metal concentration increased. The lowest performance for mycorrhization in selected plants and species was Sorghum – *G. intraradices* combination at 100 ppm with 46.2%. This mycorrhization rate could be enough for improving the efficiency of phytoremediation; but, under selected conditions, it was the least favorable option. Comparison of sorghum plants' root mycorrhization is presented in Figure 4.12.

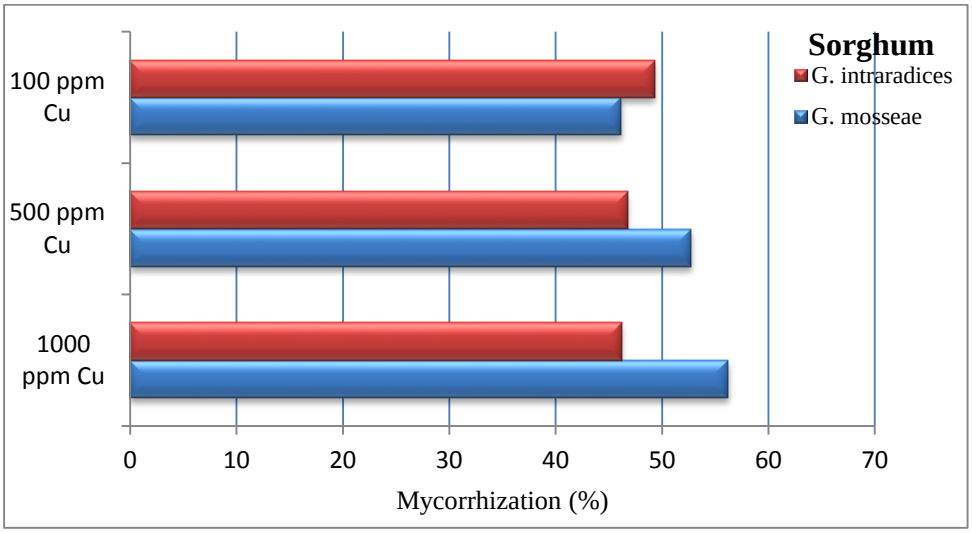

Figure 4.12. Comparison of mycorrhization rate of sorghum roots.

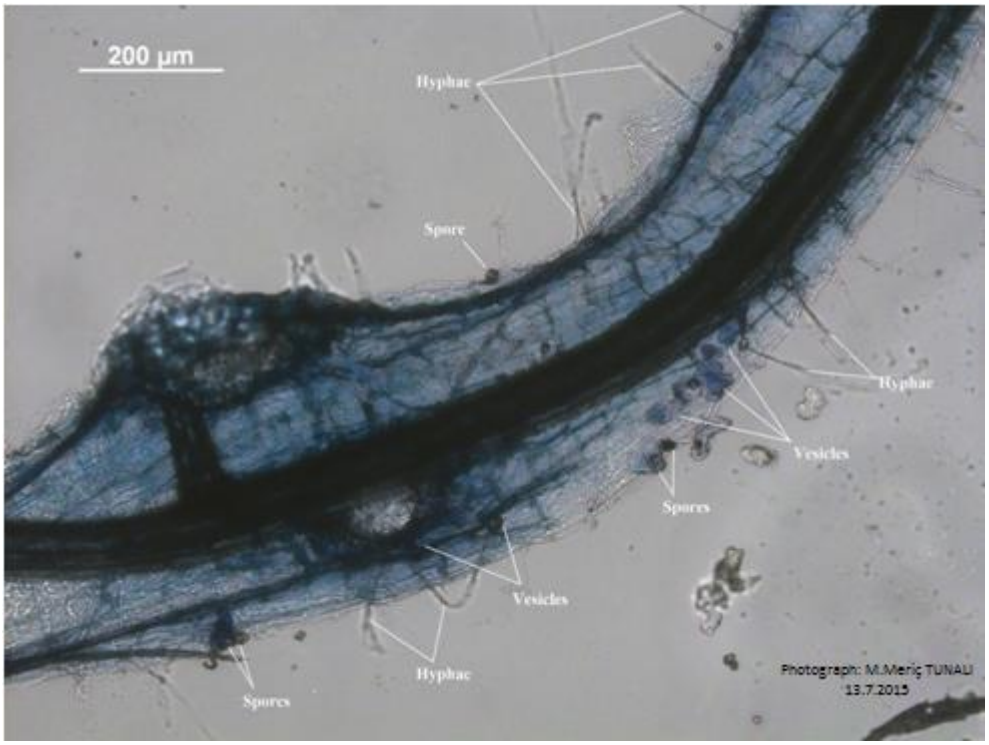

Figure 4.13. Spore, hyphal, and vesicular forms of *G. mosseae* in sunflower roots.

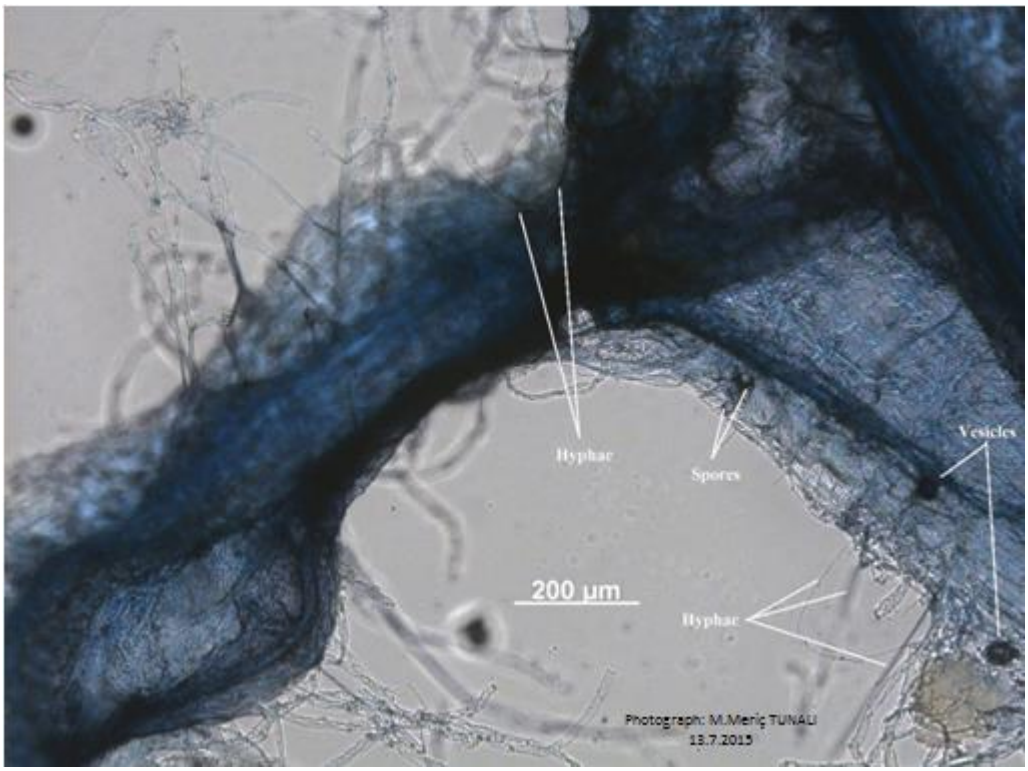

Figure 4.14. Spore, hyphal, and vesicular forms of *G. intraradices* in sorghum roots.

In Sunflower plants; different levels of heavy metals affects root Cu accumulation, and root mycorrhization significantly by $P < 0.01$. Accumulation of Cu in roots was higher in sunflowers inoculated by *G. mosseae* treated by 500 and 1000 ppm Cu. As well as different heavy metal concentration levels correlated with root mycorrhization rate showed significant difference by $P < 0.01$. The analysis data showed significant different between AMF species in terms of root mycorrhization rate under different heavy metal concentrations (500 and 100) by $P < 0.05$.

Table 4.10 shows the concentrations of the Cu measured in the roots and shoots of the plants of *S. bicolor* and *H. annuus,* which were collected at the moment of the highest biomass yield. The roots showed higher values of Cu accumulation than observed in the shoots. As a consequence, the translocation factor (shoot/root calculated in terms of concentration), which is typically >1 in the hyperaccumulator species, was <1 for all the Cu contents of sunflower and sorghum plants. Cu toxicity to plants is associated with the Cu level of 150-400 mg/kg in soil [114].

In this setup the soil contained approximately 300 mg of Cu per pot(3 kg)for 1000 ppm; hence, maximum Cu concentration available in soil was 100 mg/kg to prevent toxicity. The concentration of Cu measured in the roots of *S. bicolor* and *H. annuus* was, on average, about 28.32mg/kg and 111.74 mg/kg; respectively.

While Analysis of Variance (ANOVA) did show significant effect of heavy metal concentration levels in translocation of Cu by $P < 0.05$; this parameter was not significant in sunflowers shoot. However, Cu accumulation in rhizosphere was highly significant in both sorghum and sunflowers in terms of heavy metal levels. Meaning Cu was translocated in both plants' shoots along with the increased heavy metal concentrations. Root mycorrhization rate showed different reactions to heavy metals available in soils. Whereas, different heavy metal concentrations caused varieties in symbioses performance. *G. mosseae* demonstrated better resilience to harsh conditions. Our morphological observation evidently indicates that *G. mosseae* achieved highest level of colonization by 69.8% mycorrhization rate.

The impact of mycorrhizal species on the Cu offtake under different levels of heavy metal was also investigated. Findings indicated that increased Cu concentration in irrigation solution; Cu translocation increased with mycorrhization affect in Sorghum shoots by $P < 0.05$ but it was non-significant in Sunflowers which was inoculated by both Glomus species. Glomus species were preformed differently in both plant roots in terms of heavy metal uptake as data showed that there was different impact on Cu uptake between Glomus species in sorghum plants $P < 0.01$ and for sunflowers up to $P < 0.05$ were significant. *G. mosseae* acted better in to uptake Cu in both plants through three different heavy metal concentrations (100, 500 and 1000 ppm). Interactive effects between AMF species and different heavy metal concentrations showed in Table 4.10.

Table 4.10. Relation of Cu concentration on roots and shoots of the plants with mycorrhization

Plant - AMF	Treatment (Cu, ppm)	Plant Fresh Weight (g)	SD	Shoot Cu Concentration (mg/kg)	SD	Root Cu Concentration (mg/kg)	SD	Mycorrhization on Roots (%)
Sunf. - Control	100	14.30	0.22	10.42	2.44	18.70	0.33	N/A
Sunf. - Control	500	16.40	0.58	9.50	1.85	57.81	0.43	N/A
Sunf. - Control	1000	13.60	0.71	12.07	0.98	88.52	2.42	N/A
Sunf. - *G. mosseae*	100	15.27	0.69	7.97	0.14	29.34	2.57	53.45
Sunf. - *G. mosseae*	500	14.50	0.92	9.95	0.74	184.02	47.94	53.7
Sunf. - *G. mosseae*	1000	14.60	0.39	13.13	1.89	249.86	19.18	59.8
Sunf. - *G. intraradices*	100	16.30	0.28	7.65	0.55	27.11	2.21	49
Sunf. - *G. intraradices*	500	15.07	0.23	8.56	0.72	113.39	20.96	51.9
Sunf. - *G. intraradices*	1000	13.90	0.58	11.69	1.24	123.45	21.09	56.65
HMlevels		*NS*		*NS*		**		**
AMF		*NS*		*		**		*
HM x AMF		*NS*		*		**		*
Sorg. - Control	100	6.00	0.61	4.94	1.88	12.71	1.11	N/A
Sorg. - Control	500	5.40	0.63	8.90	0.74	14.21	0.85	N/A
Sorg. - Control	1000	3.10	0.11	5.80	0.22	29.96	0.99	N/A
Sorg. - *G. mosseae*	100	3.57	0.36	5.71	0.27	12.06	1.23	46.15
Sorg. - *G. mosseae*	500	3.77	0.84	6.19	0.78	18.01	0.60	52.75
Sorg. - *G. mosseae*	1000	4.80	0.77	12.98	0.90	73.97	3.16	56.2
Sorg. - *G. intraradices*	100	5.80	0.59	5.22	0.36	11.60	0.52	49.3
Sorg. - *G. intraradices*	500	4.33	0.58	3.92	0.62	21.40	0.75	46.75
Sorg. - *G. intraradices*	1000	7.57	0.25	6.39	0.67	42.24	4.20	46.2
HMlevels		*NS*		*		**		**
AMF		*		**		**		*
HM x AMF		*		*		**		*

Data are the means of three replicates _ standard error. Significance levels of heavy metals (HM), mycorrhizal species (AMF) and the interaction HM _ AMF are shown: *P < 0.05; **P < 0.01

4.2.3. Glomalin Related Soil Protein (GRSP)

The glycol-protein, glomalin, is produced with arbuscular mycorrhizal activity under critical conditions. The stress source for the production of glomalin in this scenario was related to heavy metal – Cu- stimulation. GRSP levels in soil samples gathered from root zone (below plant roots) of sunflower and sorghum plants are presented in Figure 4.15 and Figure 4.16.

Glomalin levels of sunflower and sorghum planted soils were escalated with increased heavy metal concentration in the soil body. Produced glomalin levels of *Glomus mosseae*, which had root mycorrhization rate were between 53.45% - 59.8% for sunflower, were 5.4 mg/kg at 100 ppm Cu, 7.6 mg/kg, at 500 ppm, and 9.7 mg/kg at 1000 ppm Cu, respectively. Results represent that glomalin was proportionally produced with increased stress caused by heavy metals. However, *Glomus intraradices'* mycorrhization with sunflower roots generated more glomalin at 500 ppm Cu. Beneficial behavior of symbiosis, determined to be under more stress at that concentration since glomalin production is a result of heavy metal intensity (Figure 4.15).

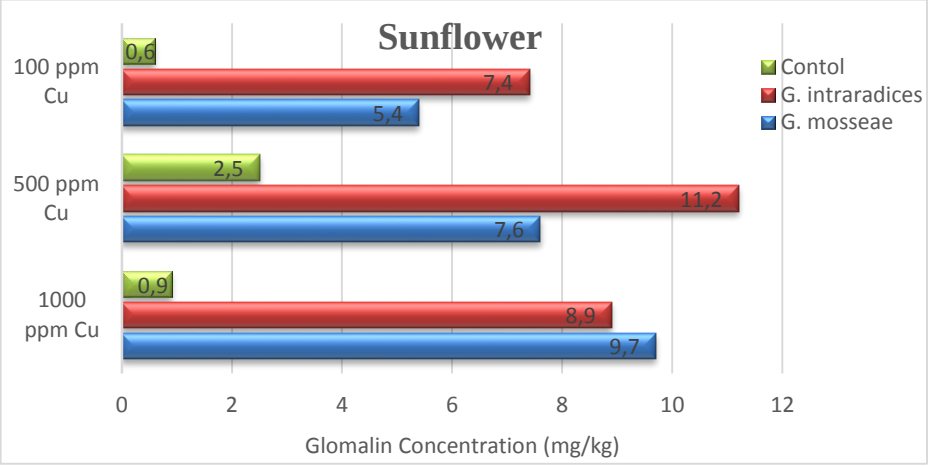

Figure 4.15. GRSP concentrations of sunflower planted soils.

Glomus intraradices, which had shown relatively low mycorrhization levels than *G. mosseae* on the plant roots, generated 6.55 mg/kg at 100 ppm Cu, 7.75 mg/kg at 500 ppm, and 8.1 mg/kg at 1000 ppm Cu, where mycorrhization rates were between 46.2% - 49.3% on sorghum roots. Linear increase in glomalin concentrations for *G. intraradices*– sorghum

is similar to *Glomus mosseae* – sunflower interaction. Correspondingly, at 500 ppm Cu concentration *Glomus mosseae's* symbiosis with sorghum plants' roots produced more glomalin. Therefore, different types AMF reacted differently depending on host plant in terms of glomalin generation (Figure 4.16).

Previous studies have shown that under suboptimal conditions for AMF hyphal growth, glomalin content increased. Using structural equation modeling [115], have inferred that a direct effect of glomalin on metal detoxification may be higher than the (residual) contribution of AMF hyphae.

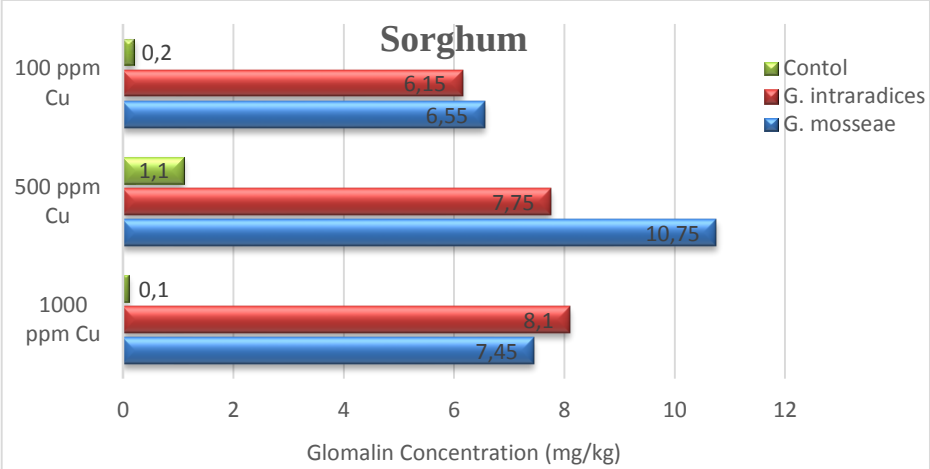

Figure 4.16. GRSP concentrations of sorghum planted soils.

Humic compounds' extraction in Bradford assay may interfere with the GRSP content; yet, correlation between glomalin related soil protein (GRSP) content and total extracts is proportional. Hence, indicating a good comparison parameter in different types of soil [116]. Stabilization of Cu in non-mycorrhizal plant roots were increased reasonably with raised concentrations of available heavy metal in soil. Moreover, there was a positive correlation between soil glomalin and Cu (total and available fractions) content. Enhanced glomalin yield was observed in Sunflower – *G. mosseae*, and Sorghum – *G. intraradices* symbiosis with increased level of heavy metal stabilized in the roots. Comparison between mycorrhization, glomalin content, and root Cu concentrations was illustrated in Table 4.11.

Table 4.11. Comparison of GRSP, mycorrhization, and stabilized Cu in roots.

Plant - AMF	Average Mycorrhization (%)	Glomalin (mg/kg)	Root Cu (mg/kg)
Sunf. Control 100	N/A	0.6	18.70
Sunf. Control 500	N/A	2.5	57.81
Sunf. Control 1000	N/A	0.9	88.52
Sunf. *G. mos*. - 100	53.45	5.4	29.34
Sunf. *G. mos*. - 500	53.7	7.6	184.02
Sunf. *G. mos*. - 1000	59.8	9.7	249.86
Sunf. *G. int*. - 100	49	7.4	27.11
Sunf. *G. int*. - 500	51.9	11.2	113.39
Sunf. *G. int*. - 1000	56.65	8.9	123.45
Sorg. Control 100	N/A	0.2	12.71
Sorg. Control 500	N/A	1.1	14.21
Sorg. Control 1000	N/A	0.1	29.96
Sorg. *G. mos*. - 100	46.15	6.55	18.96
Sorg. *G. mos*. - 500	52.75	10.75	21.04
Sorg. *G. mos*. - 1000	56.2	7.45	23.32
Sorg. *G. int*. - 100	49.3	6.15	21.11
Sorg. *G. int*. - 500	46.75	7.75	21.82
Sorg. *G. int*. - 1000	46.2	8.1	22.08

The relation between glomalin content and heavy metal stabilization on plant roots could be a the result of a mechanism to improve the fungal habitat [107], perhaps in regard to high levels of potentially phytotoxic elements in soil nutrients (such as Fe and Al)[117], or to alleviate physical/spatial constraints for hyphal development [118].

High contents of glomalin along with high contents of heavy metals could be explained by the presumably low activity of microorganisms able to degrade glomalin under these extreme pollution conditions [119], and its recalcitrance in the soil [120]. Another “protection” effect of glomalin could be its iron binding [121], agreeing with previous studies in these soils that shown high content of this elements [122]. Given the recent finding of high homology between glomalin and heavy metals like Cu [97], which are stress-related proteins, it should not be neglected that a strong stress presented by high levels of heavy metals may cause over expression of this protein.

5. CONCLUSIONS

Plant-microbe interactions application for phytoremediation is a long-term remediation effort, and it requires screening of many suitable plants and beneficial specific microbes such as mycorrhizal fungi to reduce metal concentrations to acceptable levels. Time required for remediation is dependent on the type and extent of metal contamination and the duration of the growing season. However, the most important feature of the plants is the efficiency of metal removal. In this study; analysed number of sorghum plants were: 30 for every combination (180 plants in total), and sunflower plants: 15 for every combination (90 plants in total); hence, average of every data gathered from plants and pots are presented in the results. According to these results, the highest amounts of Cu removed by *S. bicolor* equated to only 20.75 mg/kg, 27.35 mg/kg, and 92.55 mg/kg through 100, 500 and 1000ppm Cu concentrations around the rhizosphere in symbioses with *G. mosseae* while the non-mycorrhizal sorghum was able to accumulate 17.67 mg/kg, 23.11 mg/kg, and 35.76 mg/kg by 100, 500 and 1000 ppm Cu. The offtake of sunflowers (*H. annuus*) had the same order of magnitude in terms of showing active Cu uptake in plants through the interactive with *G. mosseae*. Amount of Cu removed from soil by non-mycorrhizal sunflower plants were 29.12 mg/kg for 100 ppm, 67.30 mg/kg for 500 ppm, and 100.59 mg/kg for 1000 ppm; while, mycorrhizal plants uptake were 37.33 mg/kg, 193.97 mg/kg, 262.99 mg/kg at the end of 8 weeks phytoremediation duration, respectively.

Extracted amount of Cu was negligible in comparison to the magnitude in the soil by non-mycorrhizal sorghum and sunflower; therefore, under the experimental conditions, *S. bicolor* and *H. annuus* showed a very strong potential of stabilize Cu in their rhizosphere by accumulating this metal in the cell vacuoles. On the other hand, the experimental design did consider specific translocation in plants compartments to enhance the bioavailability of the metals.

Currently, phytoextraction is far from being considered a mature technology. It is difficult to predict when sufficient knowledge will be acquired about specific molecular and physiological aspects of phytoremediation for practical application. Neither the biomass crops, nor the hyperaccumulator plants have been studied deeply enough in field conditions.

Most of data published has been extrapolated from experiments performed under conditions that are not adequate to give results applicable for the future clean-up of contaminated areas. Among the few experiments dealing with the in situ performances of hyperaccumulators, it was observed that hyperaccumulator plants under certain conditions could not express their potential. Obtained data from this application shows a very interesting behaviour of interactive affects between indigenous mycorrhizal species natives in terms of heavy metal remediation on Turkish climate conditions.

However, the fundamental microorganisms like mycorrhizal of plants useful for phytoremediation should be expressed in a wide range of environmental conditions to transform phytoremediation from a natural phenomenon to a sustainable technology for soil clean-up.

A strong positive correlation between glomalin content and Cu (total and available fractions) were found. Results from this experiment agree with such previous observations, and suggest that the HM sequestration in glomalin may be another mechanism by which AMF could improve environmental conditions for their development. In this study, at 1000 ppm Cu concentration the contribution of mycorrhizal activity of *G. mosseae* increased heavy metal stabilization on sunflower roots by 182.26% and for sorghum roots by 146.89%; while *G. intraradices* had increase this performance by 96.14% and 40.98%, respectively. The root mycorrhization rates were observed as; Sunflower - *G. mosseae*: 59.8%, Sunflower – *G. intraradices*: 56.65%, Sorghum – *G. mosseae*: 56.2%, and Sorghum – *G. intraradices*: 46.2%. It probably is the highest value reported under field conditions to date. Although the methodology used for glomalin extraction and quantification may overestimate its content in soil [123], In this study, highly significant correlations were found between root mycorrhization rate, Cu uptake and glomalin generation in roots. At 1000 ppm Cu concentration, for non-mycorrhizal sunflower plants'

root Cu concentration was 88.52 mg/kg; while, mycorrhization of *G. mosseae* was 59.8%, GRSP concentration was 9.7 mg/kg and the root Cu concentration was raised to 249.86 mg/kg (182.26% increase). For *G. intraradices s*mycorrhization rate was 56.65%, GRSP concentration was 8.9 mg/kg and root Cu concentration was enhanced by 39.46% (to 123.45 mg/kg).

This effect is ecologically very important, since even under extreme conditions the deposition of large amounts of glomalin could promote water-stable aggregates formation/stabilization, helping plant to produce root cells in terms of especially when heavy metal accumulation is mostly superficial as a result of metal smelter activity or mine tailing [122].

As mentioned earlier, phytoremediation is a relatively recent field of research and application. Currently most research is limited to laboratory and greenhouse scale studies and only a few studies have been conducted to test the efficiency of phytoremediation in actual field. Results in actual field can be different from those at laboratory or greenhouse conditions [124], because field is a real world where different factors simultaneously play their role. Factors that may affect phytoremediation in the field include variations in temperature, nutrients, precipitation and moisture, plant pathogens and herbivory, uneven distribution of contaminants, soil type, soil pH, and soil structure [125]. Phytoremediation efficiency of different plants for specific target heavy metals has to be tested in field conditions in order to realize the feasibility of this technology for commercialization. After identification of desirable traits in natural hyperaccumulators, such traits can be selected either by conventional breeding techniques or by using new methods of hybridization such as protoplast fusion or by the manipulation of gene expression in transgenic [126]. In spite of the many challenges, phytoremediation seems as a green remediation technology with an expected great potential. Research in phytoremediation is truly interdisciplinary in nature and requires background knowledge in soil chemistry, plant biology, ecology and soil microbiology as well as environmental engineering. In view of the current trends of integration of scientific knowledge worldwide, it is hoped that many challenging questions about commercial application of phytoremediation will be answered in future.

In comparison with different engineering techniques and expensive current remediation projects of heavy metal from contaminated soil and water bodies, the processes are time consuming and plausible environmental outcomes are potentially harmful to the ecosystem. Since heavy metals cannot degrade with natural processes, effective cleanup requires their immobilization, reduction or transformation to non-toxic forms. Phytotechnology involves efficient use of plants to remove, detoxify or immobilize environmental contaminants in a growth matrix (soil, water or sediments) through the natural, biological, chemical or physical activities or processes of the plants. A brief review on phytoremediation of heavy metals and its effect on plants have been compiled to provide a wide applicability of phytoremediation.

Finally, from a biotechnological perspective, further studies on the characterization of metallophytes and their associated AMF communities are needed, to select the most suitable ecotypes method in site remediation in Turkish urban areas and other soils subjected to the effect of mining activities.

6. RECOMMENDATIONS

1. Phytoremediation research is seriously should be projected interdisciplinary in polluted environment;

2. Researchers from different backgrounds should be welcomed and encouraged to utilize their skills and expertise in this field.

3. Existing natural resources like plant and microorganism diversities should be sight seen for hyperaccumulation of various heavy metals to find new effective metal hyperaccumulators.

4. Harvested biomass after phytoremediation should be handled carefully (Bioaccumulated contaminants).

5. Biomass gathered on the field can be utilized as an energy source via incineration/anaerobic digestion or can be used as base material for biodiesel production.

6. Recycling/storage options of residues for valuable and toxic substances should be applied.

7. More phytoremediation studies should be conducted in the field and analysis keeping in mind the very green nature of the technology.

8. For optimum efficiency; surface area and plant number per square meter should be calculated according to the duration of the phytoremediation, climate conditions, and specified plants' root development behaviour.

9. More studies should be conducted to better understand interactions among the four players in the rhizosphere that is among metals, soil, microbes and plant roots.

10. Advancement in spectroscopic and chromatographic techniques should be exploited to improve understanding of the fate of metal ions in plant tissues, which in turn will improve understanding of metal hyperaccumulation and tolerance in plants.

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