EVALUATION OF MECHANISMS OF INCREASING SOIL QUALITY VIA PLANT-MICROORGANISM INTERACTIONS IN A MINE TAIL

by

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ABSTRACT

EVALUATION OF MECHANISMS OF INCREASING SOIL QUALITY VIA PLANT-MICROORGANISM INTERACTIONS IN A MINE TAIL

Arbuscular mycorrhiza (AM) which belongs to the kingdom of fungi is a symbiont of the roots of the plants. It provides water, macro and micro nutrients to its host, and takes photosynthetically derived sugars from the plant in return. In this research, the role of plant-mycorrhiza interaction on the remediation of the soil from a mine tailing area in Kütahya was evaluated, furthermore, the genetic mechanisms behind this remediation were highlighted. To assess the effect of mycorrhiza on plant growth, metal absorption by the plant, soil fertility and the expression of metal transporter genes, a greenhouse experiment was set up with *Sorghum bicolor* L. and *Rhizophagus irregularis.* Results showed that the mycorrhiza might positively affected the plant growth even at the germination stage and it increased mostly the root weight afterward. Metal absorption by the mycorrhizal roots was higher because of the increase in root growth and in the expression level of YSL-15 gene in roots. Root to shoot translocation was also higher in mycorrhizal plants. Additionally, the mycorrhiza induced the soil fertility to increase, but this was not due to the glomalin production. The results suggest that the expression and the production of glomalin might depend on the type and concentration of metals in the soil. In addition, further research should evaluate the phytoremediation application in Kütahya between June and August, since our results indicate that the symbiosis strongly depended on the temperature in the soil.

ÖZET

BİR MADEN TOPRAĞINDA BİTKİ-MİKROORGANİZMA ETKİLEŞİMLERİ İLE TOPRAK KALİTESİNİ ARTIRMA MEKANİZMALARININ DEĞERLENDİRİLMESİ

Arbusküler mikorizal funguslar bitki köklerinde simbiyotik yaşayan mantarlar alemine ait mikroorganizmalardır. Arbusküler mikorizal mantarı, kökünde yaşadığı konak bitkiye su, makro ve mikro besinler sağlarken, bitkiden de karbon kaynağı olarak fotosentez ürünü olan şekerleri tedarik eder. Bu araştırmada, Kütahya' daki bir maden bölgesi toprağının remediasyonunda bitki-mikoriza ilişkisinin rolü değerlendirilmiş ve bu remediastonun ardındaki genetik mekanizmalar aydınlatılmıştır. Arbusküler mikoriza mantarının bitkinin büyümesi, bitkinin metal absorpsiyonu, toprak verimliliği ve bitkinin metal taşıyıcı genlerinin ekspresyonu üzerindeki etkilerini değerlendirmek için *Sorghum bicolor* L. bitkisi ve *Rhizophagus irregularis* mikorizası ile bir sera seti kurulmuştur. Mikorizanın bitki büyümesini çimlenme aşamasında dahi arttırdığı ve çimlenme sonrası süreçte ise özellikle kök büyümesini arttırdığı görülmüştür. Mikorizalı bitki köklerinin daha fazla büyüme göstermesi ve yine bu köklerdeki YSL-15 geninin daha fazla ekspres olması mikorizalı bitki köklerindeki metal absorpsiyonunu mikorizasız olanlara kıyasla önemli ölçüde arttırmıştır. Kökten gövdeye metal translokasyonunun mikorizalı bitkilerde daha fazla olduğu görülmüştür. Bunlara ilaveten, mikorizanın toprak verimliliğinin artmasına sebebiyet verdiği fakat bunu glomalin üretimiyle yapmadığı görülmüştür. Glomalin üretiminin ve glomalin geninin ekspresyonunun topraktaki metal türü ve konsantrasyonuna bağlı olabileceği öne sürülmüştür. Buradaki bitki ve mikoriza simbiyoz seviyesinin sıcaklığa bağlı olarak değişkenlik gösterdiği görüldüğünden, Kütahya' da yapılacak olan fitoremediasyon saha çalışmasının Haziran ve Ağustos ayları arasında yapılması önerilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Abbreviation	**Explanation**
AMF	Arbuscular Mycorrhizal Fungi
GRSP	Glomalin-Related Soil Protein
BSA	Bovine Serum Albumin
ABC	ATP-Binding Cassette
ABCC	ATP-Binding Cassette from the Subfamily C
PS	Phytosiderophore
ER	Endoplasmic Reticulum
ZIP	ZRT/IRT - Like Protein
AtNRAMP	*Arabidopsis Thaliana* Natural Resistance-Associated Macrophage Protein
P_i	Inorganic Phosphorus
CTR	Copper Transporter
ZRT	Zinc Transporter Proteins
FTR	Ferredoxin-thioredoxin reductase
PolyP	Polyphosphate
DI	Distilled Water
HM	Heavy Metal
YSL	Yellow stripe like
ICP-OES	Inductively Coupled Plasma – Optical Emission Spectrometry
AM	Arbuscular Mycorrhiza
SE	Standard Error
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction

1. INTRODUCTION

Soil health is an important factor for environmental and human health since soil is the natural resource for agriculture and the ecosystem. However, quality of the soil is getting decreased year by year in the world. Mining activity is one of the contributors of this decrease since it leads to the increased levels of metal concentrations which even reach to the toxic levels in the soil. This research aimed to determine the effects of plant-mycorrhiza interactions in phytoremediation efforts to increase the quality of the soil taken from a mine tailing area in Kütahya, to highlight the molecular genetic mechanisms involved in these effects. Within the scope of this research, heavy metal stabilization capacities were determined for both the selected plant and the plant-mycorrhiza together in this soil. Also, the contribution of mycorrhiza to the soil quality and the mechanisms of its contribution were examined. Moreover, the genetic mechanisms that have a role on this phytoremediation application in the plant were determined. In the light of the findings of this research, new suggestions for the possible phytoremediation application of this mine tailing area in Kütahya have been made. Moreover, findings of genetic mechanisms behind the remediation in this research have contributed to the improvement of the phytoremediation applications in the future. The hypothesis of this study was: Do mycorrhizal fungi contribute the plant tolerance for resisting heavy metal existing in the mine tailing soil? If yes, what would be the mechanisms of mycorrhizal fungi for this contribution? Any development of understanding and knowledge can assist scinetists to progress plant-microbe interactions at the environmental stress circumstances.

2. LITERATURE REVIEW

Soil is a finite resource and it has a critical role on persistence of life on earth. Soil is fundamental for food, feed, fuel and ecosystem. Therefore, protection of soil quality is becoming essential especially with the increasing human population over the course of many years.

Over centuries, changing technologies and the increase in human population size has resulted in increased industrial, agricultural, mining and military activities which in turn induced the contamination and pollution of soils with heavy metals and organic pollutants. This contamination and pollution are the main causes of soil degradation, which refers to the loss of soil quality via physical, chemical and biological ways. Therefore, remediation of polluted soils is crucial in terms of sustainability.

Phytoremediation is a green technology that uses both wild and genetically modified plants to remove pollutants from soil. Plants naturally use both organic compounds and heavy metals for their metabolisms. In the case of toxic concentrations of these materials in the soil, plants benefit from their own detoxification mechanisms, which contribute to the removal of pollutants from soil. Mechanisms of removal from the soil include uptake, translocation, metabolization and sequestration of toxins into the cell vacuole (Peuke and Rennenberg, 2005). Glutathione S-transferase and phytochelatin synthase are the enzymes required for vacuole storage of organic pollutants and heavy metals, respectively. Phytochelatin synthase carries out the synthesis of phytochelatins that are the metal chelating peptides required for the detoxification of heavy metals in higher plants, eukaryotic algae, nematode, cyanobacteria and fungi. Glutathione S-transferase catalyzes the conjugation of the reduced form of glutathione to xenobiotic organic pollutants, such as pesticides (Edwards et al., 2000), resulting in conjugates that are less toxic, more water soluble and suitable for vacuole storage, which can favor their subsequent degradation. ABC binding cassette transporter proteins are transmembrane proteins that contribute to the detoxification of toxic organics through efflux of toxins out of root cells and transfer of glutathione-toxin conjugates to the vacuole (Meagher, 2000). In addition, some subfamilies of ABC transporter proteins, such as ABCC type transporters, provide transfer of phytochelatin-heavy metal complex to the vacuole (Park et al., 2011). The basic steps of detoxification are shown in Figure 2.1 below:

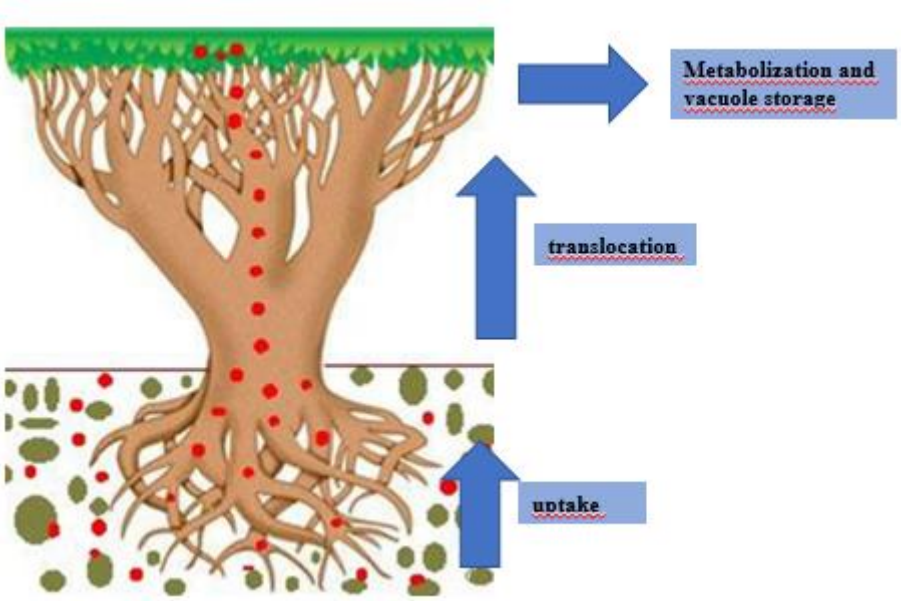


Figure 2.1. The detoxification process of pollutants and heavy metals by a plant. The first step is the uptake of organic pollutants and heavy metal ions along with water; the second step is the translocation of these ions and molecules from roots to the upper part of the plant; the third step is the metabolization and sequestration of toxins in the vacuole.

A wide range of transition metals such as Zn, Mn, Cu, Fe are required for normal plant growth and development (Hall, 2003). In plants, these metals are the micronutrients for plants with crucial roles in various metabolic pathways like DNA synthesis, photosynthesis and respiration (Rout and Sahoo, 2015). Because their contributions to metabolic homeostasis make them essential for plant growth, development and survival, plants have different mechanisms and pathways to uptake metal ions from soil via their roots.

Plants take up heavy metal ions in the water that is absorbed by the roots. Thus, plants are only able to take up the water soluble forms of these metal ions. Since insoluble forms are not available for the plant, soil pH and temperature, which determine the oxidation states of metal ions, are important. Plants have evolved two different strategies for the acquisition of essential metal ions, which are referred to as strategy I and strategy II. The first strategy is based on the reduction of metal ions in the soil since most of the essential metals like Fe, Zn, Mn are soluble in their reduced forms. Strategy I plants secrete protons to the soil from their root cells via plasma membrane-bound reductase, a process called rhizosphere acidification, and this results in the reduction of metal ions. The second strategy is based on chelationof metal ions, since metal ion complexes are more soluble and mobile than the metal ions alone. Strategy II plants, such as sorghum release phytosiderophores, which is a chelating agent for the metal ions, from their roots (Ferrol et al., 2016). The mechanism of metal uptake by strategy I and strategy II are shown in petunia and barley in Figure 2.2 below.

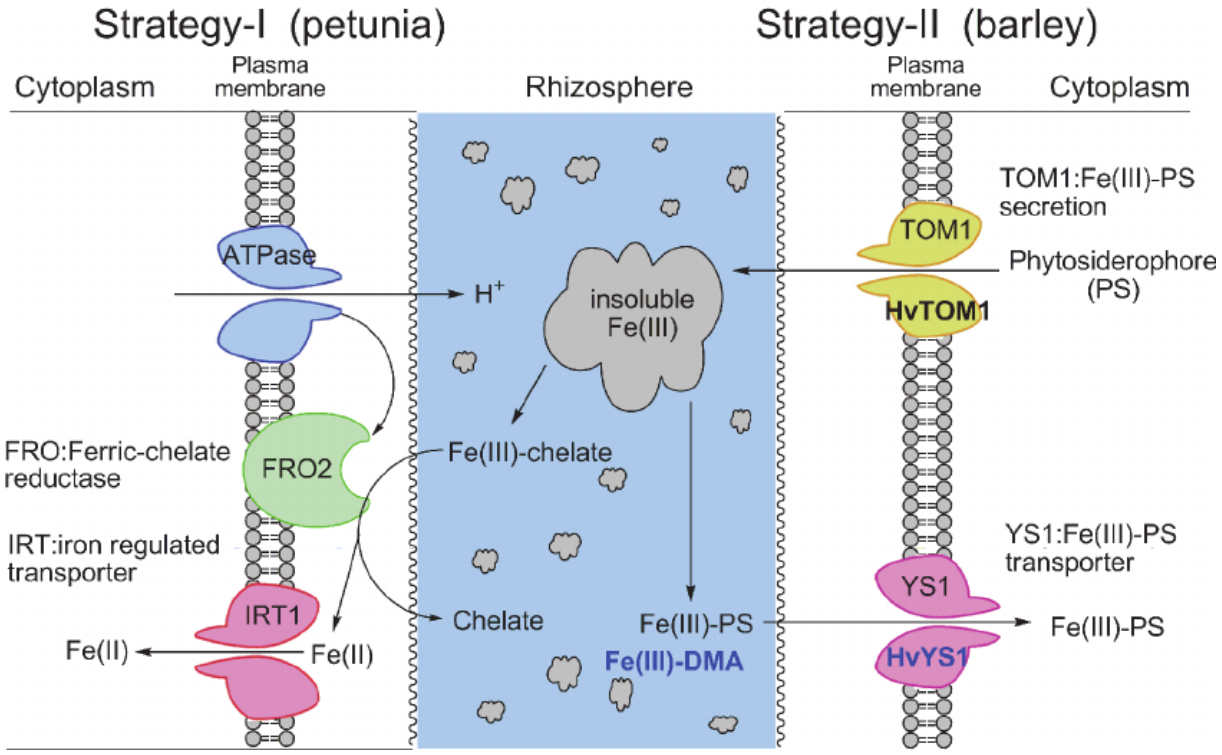


Figure 2.2. Metal uptake from the soil by strategy I and strategy II plants (Murata et al., 2015).

Plants take up the soluble forms of metal ions from soil through transmembrane metal transporter proteins on the root epidermal cell membranes. These metal transporters span the cell membrane and are also present on the membranes of golgi, endoplasmic reticulum (ER), vacuole and plastids to facilitate the heavy metal compartmentalization in the cell. The heavy metal ATPases, the cation diffusion facilitator family, the Nramps, the ZIP family and the cation antiporters are the trace metal transporters in plants (Hall, 2003). These transporters in the plant cell are shown in Figure 2.3 below.

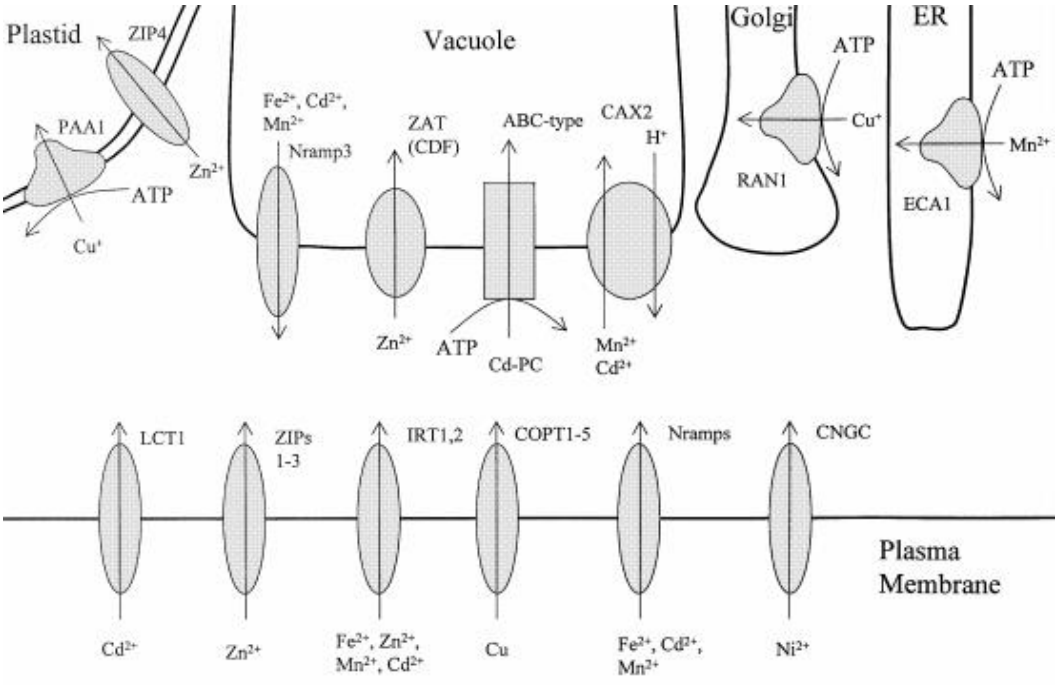


Figure 2.3. Various metal transporters found on the plasma, golgi, ER, vacuole and plastid membranes; transportation mechanisms are shown.

The expression levels of metal transporter genes affect the amount and rate of metal uptake and metal tolerance of plant cells. Various environmental and cellular signals can result in over-expression or under-expression of the metal transporter genes. Abundance or deficiency of the metal ions in soil can affect the regulation of metal transporter gene expression. For instance, it was observed that *AtNRAMP3* and *AtNRAMP4* genes which play role on Fe uptake were over-expressed in root cells under Fe starvation (Thomine and Schroeder, 2004).

The expression profiles and types of metal transporter genes differbetween plant species and organs, type of cells and organelles. As a result, bioaccumulation and translocation factors also differ from plant to plant and organ to organ. These differences also determine the phytoremediation potential of a plant. For instance, hyperaccumulator plants are preferred for phytoremediation applications since they have high capacity to absorb, translocate and accumulate heavy metals. Hyperaccumulator plants are able to overcome heavy metal stress via the upregulation of hyperaccumulator genes which include metal transporter genes and the genes that are required for absorption and sequestration of heavy metals.

Much research has been carried out on metal transporter and hyperaccumulator genes to improve and highlight their contribution to phytoremediation applications. Understanding the molecular and genetic mechanisms behind the metal accumulation, resistance and tolerance of different plants have

taken possible genetic engineering applications into consideration. For instance, it has been shown that transgenic poplar was successful at hyperaccumulating Cu and Cd, and that As and Hg resistance can be induced via bacterial gene transfer to poplar and *Arabidopsis* plant (Merkle, 2006). Another successful application of genetic engineering in phytoremediation was carried out in yeast; transgenic yeast to which iron regulated transporter gene was transferred showed ability to hyperaccumulate iron.

Plants are the key element of phytoremediation applications however supportive microorganisms are also used with the plants. These supportive microorganisms which can be bacteria or fungi, live symbiotically with the selected plants. Arbuscular mycorrhizal fungi (AMF) are one of the frequently used microorganisms in phytoremediation. AMF are mycorrhiza belonging to the phylum of Glomeromycota. They live as an obligate biotroph in the roots and the rhizosphere of many kinds of plants from grass to woody plants. The symbiosis between AMF and plants is based on the nutrient and water exchange among them. The plant provides photosynthesis products, resources for the germination of AMF spores and a place for colonization to the mycorrhizae on the other hand, AMF provide nutrients and water to its host plant. The enhanced uptake of macro and micro nutrients by plant due to the symbiosis with the AMF eventually encourages the plant growth. Besides supporting plant growth, AMF also help plant to cope with harsh conditions, like pathogens, drought, salt and heavy metal stresses. The positive effect of AMF on plant growth and protection against the pathogens in the soil is shown in Figure 2.4.

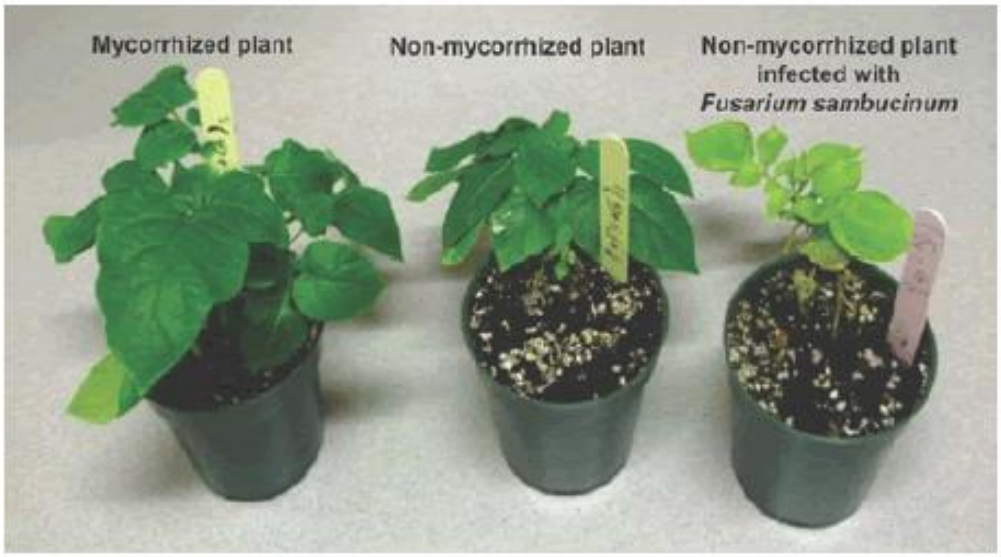


Figure 2.4. Positive effect of AMF on plant growth and protection against soil-borne pathogens (Bolduc, 2011).

AMF is essential for the uptake of phosphorus (P) by host plants. P is an essential macronutrient for plant growth and development (Mlodzinska and Zboinska, 2016). P is found in organic and mineral forms in soil. Since plants can utilize only the inorganic form of P, most of the P in the soil, up to 80% of P, is unavailable for plants (Schachtman et al., 1998). For instance, phytic acid (inositol hexaphosphate), the most common organic form of P in soil, cannot be used by plants (Richardson, 1994). AMF enhance uptake of inorganic P to host plants in multiple ways: by encouraging plant root growth, by converting the unavailable P to available forms by mycorrhizal exudates, by affecting the expression of P_i transporters on the membrane of the root cells, and by transferring inorganic phosphorus taken up by mycorrhiza directly to the plant via fungal phosphate transporters (Walder, F. et al., 2016). Modes of P aacquisition by plants from the soil is shown in Figure 2.5.

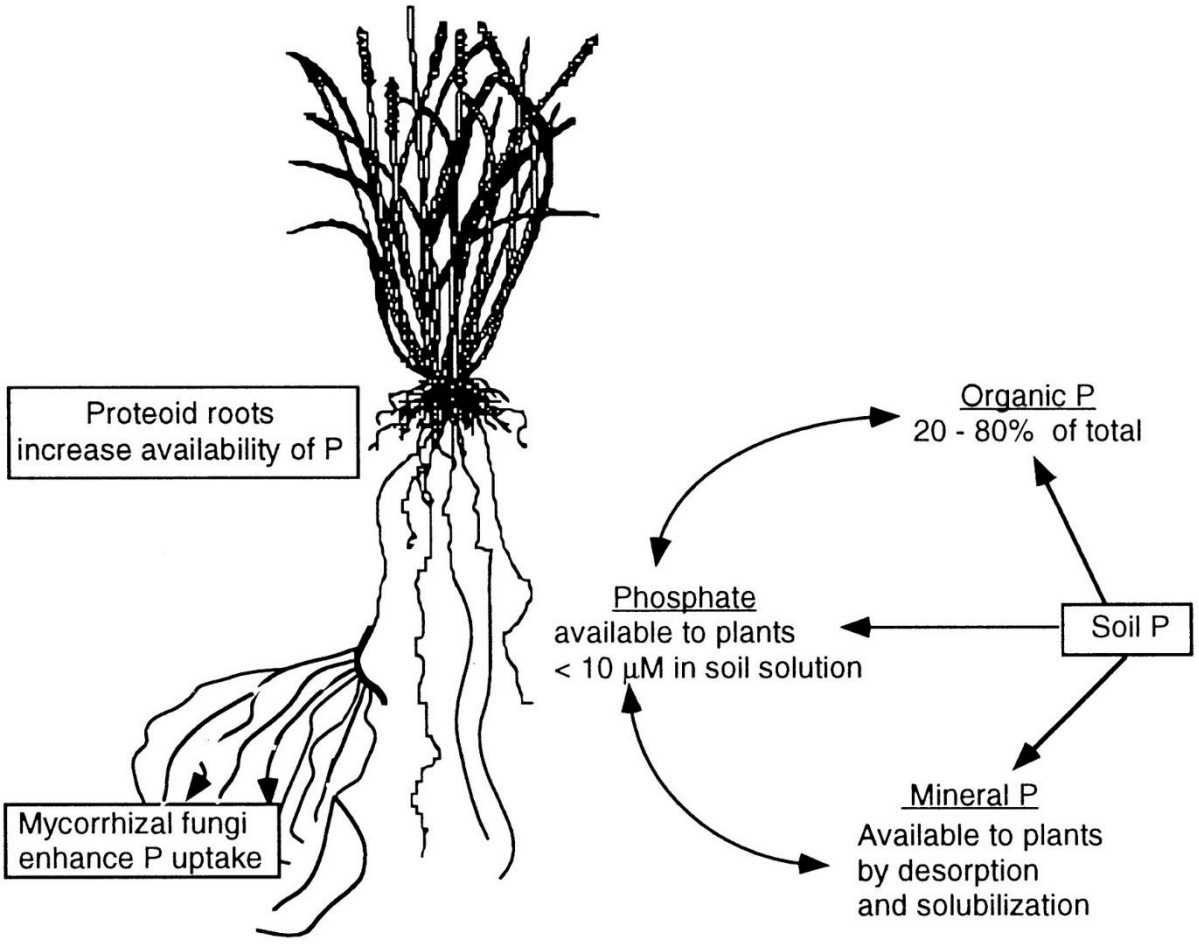


Figure 2.5. Acquisition of phosphorus from soil by plants (Schachtman et al., 1998).

Plants uptake phosphorus in the orthophosphate form, mostly as $H_2PO_4^-$. Ionization of orthophosphate depends on the pH of the soil, and $H_2PO_4^-$ is found mostly at the pH range between 4 and 6. Therefore, bioavailability of P in the soil is pH dependent. If the soil is alkaline or phosphorus deficient, plant roots pump protons to the soil to acidify the rhizosphere to aid uptake of inorganic

phosphorus in the form of $H_2PO_4^-$ or HPO_4^{-2} (Gillespie and Pope, 1991). The orthophosphate species favore at various pH values are shown in Figure 2.6 below.

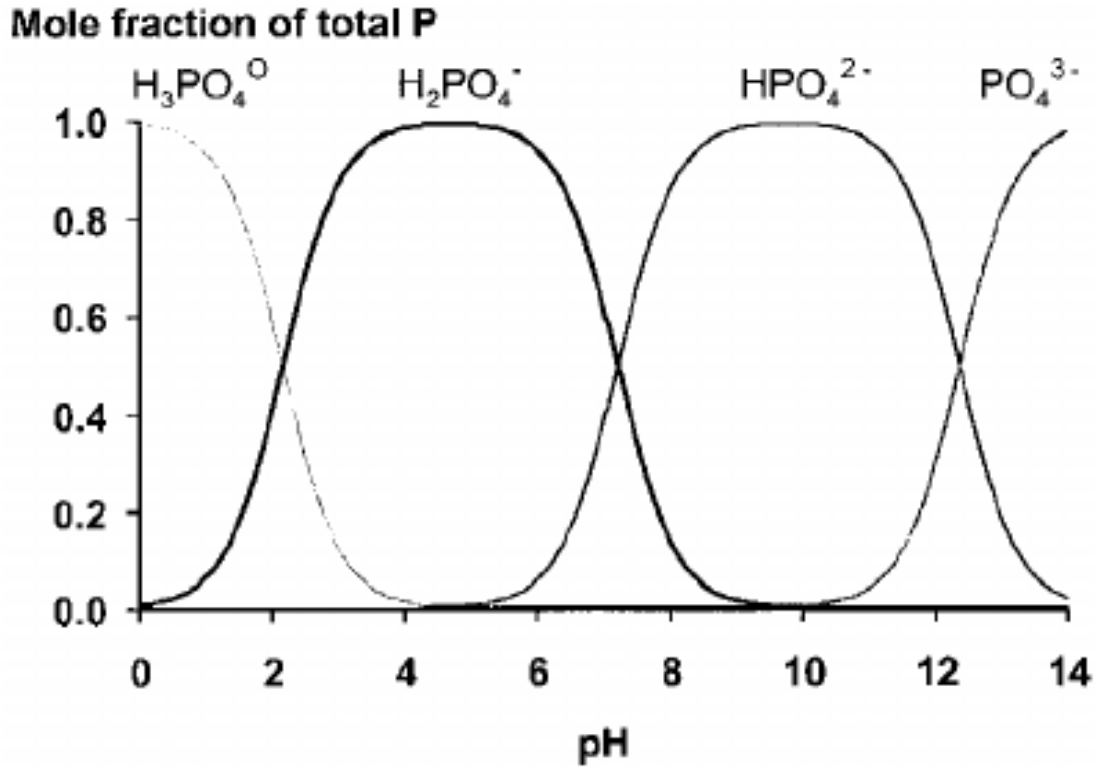


Figure 2.6. Distribution of orthophosphate species at various pH values (Hinsinger, 2000).

AMF is a type of endomycorrhizae which has fungal structures both outside and inside of the plant root. Arbuscule structures are located inside of the root epidermis cells while hyphae structures are located both inside and outside of the root. Arbuscules which are located directly in the root cells provide the nutrient and water exchange between AMF and root cells. Intraradical hyphae are located in the intercellular area and extraradical hyphae are located outside the root. Extraradical and intraradical hyphae are connected to each other to maintain the transfer of nutrient and water that are absorbed from the soil by AMF to the inside of the root and to maintain the transfer of carbon source which is provided by plant to the AMF (Aarle and Olsson, 2008). A mass of branching hyphae constructs the mycelium which has an important role in the plant-AMF symbiosis due to its high absorptive capacity. Water, nutrients, organics, aminoacids and heavy metals are absorbed by the extraradical hyphae, located outside of the root, and they are transported to the intraradical hyphae where they will be transferred to the plant through an exchange at the symbiotic interface within the root (Silva, 2000). Mycorrhizal structures within the symbiotic interface are shown in Figure 2.7 below.

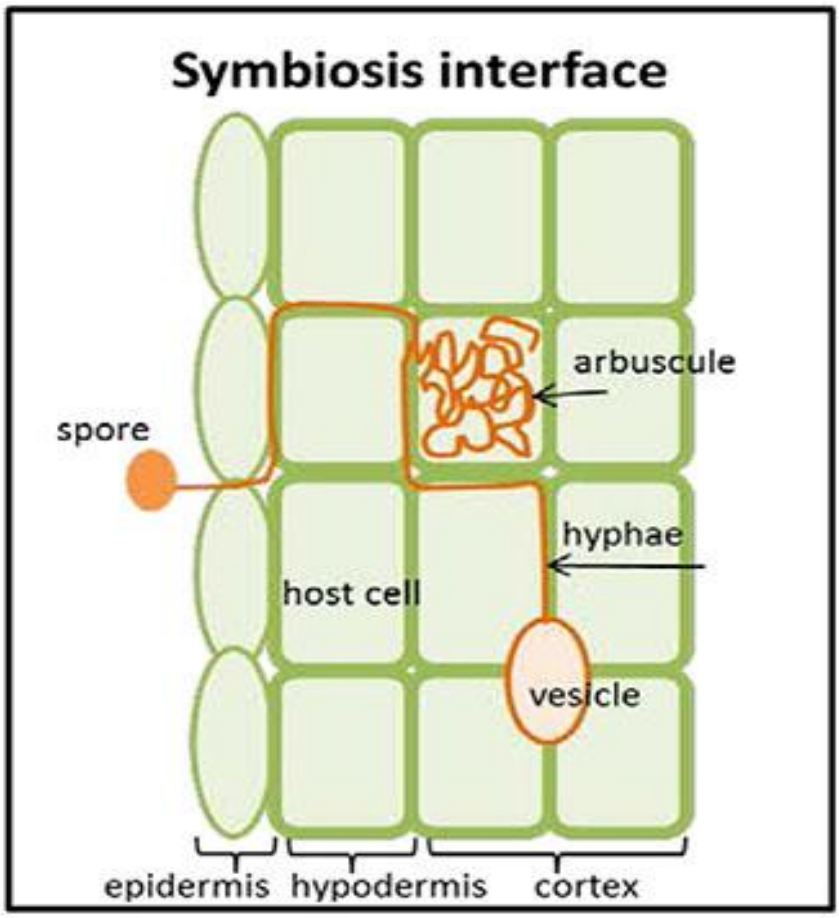


Figure 2.7. Representation of hyphae, vesicle, arbuscule and spore of the AMF within the symbiotic interface (French, 2017).

AMF plays a big role in heavy metal absorption, accumulation and translocation in plants, and it provides protection against toxic levels of heavy metals. Heavy metals are absorbed both by the plant's own pathways and by mycorrhizal pathways. As in the case of root epidermis cells, fungal cells that construct the hyphae have their own metal transporters on the cell membrane. Genome wide analysis of metal transporters in AMF species have been highlighted our understanding about the heavy metal absorption in the mycorrhizae. CTR family Cu transporter, ZIP family Zn and Fe permease and iron permease RiSmF1 are a few of known metal transporters belong to the AMF species (Tamayo et al., 2014). Heavy metals are absorbed by extraradical mycelium of AMF thanks to the high affinity metal transporters on the fungal cell membrane; fungal cells use essential heavy metals for their own metabolism and transport rest of the absorbed metals to the plant. This exchange occurs at the symbiotic interface between fungal and plant root cells. The efflux of these metal ions from fungal cell to the root cell is carried from the transmembrane proteins on the fungal membrane like heavy metal ATPases and cation diffusion facilitators. Moreover, heavy metal binding chaperones deliver metal ions to the efflux proteins on the membrane. For instance, three genes

encoding for the putative chaperones were found in the *Rhizophagus irregularis* genome (Hong et al., 2009).

Under toxic heavy metal concentrations in the soil, AMF employs different mechanisms to overcome this stress which in turn constitutes a protection zone for the plant. These mechanisms are binding of heavy metals to the AMF cell wall, chelation of metal ions in the soil by the fungal protein glomalin, storage of heavy metals in AMF spores, decreased uptake of metals through metal transporters and increased efflux of metals from the membrane (Ferrol et al., 2016). All these mechanisms decrease the toxic effects of high heavy metal concentrations and help the host plant to survive and grow under toxic conditions. Furthermore, AMF improves the P nutrition of the plant and increase plant growth, which has a dilution effect on the heavy metal concentration in the plant tissues (Singh et al., 2016). This growth dilution effect decreases the toxicity to the plant. In addition, since phosphorus and arsenate share the same transporter protein, the improved phosphate uptake due to the AMF suppresses As uptake and decreases its toxicity (Chan et al., 2015).

High concentrations of heavy metals in the soil is a stress factor for mycorrhiza. AMF have various mechanisms to cope with this stress. These mechanisms can be classified as absorption, adsorption, storage, and sequestration of the metals in soil and transfer of the metals to the plant. The AMF absorbs the metals from the soil thanks to the metal transporters on the hyphal membrane such as ZRT1 for Zn uptake, CTR for Cu uptake, FTR1 for iron uptake, SMF1 for Mn uptake. The absorbed metals can be utilized for metabolic reactions in the fungal cell, they can be transferred to the host plant, or they can be sequestered and stored within the mycorrhizal structures of spores or hyphae. After the absorption by hyphae, the heavy metals can be sequestered by phytochelatins and metallothioneins of the plant and then compartmentalized in the vacuoles by PolyP granules. Also, the absorbed metals by hyphae can be transferred to the spores and can be stored in the vacuoles of the spore cells. The heavy metals in the soil can be adsorbed on the surface of the cell walls of hyphae and spores. This adsorption immobilizes the metals in the soil. The heavy metals can also be immobilized in the soil by the mycorrhizal protein glomalin. Absorbed heavy metals by hyphae can also be transferred to the arbuscules and eventually to the host plant at the symbiotic interface while photosynthetically derived sugars are transferred to the AMF. The plant can utilize the metals for its metabolic activities and can store in the vacuoles as well. All these mycorrhizal and plant' s strategies to cope with heavy metal stress are summarized in Figure 2.8 below.

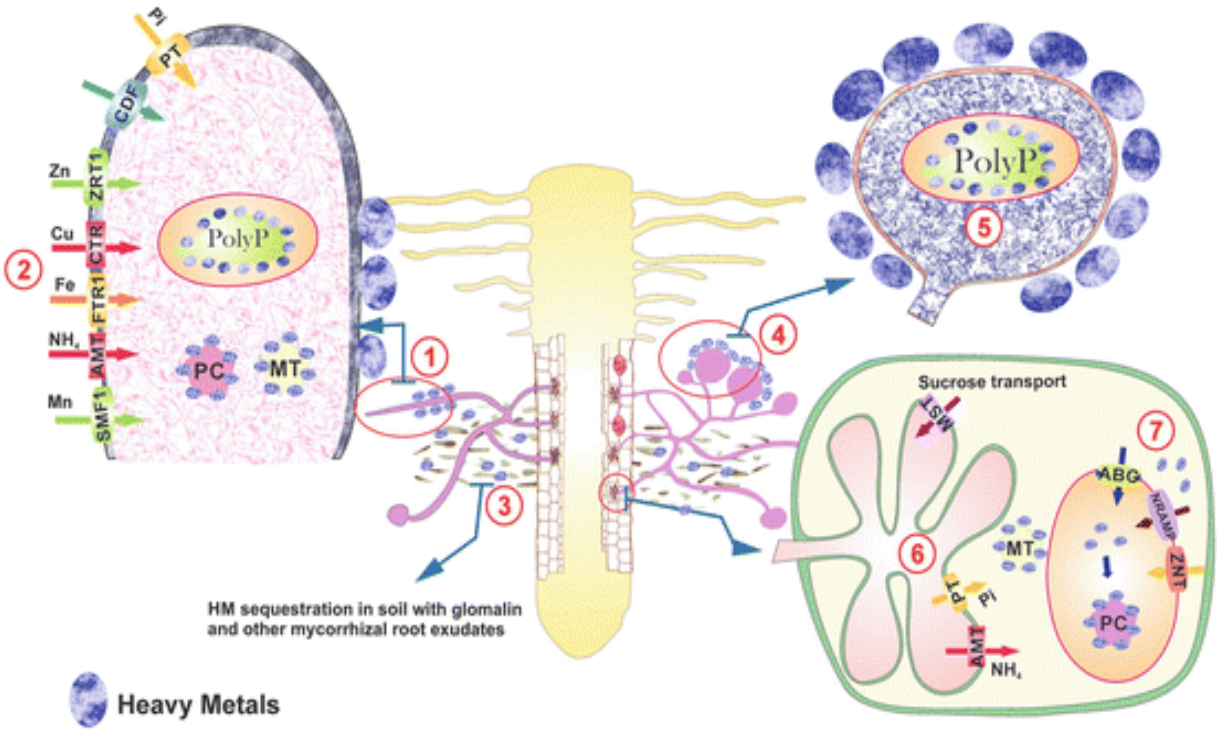


Figure 2.8. Strategies of the AMF and plant to cope with heavy metal stress. (1) Adsorption of heavy metals on the cell wall of the hypha. (2) Metal transporters on the hyphal membrane. (3) Immobilization of heavy metals by the glomalin. (4) Adsorption of heavy metals on the cell wall of the spore. (5) Sequestration of heavy metals by PolyP granules in the vacuole of the spore. (6) Nutrient and heavy metal exchange between plant and arbuscule of the AMF at the symbiotic interface. (7) Storage of heavy metals in the vacuole of the plant (Garg and Kashyap, 2017).

Iron is an essential micronutrient for plants since it is required for the respiration, photosynthesis and DNA synthesis (Rout and Sahoo, 2015). Therefore, iron uptake from the soil is crucial for the plant. The uptake of iron strongly depends on the pH of the soil which determines the availability of the iron to the plant. To take up iron, plants chelate Fe or solubilize Fe by decreasing rhizosphere pH thanks to the root exudates and proton pump mechanism. Figure 2.9 shows the Eh-pH diagram of iron to understand the forms of iron at various pH values.

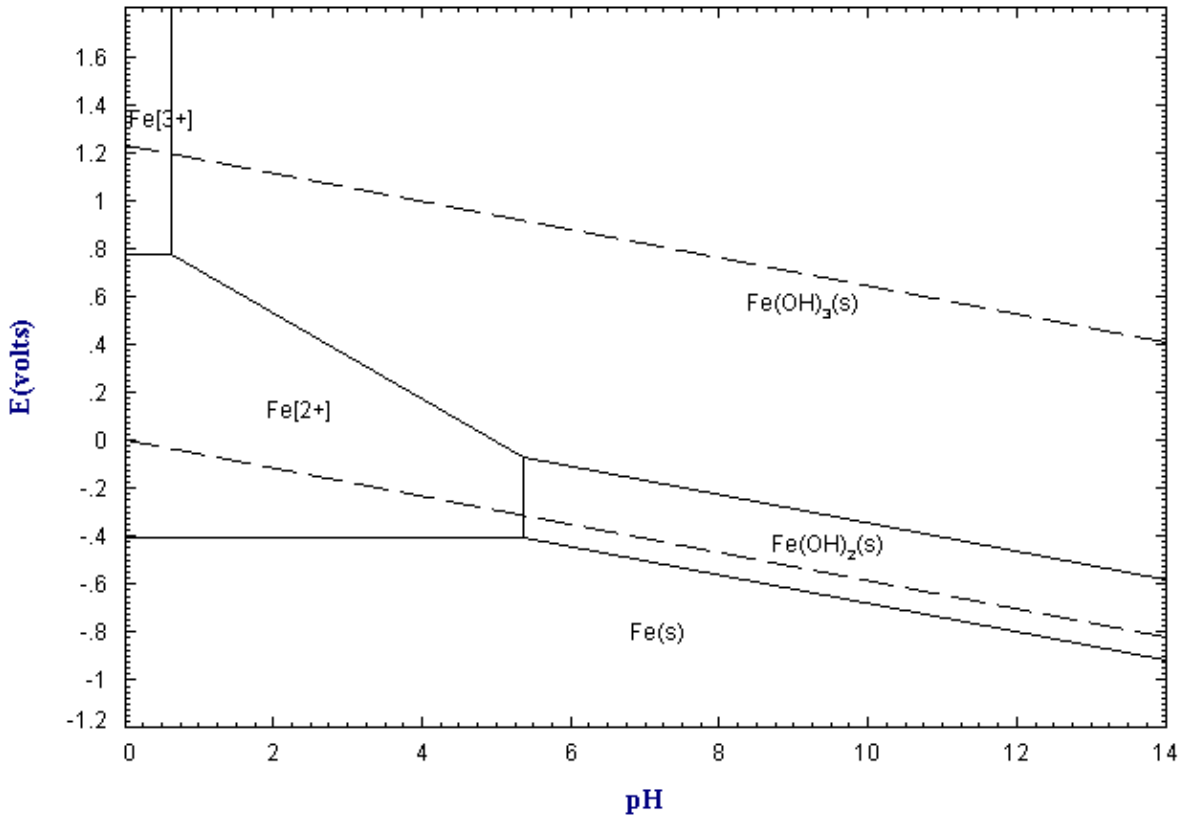


Figure 2.9. Eh-pH diagram of Fe.

Besides the pH of the soil, expression levels of iron transporters on the root membrane are also crucial for the Fe uptake from the soil. Yellow stripe like (YSL) genes encode for the metal transporters and the YSL-15 gene is especially important for the iron uptake from the soil since it encodes for the iron phytosiderophore transporter. In strategy I plants, phytosiderophores (PS) are released to make complexes with metals including the iron. YSL-15 transporter takes up the Fe-PS complex from the soil. YSL-15 has also a role in the long distance transport of iron. Under iron deficient conditions, the expression of YSL-15 gene in roots increases to uptake more iron from the soil. Therefore, the higher expression of YSL-15 in root, the greater the uptake of iron from the soil. Under Fe sufficient conditions, expression of YSL-15 increases in shoots and leaves to increase the translocation of iron.

Glomalin which is a glycoprotein produced by AMF is essential for heavy metal uptake, soil stability and fertility. It contributes to the organic content of the soil and to the aggregate formation (Nichols, 2008). The increase in soil stability and aggregation with the increasing glomalin concentrations is shown in Figure 2.10 below:

Figure 2.10. The same soil samples under different glomalin concentrations are shown.

Glomalin is a chelating agent which forms complex with heavy metal ions. These complexes provide the mobilization of heavy metals inside of the cells in the case of uptake and provide the immobilization of metals in the soil under toxic conditions. Glomalin is essential for the phytoremediation applications in terms of heavy metal uptake by AMF and plant, and of soil stability.

AMF can be used in agriculture to increase the crop yield since it promotes the plant growth. For instance, usage of AMF with potato crops in Canada increased the annual yield (Hijri, 2015). Moreover, AMF can be used to start agricultural activities in drought climates, and in salty or drought prone soils since it encourages plants to cope with these harsh conditions. Therefore, in addition to the usage of AMF for the remediation and clean-up of soils and waters, they can also be used for increasing the crop yields in agriculture.

3. MATERIALS AND METHODS

In this study, the mechanisms of increasing the quality of the soil from a mine tailing area in Kütahya by plant-mycorrhiza interactions were evaluated. The following tasks were carried out in this research;

- Soil sampling- The experimental soil was taken from the mine tailing area in Kütahya which is at the central region of Anatolia.
- Metal analysis- Analysis of the metals in this soil was carried out via ICP-OES method.
- Germination assay- Sorghum seeds were germinated in petri dishes to understand whether the seeds would germinate or not in the prepared metal solution.
- Greenhouse set up- A greenhouse experiment was set up with *Sorghum bicolor* L. and *Rhizophagus irregularis*.
- Plant growth assays- Root weight, shoot weight and height of the plants were measured for 12 weeks after planting.
- Sampling and analyses- Root, shoot and soil samples were taken at 1^{st}, 2^{nd}, 3^{rd}, 5^{th}, 8^{th}, 10^{th} and 12^{th} weeks after the planting for metal analysis by ICP-OES, determination of mycorrhization, glomalin related soil protein (GRSP), estimation of glomalin protein and gene expression.

3.1. Soil Sampling

The experimantal soil used in this research was taken from a coal mine tailing area in Tavşanlı in Kütahya. Kütahya is shown in the map of Turkey in Figure 3.1 below.

Figure 3.1. Map of Turkey. Kütahya is shown as red in the map.

3.2. Digestion Method for the Metal Analyses

All the samples in this research were digested with 10 ml 65% nitric acid and 1 ml 35% hydrogen peroxide under 800 psi pressure, at 1600 watt and 180°C in MARS 6 Microwave Accelerated Reaction System Instrument (CEM), USA. Ramp time was 5.25 minutes and hold time was 11 minutes. This digestion method was adapted from USEPA, Method 3052 (USEPA, 1996). The volume of each sample was completed to 50 ml with distilled water after the digestion.

3.3. Metal Analysis of the Soil

The experimental soil samples were taken as 0.25 gr as triplicate and digested. The concentrations of Cr, Mn, Fe, Ni, Cu, Zn, Al, Cd, Pb, Si, Co and Mo in the soil were determined by ICP-OES method after the digestion.

3.4. Germination Assay

Sorghum bicolor L. seeds were supplied from Turkish Commercial Farming Business located in İzmir. The seeds were washed with first with distilled water and then with 70% ethanol for one minute and this was repeated three times. The seeds were washed with distilled water for one minute at the end.

Whatman No.4 filter papers were put into 8 petri dishes. 4 of the petri dishes were filled with distilled water until the water wet the whole filter paper; the other 4 petri dishes were filled with heavy metal solution which contained 700 ppm Fe and 100 ppm Al as the mine tailing soil contained. 10 seeds were planted in each petri dish. The dishes were covered with aluminum foil and black plastic at the top. Germination of the seeds and growth of the seedlings were monitored for one week.

3.5. Greenhouse Set Up

The greenhouse experiment was set up on 17th of August using the experimental soil taken from Kütahya. 1.7 liter pots were used. Only 15 *Sorghum bicolor* L. seeds were planted in 24 pots which were called as AM- pots. 15 *Sorghum bicolor* L. seeds were inoculated with *Rhizophagus irregularis* in 24 pots which were called as AM+ pots. All 48 pots were covered with the sterilized sand at the top. The sand had been sterilized at 151°C for 24 hours. 150 ml of nutrient solution, Modified Strullu-Romand (MSR) medium, was added to each pot just after planting. 100 ml of nutrient solution was added to each pot three days after planting, 75 ml was added after three weeks and 50 ml was added after 6 weeks. Irrigation was done three days in a week for 4 weeks and 175 ml distilled water was added in a week. In the following two weeks, three times in a week, 75 ml daily distilled water was added. In the next 2 weeks, irrigation was done 2 days in a week and 50 ml distilled water was added at total in a week. In the last 4 weeks, 25 ml distilled water was added to each pot once a week. This watering schedule was chosen by regarding the season change by time after planting. The composition of MSR medium is shown in Table 3.1 below.

Table 3.1. Composition of MSR medium (Fortin et al., 2002).

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

3.6. Plant Growth Assays

Fresh root and shoot weights, and heights of the plants were measured at 1^{st}, 2^{nd}, 3^{rd}, 5^{th}, 8^{th}, 10^{th} and 12^{th} weeks after the plantation. Three plant samples from each pot were taken for these measurements.

3.7. Metal Analyses of the Soil, Root and Shoot Samples

Root, shoot and soil samples were taken from 3 AM+ and 3 AM- pots at the 1^{st}, 2^{nd}, 3^{rd}, 5^{th}, 8^{th}, 10^{th} and 12^{th} weeks after plantating. Three root and shoot samples were taken from each pots and soil samples were taken from the rhizosphere region of the roots. Root, shoot and soil samples were dried at 70°C for 72 hours and kept in desiccator until the metal analysis. Dried root and shoot samples were ground in stand blender and they were digested for the metal analysis. 0.25 gr of dried soil samples were taken and digested as well. The metal analyses for all digested samples were done by ICP-OES method.

3.8. Soil pH Measurements

10 gr of dried soil was taken from each soil samples that had been taken at the 1st, 2nd, 3rd, 5th, 8th, 10th and 12th weeks and dried at 70°C for 72 hours, and 25 ml of distilled water was added in falcon tubes. The tubes were mixed for three minutes and let stand for one hour. The pH values were measured from the liquid phases by pH meter.

3.9. Measurement of Glomalin Related Soil Protein (GRSP) and Estimation of the Presence of Glomalin Protein

Root and soil samples were taken from 3 AM+ and 3 AM- pots at the 1st, 2nd, 3rd, 5th, 8th, 10th and 12th weeks after the plantation. 6 roots were taken from each pots and the soil samples were taken from the rhizosphere region of the plants. 1 gr of fresh soil was taken from each soil samples and all 6 roots from each pots were taken for the GRSP extraction and measurement according to the Wright and Upadhyaya procedure (Wright and Upadhyaya, 1998). 50 ml 50 mM citric acid was added to each root and soil samples, and they were autoclaved at 121°C for 60 minutes. All samples were centrifuged at 8000 rpm for 30 minutes after autoclaving. The supernatant of each samples was filtered through Whatman No.4 filter paper and kept at -20°C.

200 µl of each soil and root extract were mixed with 1 ml Bradford Reagent (B6916 SIGMA) according to the original Bradford method (Bradford, 1976) and incubated at room temperature for 30 minutes. The absorbance of each sample was measured at 595 nm with UV-160A Spectrophotometer, Shimadzu.

0.01, 0.05, 0.1, 0.25, 0.5, 1 and 2 mg/ml Bovine Serum Albumin (A4503 SIGMA) standards were prepared with distilled water for the calibration curve construction. 1 ml Bradford Reagent (B6916 SIGMA) was mixed with 200 µl of the sample (Ratio: 50:1) according to the original Bradford method (Bradford, 1976) and incubated for 4 minutes. The absorbance of each sample was measured at 595 nm with UV-160A Spectrophotometer, Shimadzu.

Since the molecular weights of glomalin and BSA are very close, 63.1 and 66.5 kDa respectively, the protein extracts and BSA standards with various concentrations were run on SDS-PAGE gel. 9% resolving gel, 6% stacking gel and 10x running buffer were prepared. 4x SDS-PAGE loading buffer was also prepared with 200mM Tris-Cl (pH 6.8), 400 mM DDT, 8% SDS, 0.4% bromophenol blue

and 40% glycerol to load the samples into the gel. The thickness of the gel was 1.5 mm. Preparation of the gels and the running buffer are shown in Table 3.2 and Table 3.3 below.

Table 3.2. 9% resolving gel and 6% stacking gel preparation.

	9% Resolving	**6 % Stacking**
H_2O	**5 ml**	**2.9 ml**
40% AA	**2.25 ml**	**0.75 ml**
Tris Hcl pH 8.8 (6.8 for stacking)	**2.5 ml**	**1.25 ml**
10% SDS	**100 µl**	**50 µl**
10%APS	**100 µl**	**50 µl**
TEMED	**10 µl**	**5 µl**
Total volume	**10 ml**	**5 ml**

Table 3.3. Running buffer prepration.

Trizma Base	**30.3 g**
Glycine	**144.4 g**
SDS	**10 g**
H_2O	**To 1 L**

SDS-PAGE gel was run at 80 volt for 2.5 hours. Afterwards, the gel was stained with Coomassie Brillant Blue G 250 for 30 minutes and then destained for 30 minutes.

3.10. Mycorrhization Rate Counts

Two root samples were taken from each of the 3 AM+ and 3 AM- pots at the 1^{st}, 2^{nd}, 3^{rd}, 5^{th}, 8^{th}, 10^{th} and 12^{th} weeks for the determination of mycorrhization and they were kept in 70% ethanol at -20°C until the staining.

Before the staining, roots were packed in tulle and washed with 1%HCl for one minute and then distilled water for one minute, and the process was repeated for three times. The washed and packed roots were put into the falcon tubes that were filled with 10% KOH solution (w/v) and kept in water bath at 60°C for 4,5 hours. The packed roots in the tubes were transferred to new 50 ml falcon tubes which were filled with 0.05% (w/v) trypan blue solution (1:1:1 lactic acid, glycerol and water) and kept in dark for one week.

The packed roots were opened and aligned on glass slides. Cover slides were closed on the other slides and fixed by transparent nail polish. The slides were observed under the microscope. 30 fields of vision that the object occupies were observed for each slide and mycorrhization counts were done by based on these 30 fields for each sample. Microscope Axio Observer.Z1, with EC Plan-Neofluar 20x objective, and AxioCam MR5 camera was used.

3.11. RNA extraction and cDNA Synthesis

6 root samples were taken from each of the 3 AM+ and 3 AM- pots at the 1st, 2nd, 3rd, 5th, 8th, 10th and 12th weeks. The root samples were homogenized in Trizol reagent by homogenizer at 6000 rpm for 40 seconds for 2 times. Direct-zol^{TM} RNA MiniPrep Plus w Kit from ZymoResearch was used for the RNA extraction from the homogenized roots. Quality and the concentration of the extracted RNAs were checked on the nanodrop spectrophotometer. 500ng of each RNA sample was converted to cDNA immediately after the RNA extraction by the SensiFAST cDNA Synthesis Kit from Bioline. Oligo dT and random hexamer primers were mixed in this kit. Therefore, the kit can reverse transcribe both eukaryotic and prokaryotic RNAs, and the ribosomal RNAs. The cDNA samples were stored at -20°C and the RNA samples were stored at -80°C.

3.12. Determination of the Presence of Glomalin Transcripts

For the determination of the transcripts of glomalin protein, 5 different primers were designed in Primer3 software (Untergasse et al., 2012). Two of the 5 primers were designed within the third exon sequence of the glomalin gene and the other 3 primers were designed from the exon junctions. Gradient PCR experiments were performed with these primers and the cDNA samples obtained from the root RNAs at each sampling week. MyTaq DNA polymerase from Bioline was used for PCR experiments. Each PCR mix was prepared with 10 µl reaction buffer, 1 µl cDNA, 1 µl of forward and reverse primers respectively, 0.5 µl Taq polymerase and 36.5 µl nuclease free water. The PCR products were run on the agarose gels and stained with etidium bromide. 1% agarose gels were run at 110 volt for 35 minutes; 2% agarose gels were run at 120 volt for 40 minutes; 3% agarose gels were run at 120 volt for 50 minutes.

Gradient PCRs were also performed with the AML and qGint primers which target mycorrhizal genes to confirm the existence of mycorrhizal transcripts in the cDNA samples. The sequences of the glomalin, AML and qGint primers are shown in Table 3.4.

Table 3.4. Sequences of the primers for glomalin mRNAs.

Name	Sequence
glex3_1F	ATGCTGGTGAAGAAGGTGCC
glex3_1R	CAAGATCGCCATACTCGCCC
glex3_2F	TAAATTGTCGGGCGGCGTAG
glex3_2R	AACAGTGCCTTCCTCAACCG
glexjunc12_F	AAGGACGGTGTCACTGTAGC
glexjunc12_R	CAGCCAGCGGCAACATTTTT
glexjunc23_1F	AAATGTTGCCGCTGGCTGTA
glexjunc23_1R	CCATTGGCCGAGATCGTAGC
glexjunc23_2F	CAGGTTGCTACGATCTCGGC
glexjunc23_2R	GTGATGACGCCTTCCTTCCC
AML_F	ATCAACTTTCGATGGTAGGATAGA
AML_R	GAACCCAAACACTTTGGTTTC
qGint_F	GTATGCCTGTTTGAGGGTCAGTATT
qGint_R	AAACTCCGGAACGTCACTAAAGAG

3.13. Quality Control for the RNA and cDNA Samples – Control for the Integrity of the cDNA

To control the quality of the extracted RNA and reverse transcribed cDNA samples, 5^{th} week RNA and cDNA samples were run on 1% agarose gel.

For the control of the integrity of the cDNA samples, PCR was done with the primer targeting the sorghum mitochondrion since the mitochondrial genes should have been expressed as long as the plant was alive, therefore, there should have been cDNA fragments of these transcripts. The PCR products were run on 2% agarose gel.

3.14. Gene Expression Analyses

For the gene expression analysis, three primers were selected as candidates for the housekeeping gene and 5 primers were selected as candidates for the target gene. The sequences of the PP2A, EIF4a and CYP primers were taken from Reddy et al. (Reddy et al., 2016). The sequence of the Alt_{SB} primer was taken from Magalhaes et al. (Magalhaes et al., 2007). Yellow stripe 1 and YSL 15 primeres were designed in Primer-BLAST software (Ye et al., 2012). The primers and their targets are as:

- PP2A primer for housekeeping gene: targets the gene of the catalytic subunit of the sorghum bicolor serine/threonine-protein phosphatase
- EIF4a primer for housekeeping gene: targets the gene of the sorghum bicolor initiation factor 4A
- CYP primer for housekeeping gene: targets the gene of the sorghum bicolor 70 kDa peptidyl-prolyl isomerase
- Alt_{SB} primer for target gene: targets the gene of the sorghum bicolor MATE transporter
- Yellow stripe 1-218 and Yellow stripe 1-242 primers for target gene: targets the gene of the sorghum bicolor iron-phytosiderophore transporter yellow stripe 1
- YSL 15-116 and YSL 15-146 primers for target gene: targets the gene of the sorghum bicolor iron-phytosiderophore transporter yellow stripe like 15

The sequences of the primers are shown in Table 3.5 below:

Table 3.5. Sequences of the primers for candidate reference and target genes.

Name	Sequence
PP2A-F	AACCCGCAAAACCCCAGACTA
PP2A-R	TACAGGTCGGGCTCATGGAAC
EIF4a-F	CAACTTTGTCACCCGCGATGA
EIF4a-R	TCCAGAAACCTTAGCAGCCCA
AltSB-F	GTGCTGGATCCGATCCTGAT
AltSB-R	CACTGCCGAAGAAACTTCCA
Yellowstripe 1-218-F	CATCTTCCGTGCTCGCTTTG
Yellowstripe 1-218-R	CTCTCGGAGAAGGAGAACCCA
YSL15-116-F	GCAAGTGCGTGCATTTCTGA
YSL15-116-R	TGAGGCCAAACGTAGGGAAC
CYP-F	GTATCTGTGCTCGCCGTCTCT
CYP-R	TTCACCCAACTCCTCAACCCC
Yellowstripe 1-242-F	GGACCTTCCCATCTTCCGTG
Ylwstripe 1-242-R	TCTCAAGTATTGTGGCTCTCGG
YSL15-146-F	ACATGTGCGTAGGGACCTTG
YSL15-146-R	CTTGGCGAGAGAAAGCAACG

Gradient PCRs were performed with these 8 primers. Best candidate primers and the best annealing temperatures of them were determined by based on these PCRs.

The best primers for a housekeeping gene were determined to be CYP, PP2A and the primers for target genes were determined to be YSL 15-116, Yellow stripe 1-218. A qRT-PCR experiment was performed at the annealing temperature of 61°C with these 4 primers by using Ampliqon RealQ Plus 2x Master Mix, green (without ROX) in Thermo Scientific™ PikoReal™ Real-Time PCR System. The protocol for this experiment was as following:

95°C; time: 15 minutes
95°C; time: 15 seconds
61°C; time: 30 seconds
72°C; time: 30 seconds
} 50 cycles

For melting curve: Starting temperature: 60°C; end temperature: 95°C

Another qRT-PCR was performed with Yellow stripe 1 and PP2A primers at the annealing temperature of 61°C. Used materials and the equipment were the same as in the previous qRT-PCR. The protocol for this experiment was as follows:

95°C; time: 15 minutes
95°C; time: 20 seconds
61°C; time: 30 seconds
72°C; time: 30 seconds
} 50 cycles

For melting curve: Starting temperature: 60°C; end temperature: 95°C

Another qRT-PCR was performed with Yellow stripe 1 and PP2A primers at the annealing temperature of 62°C. Used materials and the equipment were the same. The protocol for this experiment was as following:

95°C; time: 15 minutes
95°C; time: 15 seconds
62°C; time: 30 seconds
72°C; time: 30 seconds
} 50 cycles

For melting curve: Starting temperature: 70°C; end temperature: 95°C

The last qRT-PCR was performed with YSL 115-116 and PP2A primers at the annealing temperature of 56°C. Used materials and the equipment were the same. The protocol for this experiment was as following:

95°C; time: 15 minutes
95°C; time: 15 seconds
56°C; time: 30 seconds
72°C; time: 30 seconds
} 50 cycles

For melting curve: Starting temperature: 70°C; end temperature: 95°C

The expression levels of the target genes were normalized to the expression levels of the housekeeping genes. At the end, one target and one housekeeping gene were selected as YSL 15 and PP2A. The relative expression levels of YSL 15 of AM- and AM+ roots were calculated.

4. RESULTS

4.1. Metal Analysis of the Soil

Ininial, assessment of the experimental soil was implemented by metal analysis of the mine tailing via ICP-OES equipment. Technically, twelve metals were assessed, and their concentrations in the mine tailing soil sample is shown in Table 4.1. Iron and Aluminium were found to have the highest concentrations among these 12 metals.

Table 4.1. The results of metals anaysis in the experimental soil.

	Sample 1	Sample 2	Sample 3	Standard Deviation	Mean
Sample size	**0.2573 g**	**0.2588 g**	**0.256 g**	**0.00140119**	**0.257367**
Cr in ppm	**4.35**	**4.74**	**4.8**	**0.244335834**	**4.63**
Mn in ppm	**7.27**	**4.08**	**3.45**	**2.047982747**	**4.933333**
Fe in ppm	**703**	**677**	**609**	**48.5386444**	**663**
Ni in ppm	**9.94**	**11.1**	**9.35**	**0.890337015**	**10.13**
Cu in ppm	**0.336**	**0.35**	**0.298**	**0.026907248**	**0.328**
Zn in ppm	**0.16**	**0.171**	**0.129**	**0.021779195**	**0.153333**
Al in ppm	**52.6**	**95.6**	**80.5**	**21.81520876**	**76.23333**
Cd in ppm	**0.002**	**0.003**	**0.003**	**0.00057735**	**0.002667**
Pb in ppm	**0.029**	**0.029**	**0.025**	**0.002309401**	**0.027667**
Si in ppm	**3.62**	**3.88**	**3.87**	**0.147309199**	**3.79**
Co in ppm	**0.359**	**0.425**	**0.342**	**0.043844422**	**0.375333**
Mo in ppm	**0.008**	**0.033**	**0.009**	**0.014153916**	**0.016667**

4.2. Germination Assay

Sorghum seeds were germinated on the filter papers in petri dishes in a solution which contained Fe and Al in distilled water. To stimulate the effect of the mine tailing pollution, the concentrations of iron and aluminium used in the experimental solution were the same as the concentrations found in the soil. Both the seeds in iron (Fe) and aluminium (Al) solution and the seeds in distilled water germinated in a few days in the petri dishes. Preliminary results indicated that, sorghum seeds were able to germinate at the Fe and Al concentrations level of the mine tailing soil samples. Photo of the seeds at the fist days of seed germination is shown in Figure 4.1.

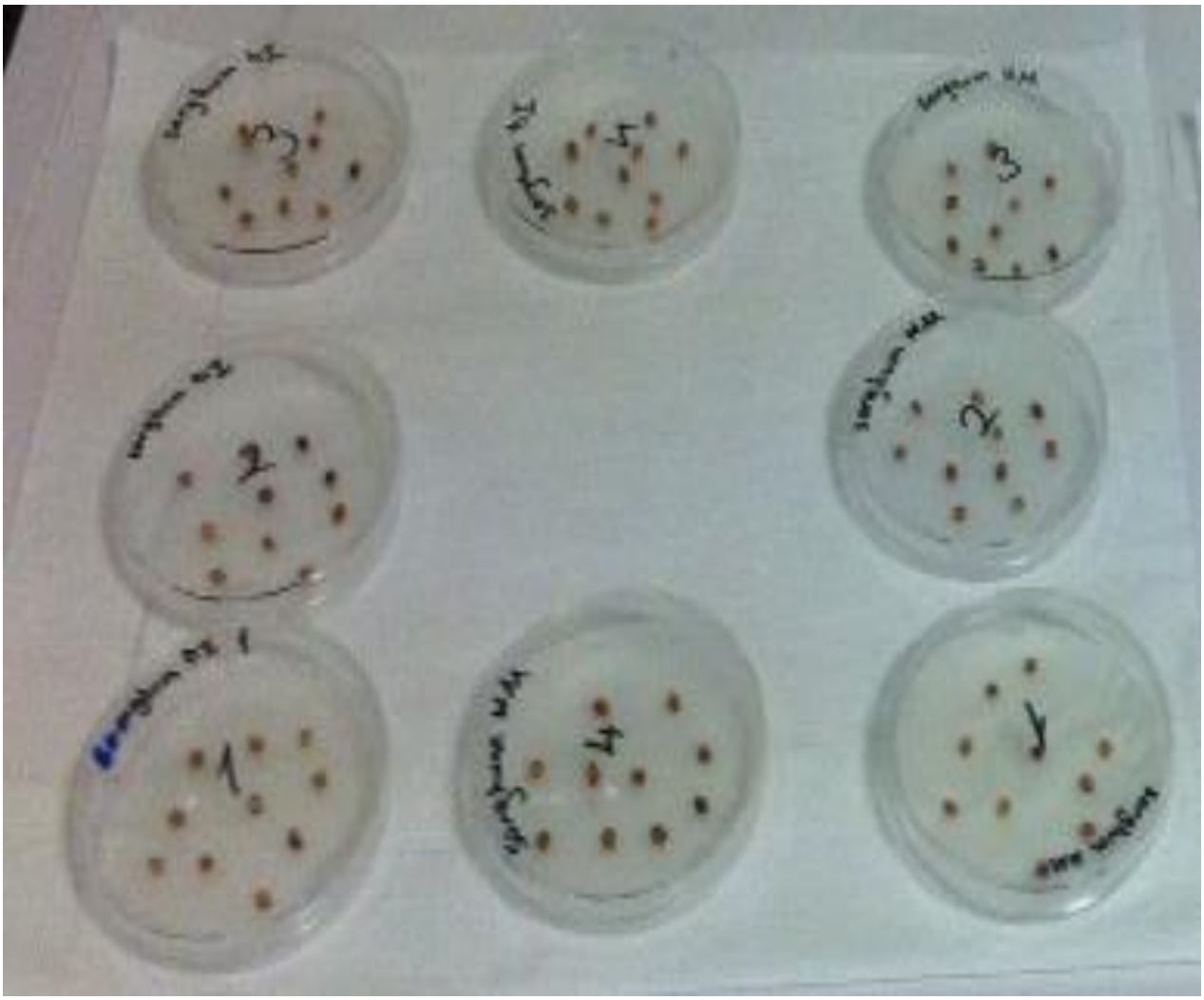


Figure 4.1. Germination assay of sorghum seeds. Four petri dishes which were labelled as ‘sorghum DI’ contained distilled water whereas the rest four petri dishes which were labelled as ‘sorghum HM’ contained Fe and Al solution.

4.3. Plant Growth Assays

Growth of the plants was followed for twelve weeks after planting by root and shoot weights, and height measurements.

4.3.1. Root and Shoot Weights

For the root and shoot weight measurements, plants from three AM+ pots and three AM- pots were assessed at each sampling week. Fresh root and shoot weights of three plants from each pot were determined. Root weights of plants with and without AMF measured every week are shown in Figure 4.2 and Figure 4.3.

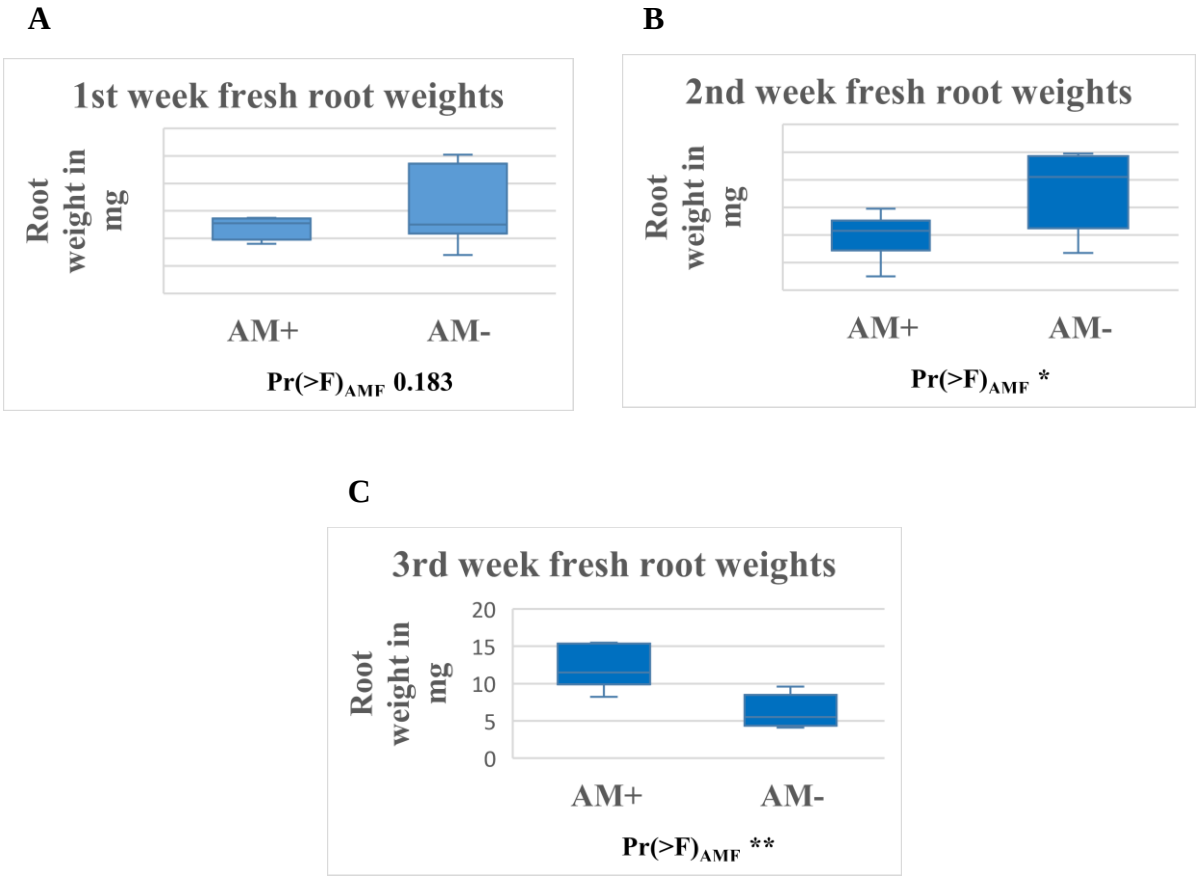


Figure 4.2. Root weights of plants with and without AMF measured every sampling week. ±SE based on t test was used to assess the difference between fresh weights of mycorrhizal and non-mycorrhizal roots. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05. (A) 1st week fresh root weights. (B) 2nd week fresh root weights. (C) 3rd week fresh root weights.

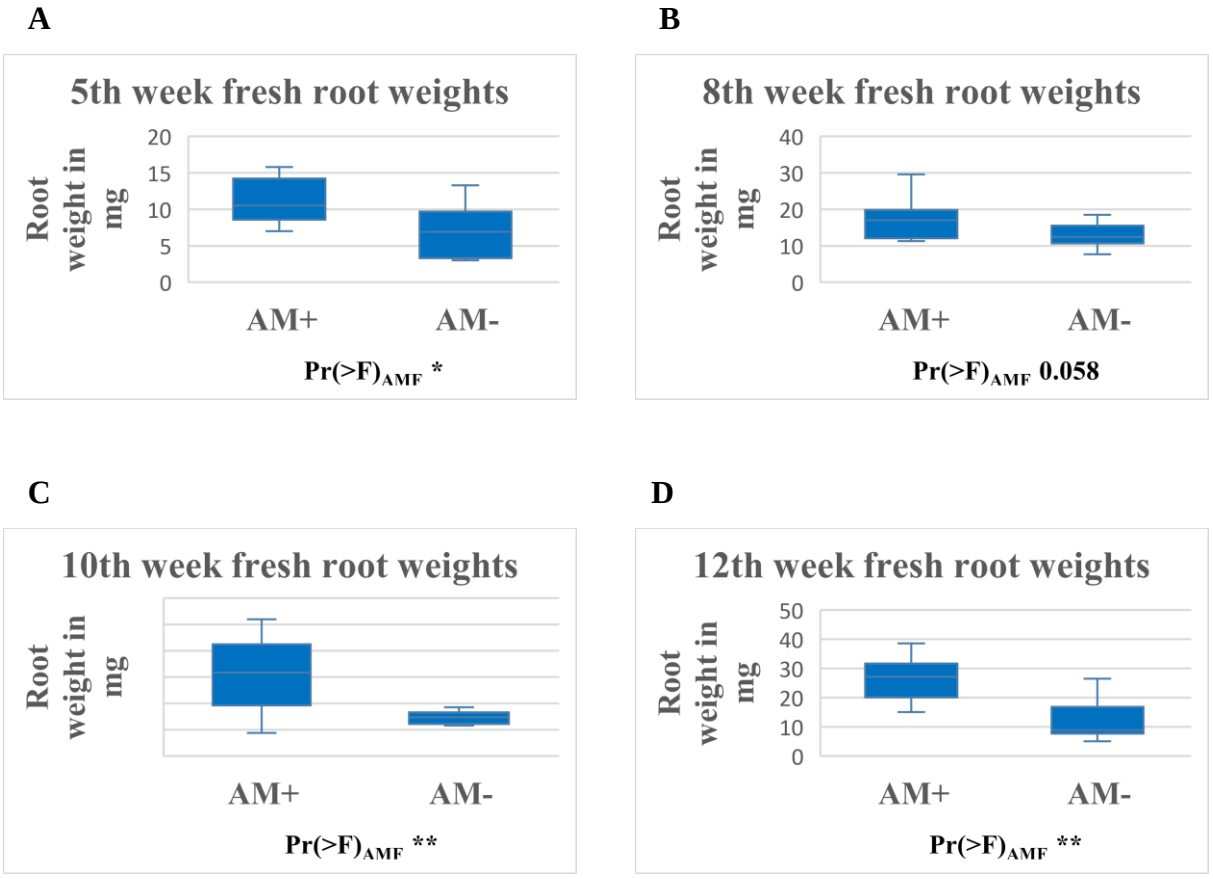


Figure 4.3. Root weights of plants with and without AMF measured every sampling week. ±SE based on t test was used to assess the difference between fresh weights of mycorrhizal and non-mycorrhizal roots. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05. (A) 5th week fresh root weights. (B) 8th week fresh root weights. (C) 10th week fresh root weights. (D) 12th week fresh root weights.

As shown in Figure 4.2 and Figure 4.3, there is statistically significant difference between mycorrhizal (AM+) and non-mycorrhizal (AM-) root weights at 2nd, 3rd, 5th, 10th and 12th weeks.

Shoot weights of AM+ and AM- groups at each sampling week are shown as bar plots in Figure 4.4 and Figure 4.5.

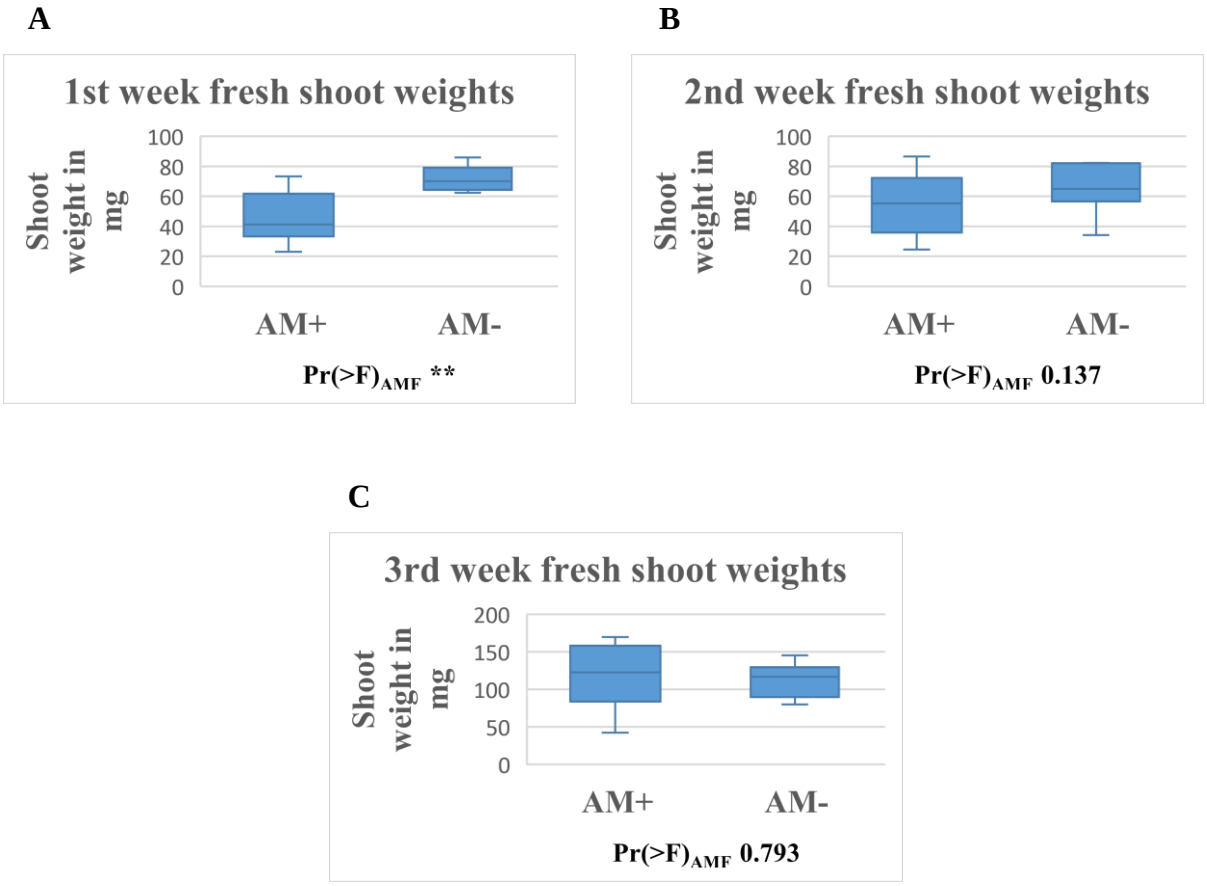


Figure 4.4. Shoot weights of plants with and without AMF measured every sampling week. ±SE based on t test was used to assess the difference between fresh weights of the shoots of mycorrhizal and non-mycorrhizal plants. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05. (A) 1st week fresh shoot weights. (B) 2nd week fresh shoot weights. (C) 3rd week fresh shoot weights.

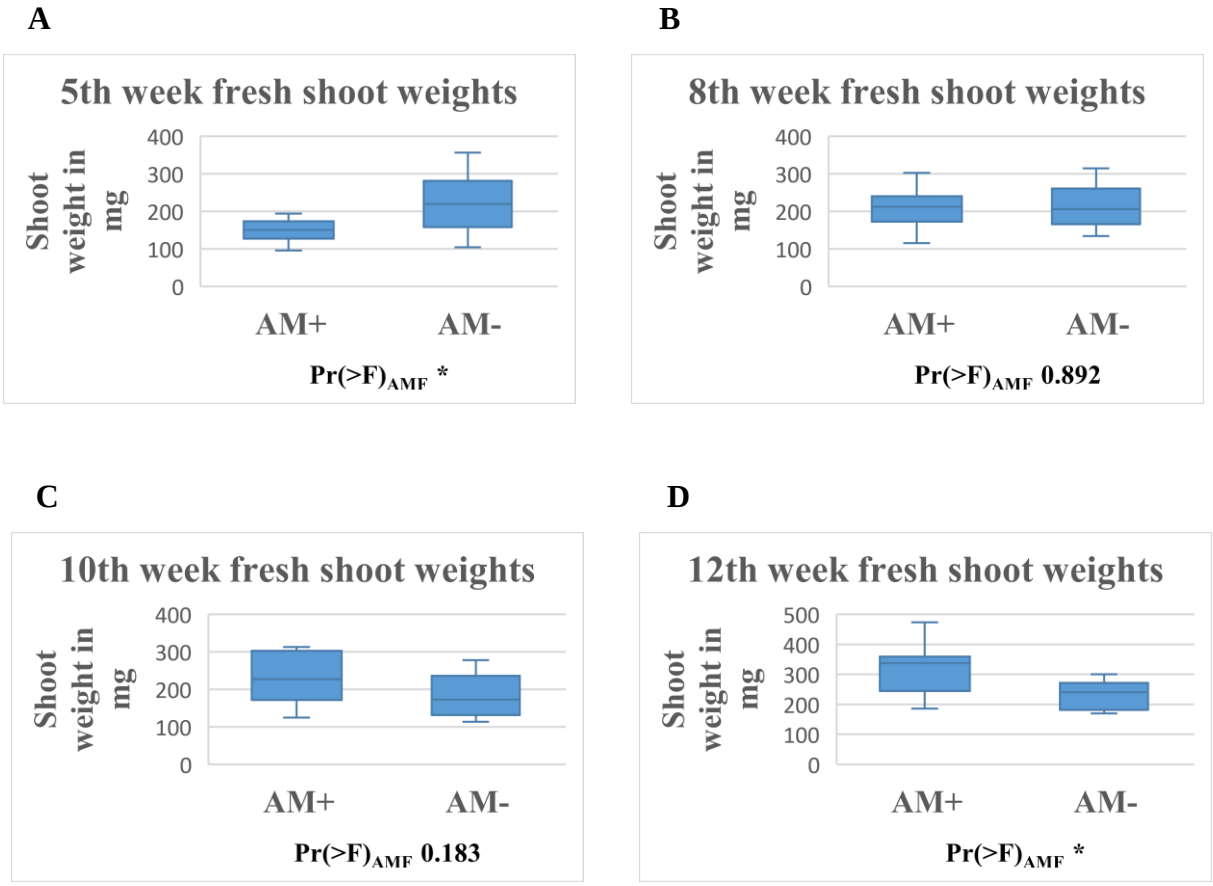


Figure 4.5. Shoot weights of plants with and without AMF measured every sampling week. ±SE based on t test was used to assess the difference between fresh weights of the shoots of mycorrhizal and non-mycorrhizal plants. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05. (A) 5th week fresh shoot weights. (B) 8th week fresh shoot weights. (C) 10th week fresh shoot weights. (D) 12th week fresh shoot weights.

As shown in Figure 4.4 and Figure 4.5, there is statistically significant difference between AM+ and AM- shoot weights at 1st, 5th and 12th weeks.

Root weight measurment was achieved at the different weeks after sowing and is shown in Figure 4.6. This figure showed that weights of mycorrhizal roots are higher than the weights of non-mycorrhizal roots from the 3rd week. Based on the statistical analysis it could be concluded that both AMF and time have significant effects on root weight. Also, the standard error in the AM+ group is higher than in AM- group in most of the weeks.

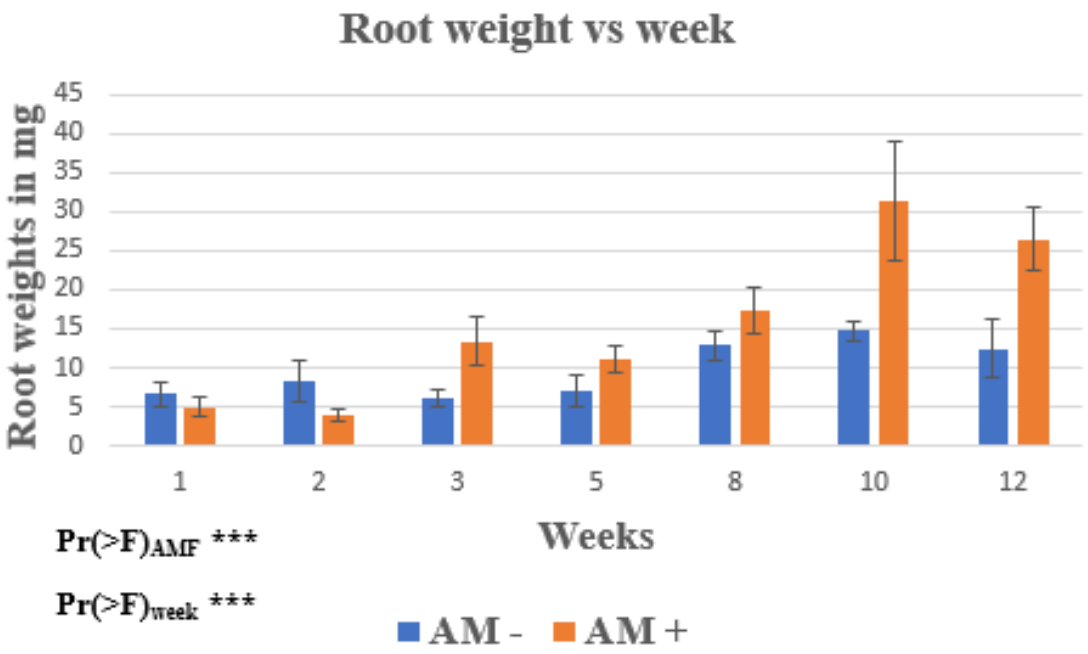


Figure 4.6. Fresh root weights of AM+ and AM- groups are indicated as columns with ±SE at each sampling week. Two-way ANOVA was used to test the effect of AMF and week on root weight. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

Shoot weight measurment at the different weeks is shown in Figure 4.7. From the statistical analysis it is concluded that only the time has a significant effect on shoot weight; the presence of AMF has no significant effect on shoot weight.

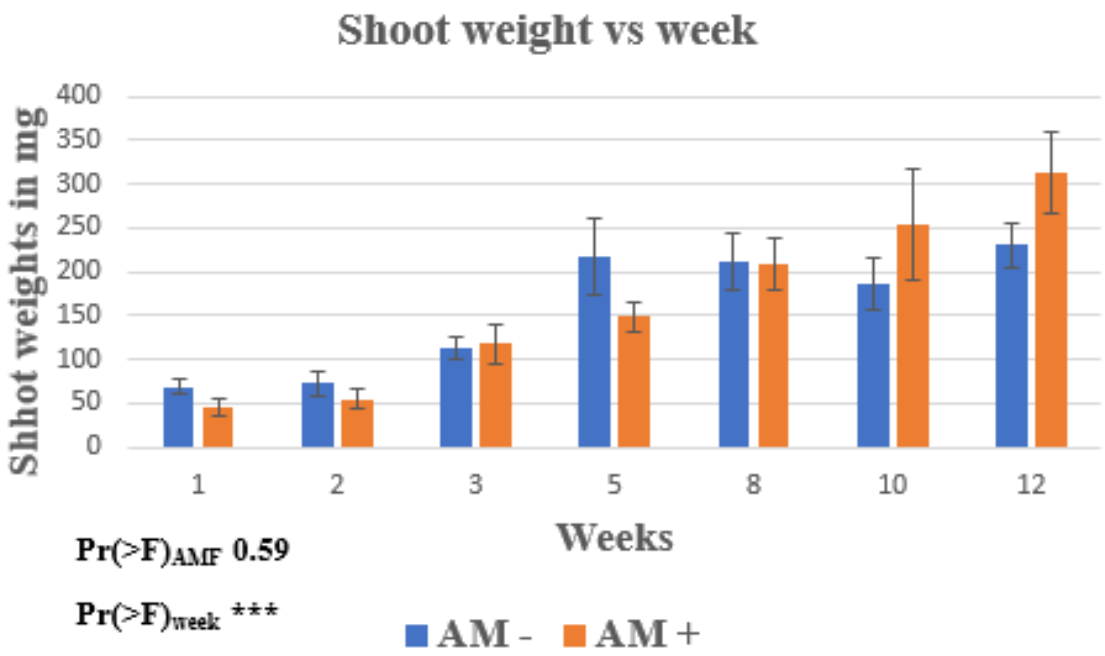


Figure 4.7. Fresh shoot weights of AM+ and AM- groups are indicated as columns with ±SE at each sampling week. Two-way ANOVA was used to test the effect of AMF and week on shoot weight. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

4.3.2. Heights of the Plants

Heights of the plants were measured every week during towelvs weeks of the experiment. Three plants were assessed from each pot. Heights of plants in AM+ and AM- groups at the end of each week are shown as bar plots in Figure 4.8 and Figure 4.9.

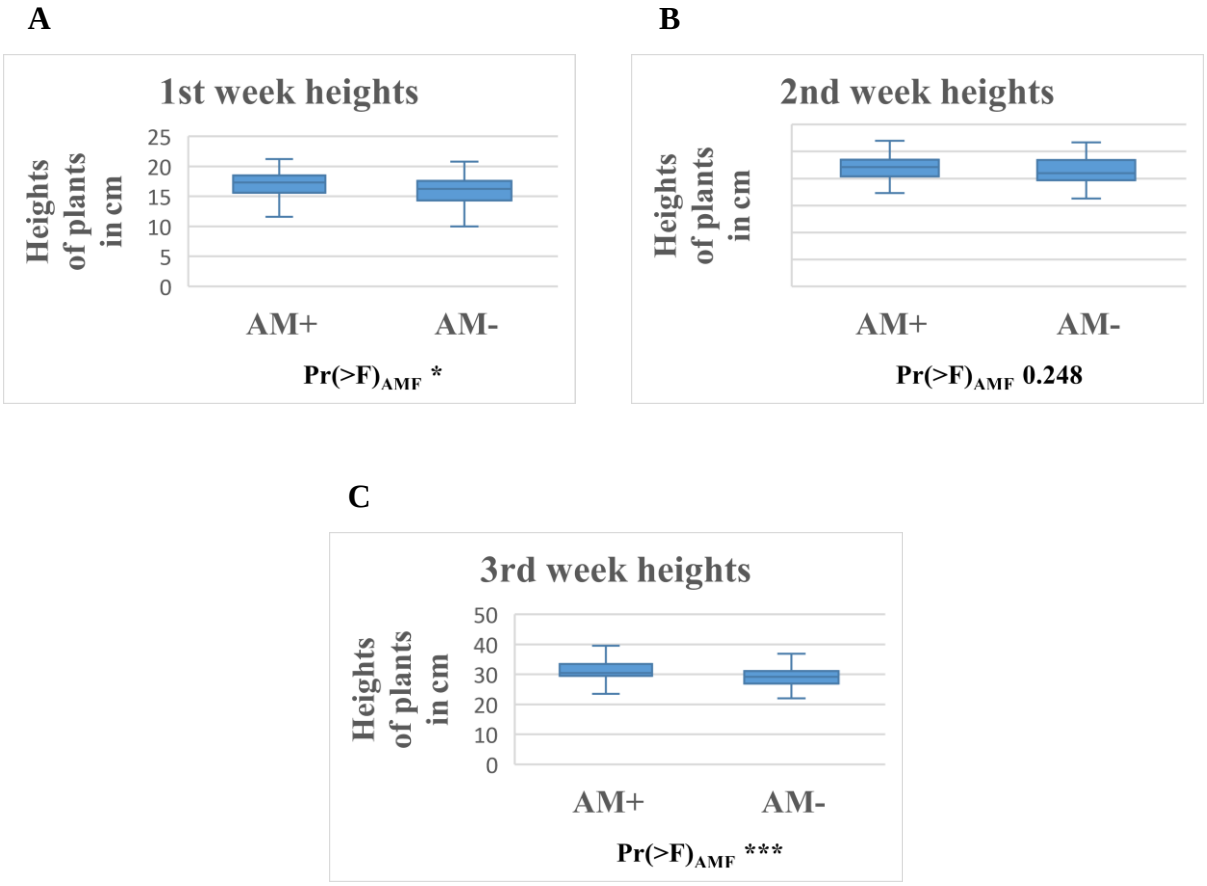


Figure 4.8. Heights of AM+ and AM- plants are indicated as bar plots with ±SE for each sampling week. Independent samples t test was used to assess the difference between heights of AM+ and AM- plants. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05. (A) 1st week heights. (B) 2nd week heights. (C) 3rd week heights.

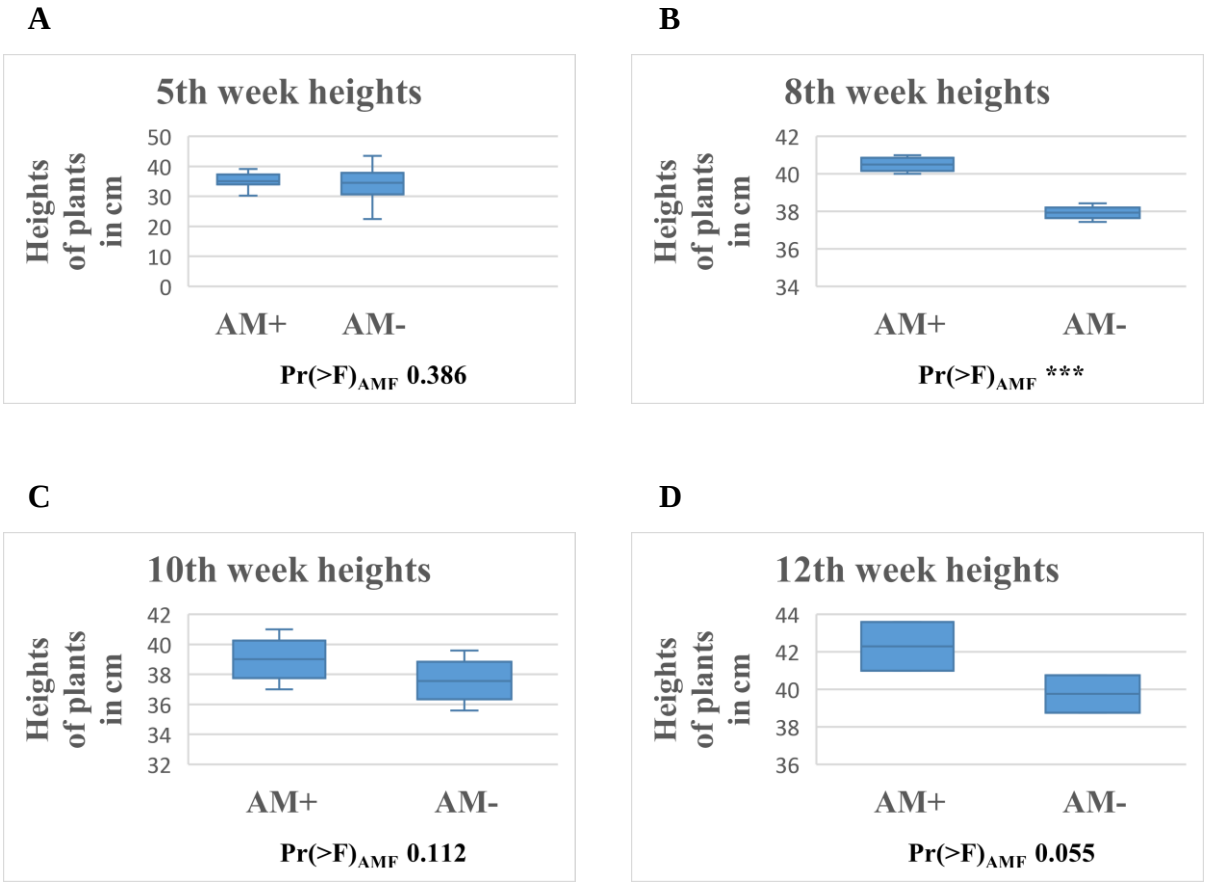
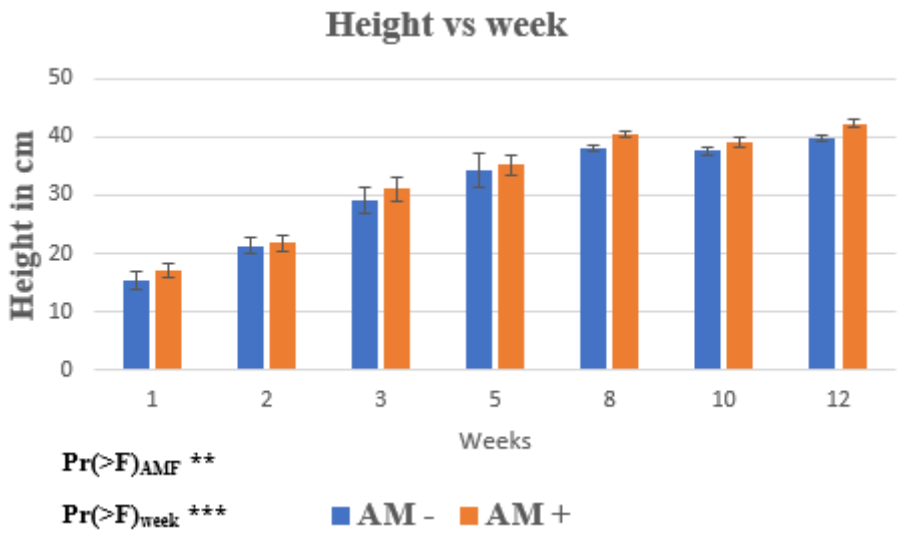


Figure 4.9. Heights of AM+ and AM- plants are indicated as bar plots with ±SE for each sampling week. Independent samples t test was used to assess the difference between heights of AM+ and AM- plants. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05. (A) 5th week heights. (B) 8th week heights. (C) 10th week heights. (D) 12th week heights.

As shown in Figure 4.8 and Figure 4.9, there is statistically significant difference between heights of plants in AM+ and AM- groups at 1st, 3rd and 8th weeks.

Heights of plants measurment at the different weeks is shown in Figure 4.10.A. The photo of the plants in greenhouse at 10th day after plantation is also shown in 4.10.B. In the height graph, standard error in AM+ group is higher than in AM- group at each week. From the statistical analysis it is concluded that both AMF and time have significant effects on height of the plants. However, the significance level of AMF effect on height is lower than the significance level of AMF on root weight.

A



B

Figure 4.10. (A) Heights of plants in AM+ and AM- groups are indicated as columns with ±SE at each sampling week. Two-way ANOVA was used to test the effect of AMF, week and their interaction on height. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05. (B) 10th day in greenhouse after plantation. Mycorrhizal and non-mycorrhizal plants are shown with black brackets.

Root and shoot weights, and heights of the plants were determined. The effect of AMF, time and their interaction on plant growth were assessed. The interaction had no significant effect on plant growth. AMF had a statistically significant effect on root weight and height but had no significant effect on shoot weight whereas time had significant effect on root and shoot weight, and height as well. From the statistical point of view, it is concluded that AMF encouraged mostly the growth of

the root of the plant. Moreover, the significant positive effect of AMF on root growth had started to be seen from the 3rd week. In addition to this, the photo of the plants in greenhouse at 10th day after plantation shows an obvious morphological difference between the plants with and without AMF since the numbers of plants in AM+ pots were clearly higher than the numbers of plants in AM- pots. Besides the effect of AMF and time, the effect of their interaction on plant growth was also assessed by two-way ANOVA analysis. However, the interaction had no significant effect on plant growth.

4.4. Mycorrhization Rates

Mycorrhization rate is the determination of symbiosis level of AMF with sorghum roots. The rates were measured for both plants with and without by microscopic counts. Determined mycorrhization rates were based on the presence or absence of AMF within the field of view that the object occupies but not on the number of each mycorrhizal structure. This gives a knowledge about the percentage of presence of AMF along the root, however does not tell anything about the intensity of mycorrhization. Hyphaes, arbuscules, vesicles and spores were included in these counts. Microscopic count results are shown in Table 4.2 below.

Table 4.2. Root mycorrhization rate at each week for both plants with and without AMF.

Sample name	# of fractions that include hyphae	# of fractions that include arbuscule	# of spores	# of fractions that include vesicle	# of fractions that include mycorrhizal structures among 30 fractions	mycorrhization rate %
1st week AM-	**2**	**1**	**15**		**2**	**6.67**
1st week AM+	**0**	**0**	**34**		**0**	**0**
2nd week AM-1	**0**	**0**	**11**		**0**	**0**
2nd week AM-2	**1**	**0**	**16**		**1**	**3.33**
2nd week AM-3	**0**	**0**	**9**		**0**	**0**
2nd week AM+1	**0**	**0**	**5**		**0**	**0**
2nd week AM+2	**0**	**0**	**11**		**0**	**0**
2nd week AM+3	**2**	**1**	**27**		**3**	**10**
3rd week AM-1	**0**	**0**	**17**		**0**	**0**
3rd week AM-2	**0**	**0**	**14**		**0**	**0**

Table 4.2. Continued.

3rd week AM+2	**2**	**0**	**37**		**2**	**6.67**
3rd week AM+3	**12**	**6**	**21**		**17**	**56.67**
5th week AM-1	**0**	**0**	**3**		**0**	**0**
5th week AM-2	**8**	**0**	**2**		**8**	**26.67**
5th week AM-3	**2**	**0**	**4**		**2**	**6.67**
5th week AM+1	**6**	**0**	**16**	**8**	**6**	**20**
5th week AM+2	**30**	**10**	**0**	**10**	**30**	**100**
5th week AM+3	**30**	**12**	**0**	**12**	**30**	**100**
8th week AM-1	**0**	**0**	**10**		**0**	**0**
8th week AM-2	**1**	**0**	**9**		**1**	**3.33**
8th week AM-3	**0**	**0**	**4**		**0**	**0**
8th week AM+1	**4**	**0**	**5**	**4**	**4**	**13.33**
8th week AM+2	**18**	**0**	**8**	**5**	**18**	**60**
8th week AM+3	**0**	**0**	**6**	**3**	**0**	**0**
10th week AM-1	**0**	**0**	**5**		**0**	**0**
10th week AM-2	**0**	**0**	**0**		**0**	**0**
10th week AM-3	**0**	**0**	**6**		**0**	**0**
10th week AM+1	**0**	**0**	**4**		**0**	**0**
10th week AM+2	**3**	**0**	**4**		**3**	**10**
10th week AM+3	**6**	**0**	**2**		**6**	**20**
12th week AM-1	**0**	**0**	**7**		**0**	**0**
12th week AM-2	**0**	**0**	**1**		**0**	**0**
12th week AM-3	**0**	**0**	**1**		**0**	**0**
12th week AM+1	**14**	**0**	**3**		**14**	**46.67**
12th week AM+2	**1**	**0**	**8**		**1**	**3.33**
12th week AM+3	**4**	**0**	**2**	**4**	**6**	**20**

Mycorrhization and spores were seen both in AM+ and AM- groups. The presence of AMF in non-mycorrhizal plant roots points out the naturally found mycorrhiza in this mine tailing soil. The number of spores in AM+ group decreased after 3rd week which is an indication of the germination of mycorrhiza. Moreover, the sudden increase in mycorrhization rates between 3rd and 5th week correlates with the decrease in number of spores after 3rd week.

Mycorrhization rate versus week graph for AM+ group is shown in Figure 4.11. It is seen that mycorrhization had started newly at 2nd week, however, it had completely initiated at 3rd week. Symbiosis reached its top level at 5th week and decreased until 10th week which had the lowest mycorrhization rate. A small increase in mycorrhization occured until 12th week.

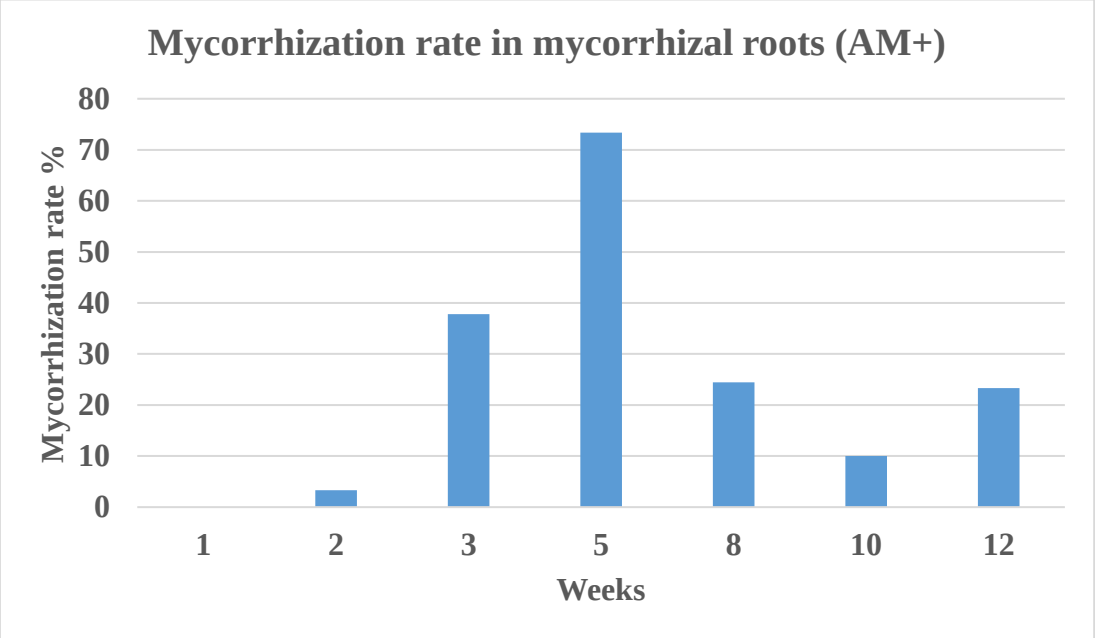


Figure 4.11. Mycorrhization rates in percentages are shown for each sampling week.

To compare whether there is an effect of temperature on decrease in mycorrhization rates at 10th week or not the temperatures in İstanbul were assessed. Average daytime and night temperatures until 8th week and between 8th and 10th weeks are shown in Table 4.3. There is an average 5 degrees decrease in both daytime and night temperatures from 8th to 10th week.

Table 4.3. Average daytime and night temperatures.

	Daytime	Night
0-8th	27 °C	18 °C
8th-10th	22 °C	13 °C

It was noted that a sudden and dramatic decrease in both daytime and night temperatures between 8th and 10th week occured in greenhouse. However, hydrogen lamps were inserted after 10th week.

During the microscopic observations it was seen that the intensities of hyphaes and arbuscules were too high at 5th and decreased at 8th week. Also, whereas the hyphaes were surrounded around the roots at 5th, it appears as crowded balls along the roots at 8th week and the blue dye was concentrated in these hyphae balls. However, at 10th week an apparent decrease in root mycorrhization intensities was detected. Two bright field images of AM+ roots from 8th and 5th weeks are shown in Figure 4.12 and Figure 4.13 respectively.

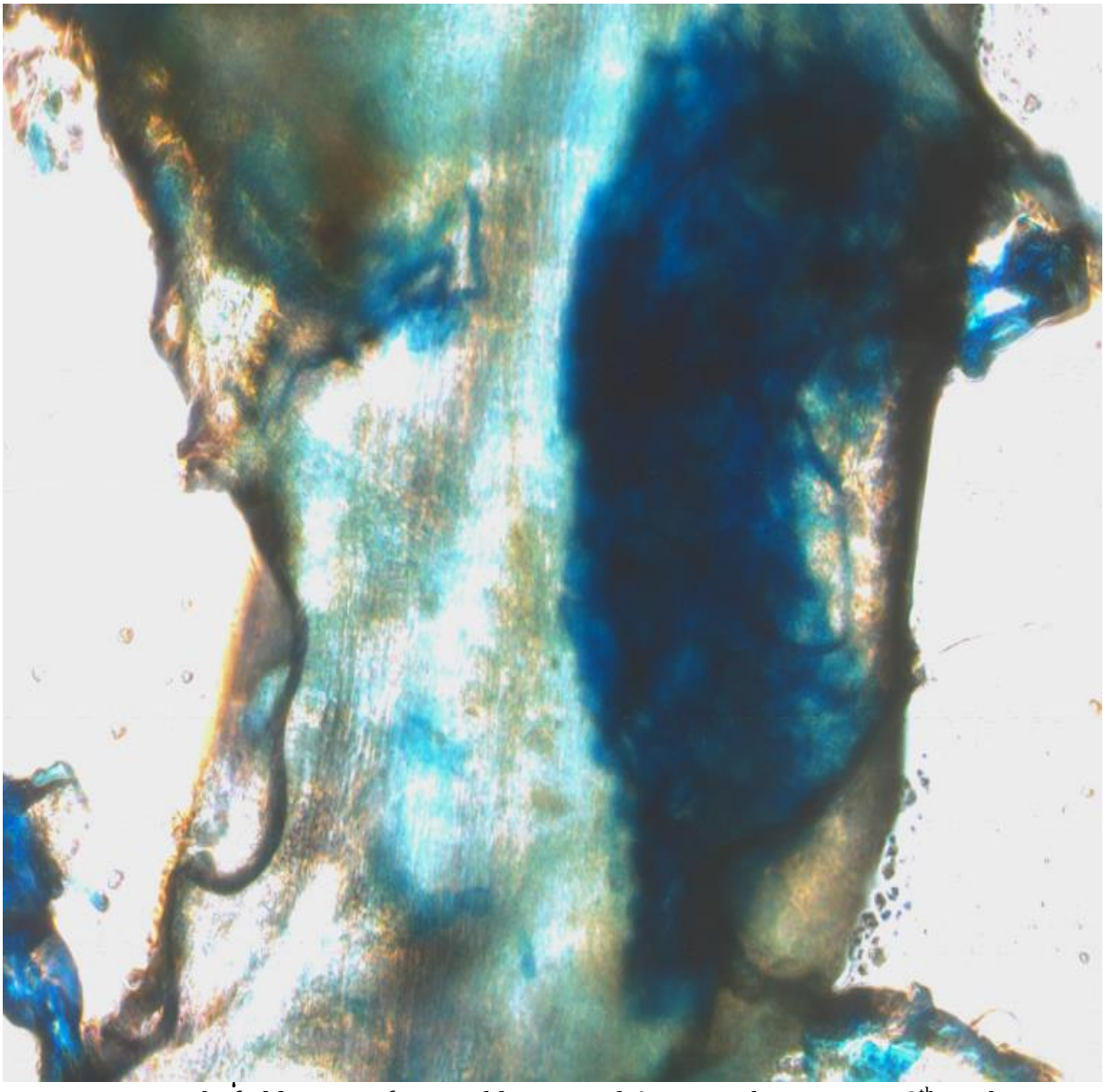


Figure 4.12. Bright field image of trypan blue stained AM+ sorghum roots at 8th week.

The hyphaes are seen as a crowded ball at the right side of the root in Figure 4.12. Also, the concentrated blue dye is clearly seen within this ball.

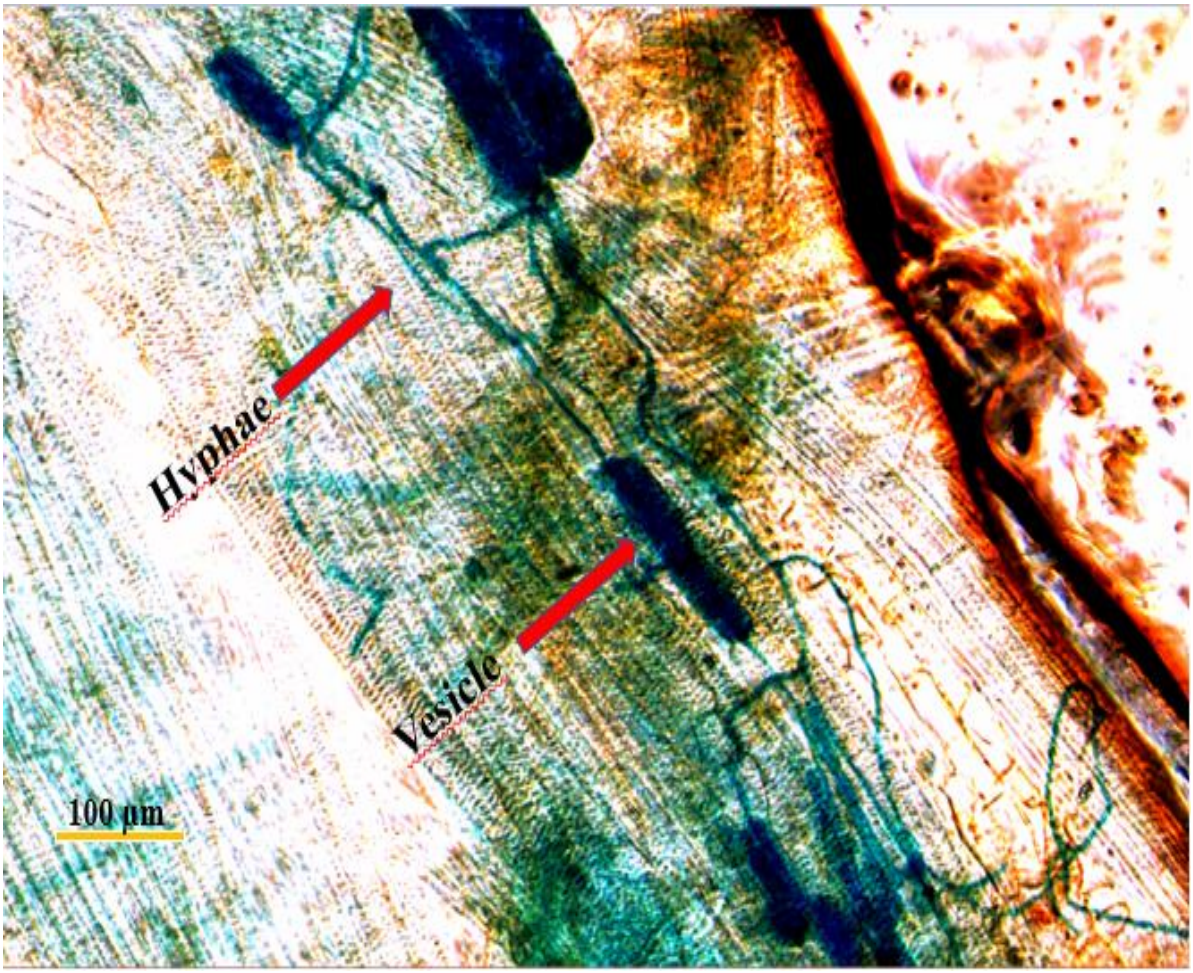


Figure 4.13. Bright field image of trypan blue stained AM+ sorghum roots at 5th week. Hyphaes and vesicles are also shown with red arrows.

As seen in Figure 4.13, the hyphae network and vesicles are very well shaped at the 5th week AM+ root.

Dark blue round shape figures were seen at 12th AM+ roots under the microscope. There were tiny filaments around these figures. The size of round shape figures was smaller than a mature spore of mycorrhiza. Also, the tiny filaments around them were like fragmented hyphae pieces. The bright field image of 12th AM+ root with these figures is shown in Figure 4.14 below.

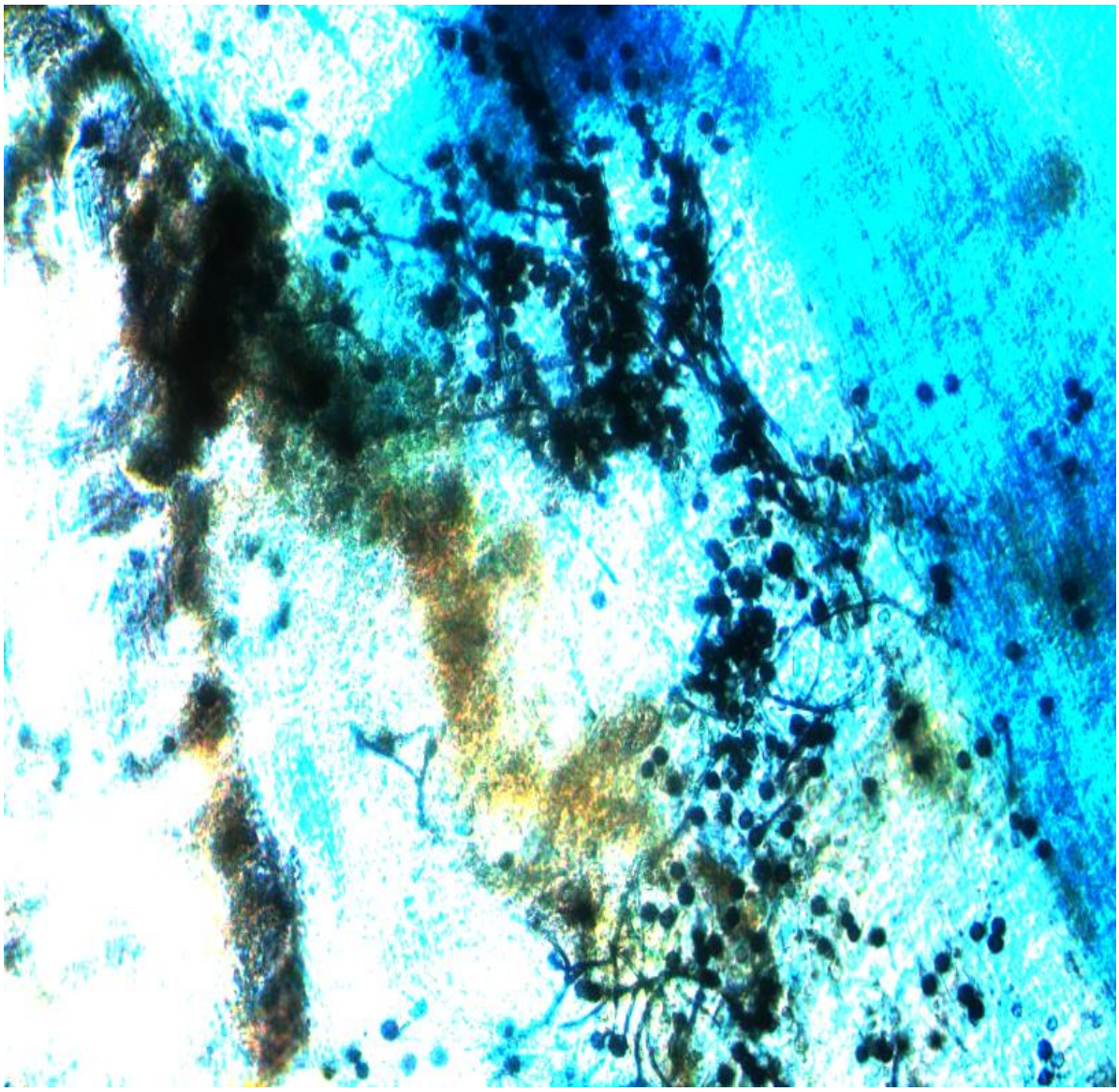


Figure 4.14. Bright field image of tryphan blue stained AM+ sorghum roots at 12th week.

In mycorrhization rate results, it was noted that mycorrhization prominently initiated at 3rd week. On the other hand, in plant growth assays the most significant effect of mycorrhiza was seen in root weight and the weights of AM+ roots were significantly higher than the weights of AM- roots from the 3rd week. This points out a possible relationship between root growth and mycorrhization.

4.5. Protein Measurements and Determination of Rhizosphere Glomalin Related Soil Protein (GRSP)

4.5.1. Bradford Assay

To assess the contribution of AMF to soil fertility protein extraction from the soil and root samples was done at each sampling week. This extraction procedure was in harsh conditions, which means high temperature and pressure, and specific to GRSP extraction. Bradford Assay was performed and the absorbances of the extracts were recorded at 595 nm to determine the protein concentrations within the extracts. The absorbance values and the amounts of fresh soil and root samples which were taken for the extraction are shown in Table 4.4.

Table 4.4. Absorbance of extacted samples at 595 nm after Bradford Reagent addition and the sample amounts.

Sample name	Absorbance at 595 nm	Amount of sample in mg
1st week AM+ soil-1	0.037	250
1st week AM+ soil-2	0.03	250
1st week AM+ soil-3	0.035	250
1st week AM- soil-1	0.021	250
1st week AM- soil-2	0.027	250
1st week AM- soil-3	0.026	250
1st week AM+ root	0.013	16
1st week AM- root	0.007	24.8
2nd week AM+ soil-1	0.06	250
2nd week AM+ soil-2	0.052	250
2nd week AM+ soil-3	0.036	250
2nd week AM- soil-1	0.041	250
2nd week AM- soil-2	0.036	250
2nd week AM- soil-3	0.04	250
2nd week AM+ root-1	0.011	13.4
2nd week AM+ root-2	0.015	15.8
2nd week AM+ root-3	0.015	11.8
2nd week AM- root-1	0.012	16.8
2nd week AM- root-2	0.012	32.4
2nd week AM- root-3	0.01	25.2
3rd week AM+ soil-1	0.033	250
3rd week AM+ soil-2	0.04	250
3rd week AM+ soil-3	0.035	250
3rd week AM- soil-1	0.031	250

Table 4.4. Continued.

3rd week AM- root-2	**0.015**	**27.2**
3rd week AM- root-3	**0.01**	**14.6**
5th week AM+ soil-1	**0.02**	**250**
5th week AM+ soil-2	**0.016**	**250**
5th week AM+ soil-3	**0.018**	**250**
5th week AM- soil-1	**0.015**	**250**
5th week AM- soil-2	**0.026**	**250**
5th week AM- soil-3	**0.021**	**250**
5th week AM+ root-1	**0.017**	**28.4**
5th week AM+ root-2	**0.011**	**37**
5th week AM+ root-3	**0.013**	**31**
5th week AM- root-1	**0.011**	**18**
5th week AM- root-2	**0.011**	**21.4**
5th week AM- root-3	**0.015**	**33.8**
8th week AM+ soil-1	**0.02**	**250**
8th week AM+ soil-2	**0.02**	**250**
8th week AM+ soil-3	**0.016**	**250**
8th week AM- soil-1	**0.025**	**250**
8th week AM- soil-2	**0.02**	**250**
8th week AM- soil-3	**0.016**	**250**
8th week AM+ root-1	**0.015**	**53.4**
8th week AM+ root-2	**0.017**	**79**
8th week AM+ root-3	**0.024**	**63.6**
8th week AM- root-1	**0.016**	**49.4**
8th week AM- root-2	**0.02**	**38.6**
8th week AM- root-3	**0.015**	**40.8**
10th week AM+ soil-1	**0.011**	**250**
10th week AM+ soil-2	**0.021**	**250**
10th week AM+ soil-3	**0.017**	**250**
10th week AM- soil-1	**0.016**	**250**
10th week AM- soil-2	**0.011**	**250**
10th week AM- soil-3	**0.014**	**250**
10th week AM+ root-1	**0.014**	**80.2**
10th week AM+ root-2	**0.017**	**132.4**
10th week AM+ root-3	**0.015**	**135.8**
10th week AM- root-1	**0.009**	**50.2**
10th week AM- root-2	**0.018**	**42.4**
10th week AM- root-3	**0.011**	**62.4**

Table 4.4. Continued.

12th week AM+ soil-1	**0.028**	**250**
12th week AM+ soil-2	**0.016**	**250**
12th week AM+ soil-3	**0.018**	**250**
12th week AM- soil-1	**0.021**	**250**
12th week AM- soil-2	**0.016**	**250**
12th week AM- soil-3	**0.018**	**250**
12th week AM+ root-1	**0.014**	**78.2**
12th week AM+ root-2	**0.023**	**60.8**
12th week AM+ root-3	**0.017**	**93**
12th week AM- root-1	**0.021**	**20**
12th week AM- root-2	**0.017**	**34.6**
12th week AM- root-3	**0.013**	**28**

To calculate the protein concentrations, a calibration curve was constructed with bovine serum albumin (BSA) standards via Bradford Assay. Absorbance values of the standards and constructed calibration curve are shown in Table 4.5 and Figure 4.15 respectively.

Table 4.5. Absorbance values of indicated BSA samples at 595 nm.

Absorbance at 595nm	**concentration of BSA**
0.01	**0.005**
0.05	**0.017**
0.1	**0.034**
0.25	**0.083**
0.5	**0.175**
1	**0.389**
2	**0.717**

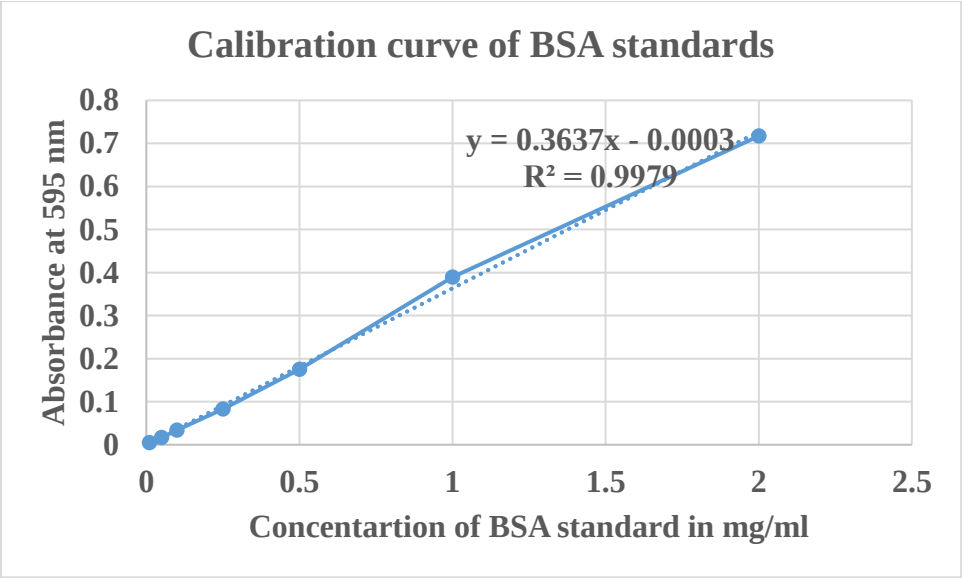


Figure 4.15. Calibration curve of BSA standards.

A nearly perfect linear curve was constructed as shown in the figure. The R^2 value is 0.9979 which indicates how the measured values perfectly fit the linear trendline. Protein concentrations of the extracts were calculated according to this calibration curve. Moreover, total amount of protein per sample and amount of protein per kg sample were also calculated by basing on the volume of citric acid per sample in which the extracts were present and the amount of taken sample respectively. The calculated values are shown in Table 4.6.

Table 4.6. Calculated values for protein concentrations of samples, total amont of protein per sample and amount of protein per kg sample are indicated.

Sample name	Concentration of protein in sample in mg/ml	Total amount of protein in 50 ml sample in mg	Amount of mg protein per kg sample
1st week AM+ soil-1	0.102557053	5.127852626	20511.4105
1st week AM+ soil-2	0.083310421	4.165521034	16662.08414
1st week AM+ soil-3	0.097058015	4.852900742	19411.60297
1st week AM- soil-1	0.058564751	2.928237558	11712.95023
1st week AM- soil-2	0.075061864	3.753093209	15012.37283
1st week AM- soil-3	0.072312345	3.615617267	14462.46907
1st week AM+ root	0.0365686	1.828430025	114276.8765
1st week AM- root	0.020071487	1.003574374	40466.70865
2nd week AM+ soil-1	0.165795986	8.289799285	33159.19714
2nd week AM+ soil-2	0.143799835	7.189991751	28759.96701
2nd week AM+ soil-3	0.099807534	4.990376684	19961.50674
2nd week AM- soil-1	0.113555128	5.677756393	22711.02557
2nd week AM- soil-2	0.099807534	4.990376684	19961.50674

Table 4.6. Continued.

2nd week AM- soil-3	**0.110805609**	**5.540280451**	**22161.1218**
2nd week AM+ root-1	**0.031069563**	**1.553478141**	**115931.2046**
2nd week AM+ root-2	**0.042067638**	**2.103381908**	**133125.4372**
2nd week AM+ root-3	**0.042067638**	**2.103381908**	**178252.7041**
2nd week AM- root-1	**0.033819082**	**1.690954083**	**100652.0288**
2nd week AM- root-2	**0.033819082**	**1.690954083**	**52189.94083**
2nd week AM- root-3	**0.028320044**	**1.4160022**	**56190.56348**
3rd week AM+ soil-1	**0.091558977**	**4.577948859**	**18311.79544**
3rd week AM+ soil-2	**0.110805609**	**5.540280451**	**22161.1218**
3rd week AM+ soil-3	**0.097058015**	**4.852900742**	**19411.60297**
3rd week AM- soil-1	**0.08605994**	**4.302996976**	**17211.9879**
3rd week AM- soil-2	**0.069562827**	**3.478141325**	**13912.5653**
3rd week AM- soil-3	**0.116304647**	**5.815232334**	**23260.92934**
3rd week AM+ root-1	**0.028320044**	**1.4160022**	**30517.28878**
3rd week AM+ root-2	**0.042067638**	**2.103381908**	**75661.21972**
3rd week AM+ root-3	**0.055815232**	**2.790761617**	**76669.27518**
3rd week AM- root-1	**0.031069563**	**1.553478141**	**73975.14959**
3rd week AM- root-2	**0.042067638**	**2.103381908**	**77330.21721**
3rd week AM- root-3	**0.028320044**	**1.4160022**	**96986.45203**
5th week AM+ soil-1	**0.055815232**	**2.790761617**	**11163.04647**
5th week AM+ soil-2	**0.044817157**	**2.24085785**	**8963.4314**
5th week AM+ soil-3	**0.050316195**	**2.515809733**	**10063.23893**
5th week AM- soil-1	**0.042067638**	**2.103381908**	**8413.527633**
5th week AM- soil-2	**0.072312345**	**3.615617267**	**14462.46907**
5th week AM- soil-3	**0.058564751**	**2.928237558**	**11712.95023**
5th week AM+ root-1	**0.047566676**	**2.378333792**	**83744.14759**
5th week AM+ root-2	**0.031069563**	**1.553478141**	**41985.89571**
5th week AM+ root-3	**0.0365686**	**1.828430025**	**58981.6137**
5th week AM- root-1	**0.031069563**	**1.553478141**	**86304.34118**
5th week AM- root-2	**0.031069563**	**1.553478141**	**72592.43651**
5th week AM- root-3	**0.042067638**	**2.103381908**	**62230.23397**
8th week AM+ soil-1	**0.055815232**	**2.790761617**	**11163.04647**
8th week AM+ soil-2	**0.055815232**	**2.790761617**	**11163.04647**
8th week AM+ soil-3	**0.044817157**	**2.24085785**	**8963.4314**
8th week AM- soil-1	**0.069562827**	**3.478141325**	**13912.5653**
8th week AM- soil-2	**0.055815232**	**2.790761617**	**11163.04647**
8th week AM- soil-3	**0.044817157**	**2.24085785**	**8963.4314**
8th week AM+ root-1	**0.042067638**	**2.103381908**	**39389.17431**
8th week AM+ root-2	**0.047566676**	**2.378333792**	**30105.49103**
8th week AM+ root-3	**0.066813308**	**3.340665384**	**52526.18528**

Table 4.6. Continued.

8th week AM- root-1	**0.044817157**	**2.24085785**	**45361.49494**
8th week AM- root-2	**0.055815232**	**2.790761617**	**72299.52375**
8th week AM- root-3	**0.042067638**	**2.103381908**	**51553.47814**
10th week AM+ soil-1	**0.031069563**	**1.553478141**	**6213.912565**
10th week AM+ soil-2	**0.058564751**	**2.928237558**	**11712.95023**
10th week AM+ soil-3	**0.047566676**	**2.378333792**	**9513.335166**
10th week AM- soil-1	**0.044817157**	**2.24085785**	**8963.4314**
10th week AM- soil-2	**0.031069563**	**1.553478141**	**6213.912565**
10th week AM- soil-3	**0.039318119**	**1.965905966**	**7863.623866**
10th week AM+ root-1	**0.039318119**	**1.965905966**	**24512.54322**
10th week AM+ root-2	**0.047566676**	**2.378333792**	**17963.24616**
10th week AM+ root-3	**0.042067638**	**2.103381908**	**15488.82112**
10th week AM- root-1	**0.025570525**	**1.278526258**	**25468.65056**
10th week AM- root-2	**0.050316195**	**2.515809733**	**59335.13522**
10th week AM- root-3	**0.031069563**	**1.553478141**	**24895.48303**
12th week AM+ soil-1	**0.077811383**	**3.89056915**	**15562.2766**
12th week AM+ soil-2	**0.044817157**	**2.24085785**	**8963.4314**
12th week AM+ soil-3	**0.050316195**	**2.515809733**	**10063.23893**
12th week AM- soil-1	**0.058564751**	**2.928237558**	**11712.95023**
12th week AM- soil-2	**0.044817157**	**2.24085785**	**8963.4314**
12th week AM- soil-3	**0.050316195**	**2.515809733**	**10063.23893**
12th week AM+ root-1	**0.039318119**	**1.965905966**	**25139.46249**
12th week AM+ root-2	**0.064063789**	**3.203189442**	**52684.03687**
12th week AM+ root-3	**0.047566676**	**2.378333792**	**25573.48163**
12th week AM- root-1	**0.058564751**	**2.928237558**	**146411.8779**
12th week AM- root-2	**0.047566676**	**2.378333792**	**68737.97086**
12th week AM- root-3	**0.0365686**	**1.828430025**	**65301.07231**

A graph of protein content in roots versus week is shown in Figure 4.16 below.

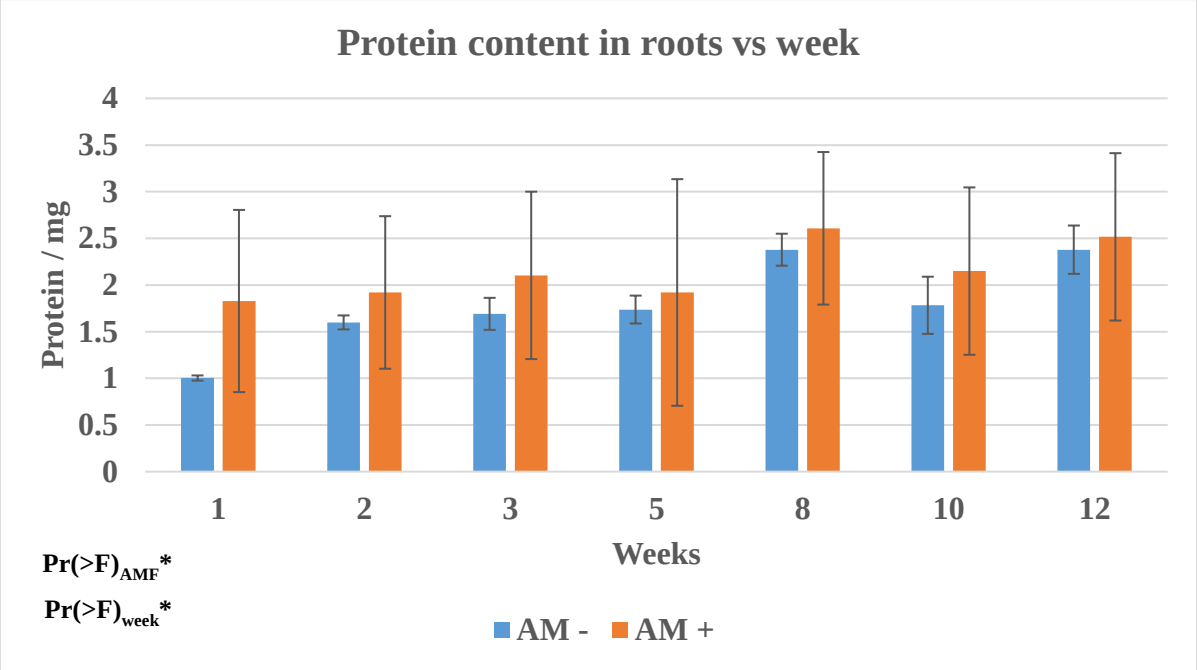


Figure 4.16. Total amount of protein in root versus week. Protein content of AM+ and AM- roots are indicated as columns with ±SE at each sampling week. Two-way ANOVA was used to test the effect of AMF and week on protein content in roots. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

Protein content in AM+ roots was higher than in AM- roots at each week. Both AMF and time have statistically significant effects on protein content in roots, however, the interaction of AMF and time has no significant effect. Also, the standard errors of AM+ group are generally higher than the standard errors of the AM- group.

To compare the AM+ and AM- groups in terms of the protein content in roots, the time effect was excluded and an independent samples t-test was performed by constructing two groups, AM+ and AM-. The two groups were not statistically significantly different from each other. However, as seen in Figure 4.16, the effect of AMF on protein content in roots was statistically significant in a two-way ANOVA test. This shows that the significance of the effect of AMF showed differences among different sampling weeks; the effect was probably significant at some weeks; whereas it was not significant at the other weeks. Therefore, there is no absolute effect of AMF on protein content of the roots.

Amount of protein per kg root versus week graph is shown in Figure 4.17.

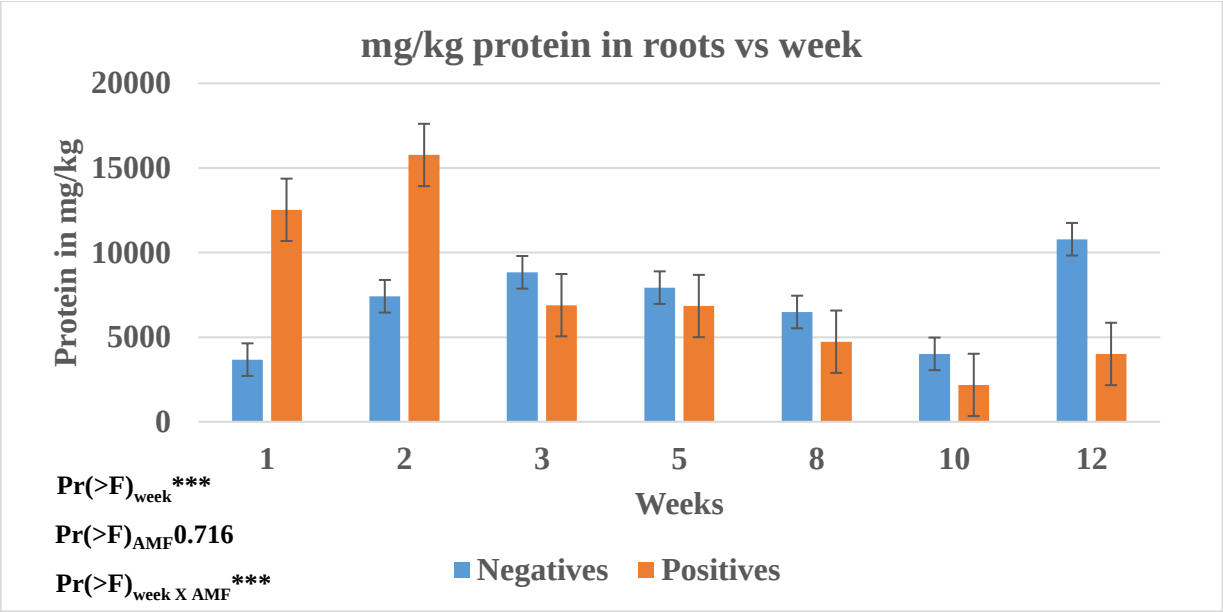


Figure 4.17. Amount of protein per kg root versus week graph. Protein content per kg root of AM+ and AM- groups are indicated as columns with ±SE at each sampling week. Two-way ANOVA was used to test the effect of AMF, week and their interaction on protein content per kg root. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

Both time and time-AMF interaction have statistically significant effects on protein amount per kg root. However, presence of AMF has no significant effect on protein amount per kg root individually. Also, the standard errors of AM+ group are generally higher than the standard errors of the AM- group.

Protein content in soil samples versus week graph is shown in Figure 4.18. It is concluded that only the time has a statistically significant effect on protein content in soil samples. As seen in the graph, protein content of AM+ and AM- soil samples are very close to each other at each sampling week which is also demonstrated by the non-significant value of AMF effect.

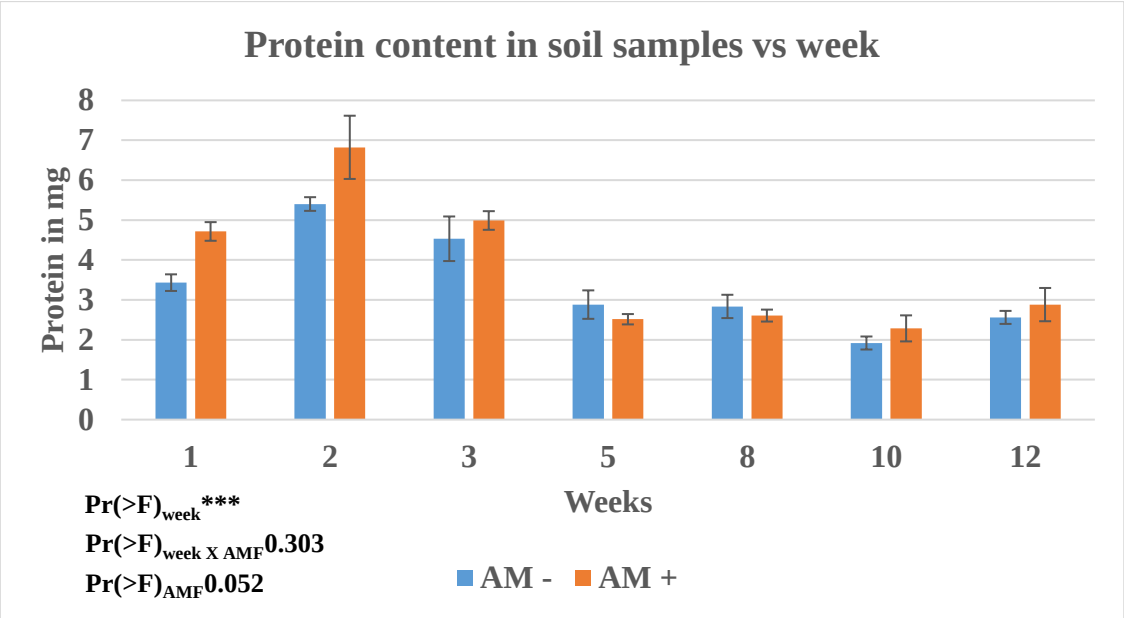


Figure 4.18. Amount of protein in soil samples versus week graph. Protein content in soil samples of AM+ and AM- groups are indicated as columns with ±SE at each sampling week. Two-way ANOVA was used to test the effect of AMF, week and their interaction on protein content in soil. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

Protein content per kg soil versus week graph is shown in Figure 4.19. It is concluded that only the time has a statistically significant effect on protein content per kg soil samples. As seen in the graph, protein content per AM+ and AM- soil samples are very close to each other at each sampling week which is also demonstrated by the non-significant value of AMF effect. However, at the average total there was a 1725.04 mg protein per kg soil difference between AM+ and AM- groups; protein content per kg AM+ soil was higher.

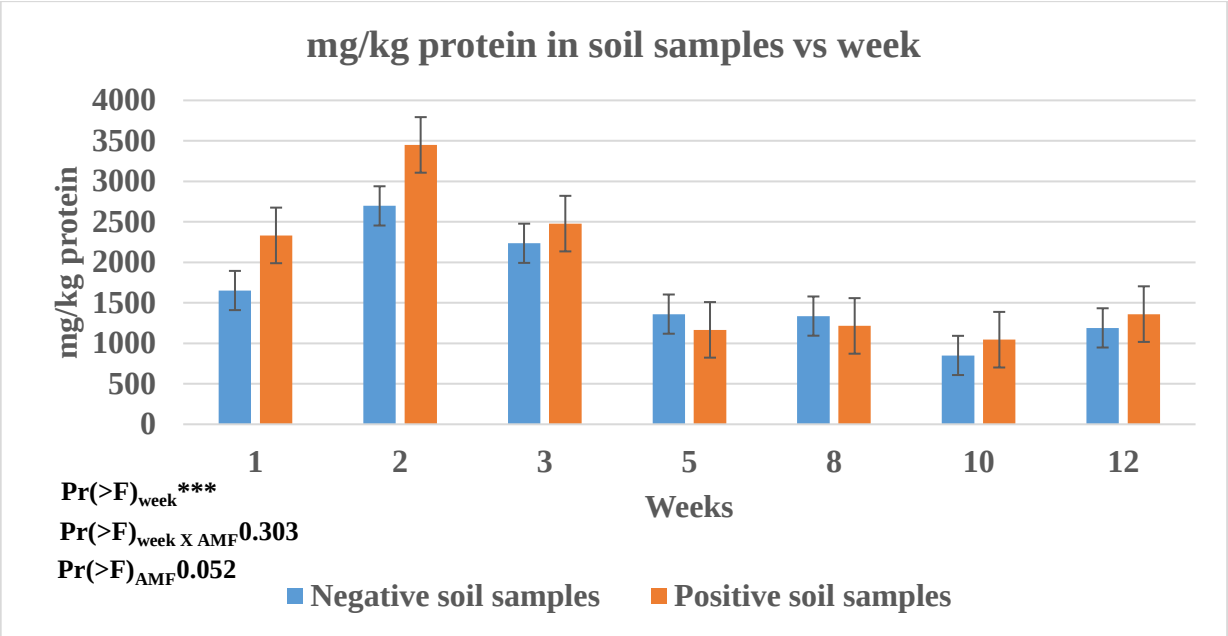


Figure 4.19. Amount of protein per kg soil versus week graph. Protein content per kg soil of AM+ and AM- groups are indicated as columns with ±SE at each sampling week. Two-way ANOVA was used to test the effect of AMF, week and their interaction on protein content per kg root. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

Time had a statistically significant effect on protein contents in the roots and soil samples, and protein contents per kg root and soil samples. On the other hand, the presence of mycorrhiza showed a statistically significant effect on only protein content in the roots, however, this effect was not seen on the protein content per kg root. The effect of interaction between AMF and time was statistically significant for the protein content in soil samples and per kg soil. Also, the standard errors of amount of protein in root and per kg root values were higher in AM+ groups than the standard errors in AM- groups.

4.5.2. SDS-PAGE Experiments

Bovine serum albumin and glomalin protein have very close molecular weights; 66.5 and 63.1 kDa respectively. To estimate the presence of glomalin in the protein extract three SDS-PAGE experiments were performed with bovine serum albumin standards with various concentrations and the extracts. However, no bands were observed in all SDS-PAGE gels eventhough the bands of the standards were seen clearly.

Two gel images from SDS-PAGE experiments are shown in Figure 4.20.

A **B**

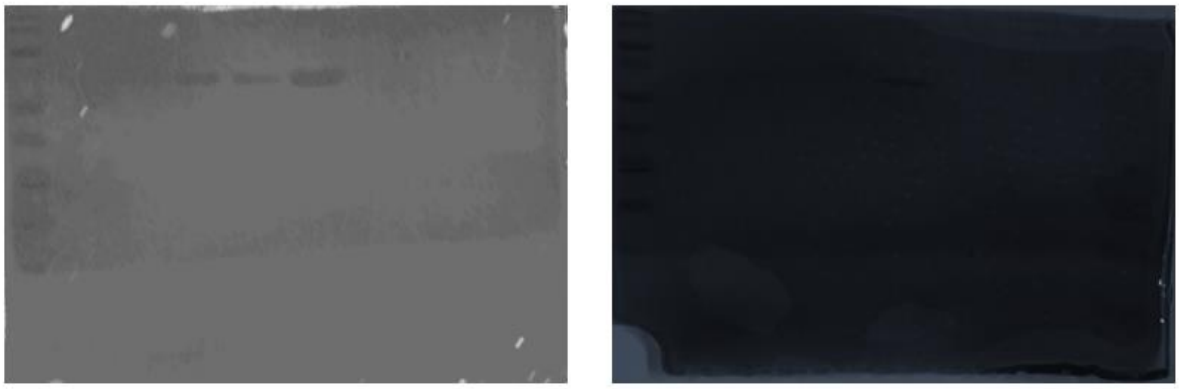


Figure 4.20. SDS-PAGE gel images. (A) Samples in the gel are ladder, BSA 25 ug/ml, BSA 50 ug/ml, BSA 125 ug/ml, BSA 500 ug/ml, 1st week AM- soil-1, 1st week AM+ soil-1, 1st week AM+ soil-2, 1st week AM+ root from left to right. (B) Samples in the gel are ladder, BSA 25 ug/ml, BSA 50 ug/ml, BSA 125 ug/ml, 5th week AM+ soil-1, 5th week AM- soil-1, 8th week AM+ soil-1, 8th week AM- soil-1 from left to right.

No band was observed in the protein extract samples obtained at various sampling weeks. However, the presence of the bands of ladders and the BSA standards indicates that there was no problem with the gels and the experiment.

4.5.3. Gradient PCRs for Glomalin Transcripts

Total RNA was extracted from the plant roots at each sampling weeks and the RNAs were converted to cDNA samples. To search for the glomalin transcripts gradient PCRs were performed with 5 different primers (glexjunc-23-2, glexjunc-23-1, glex3-1, glex3-2, glexjunc-12) specific to glomalin mRNA sequence. No product band was observed in any of the gels. Also, primer dimer formation was observed for four of the 5 primer pairs; dimer formation was not observed only for the glexjunc-12 primer. Three gel images from the gradient PCRs with glexjunc-23-2, glexjunc-23-1, glex3-1, glex3-2 primers are shown in Figure 4.21. PCR products of glexjunc-12 and glexjunc-23-2 primers are also shown in Figure 4.22.

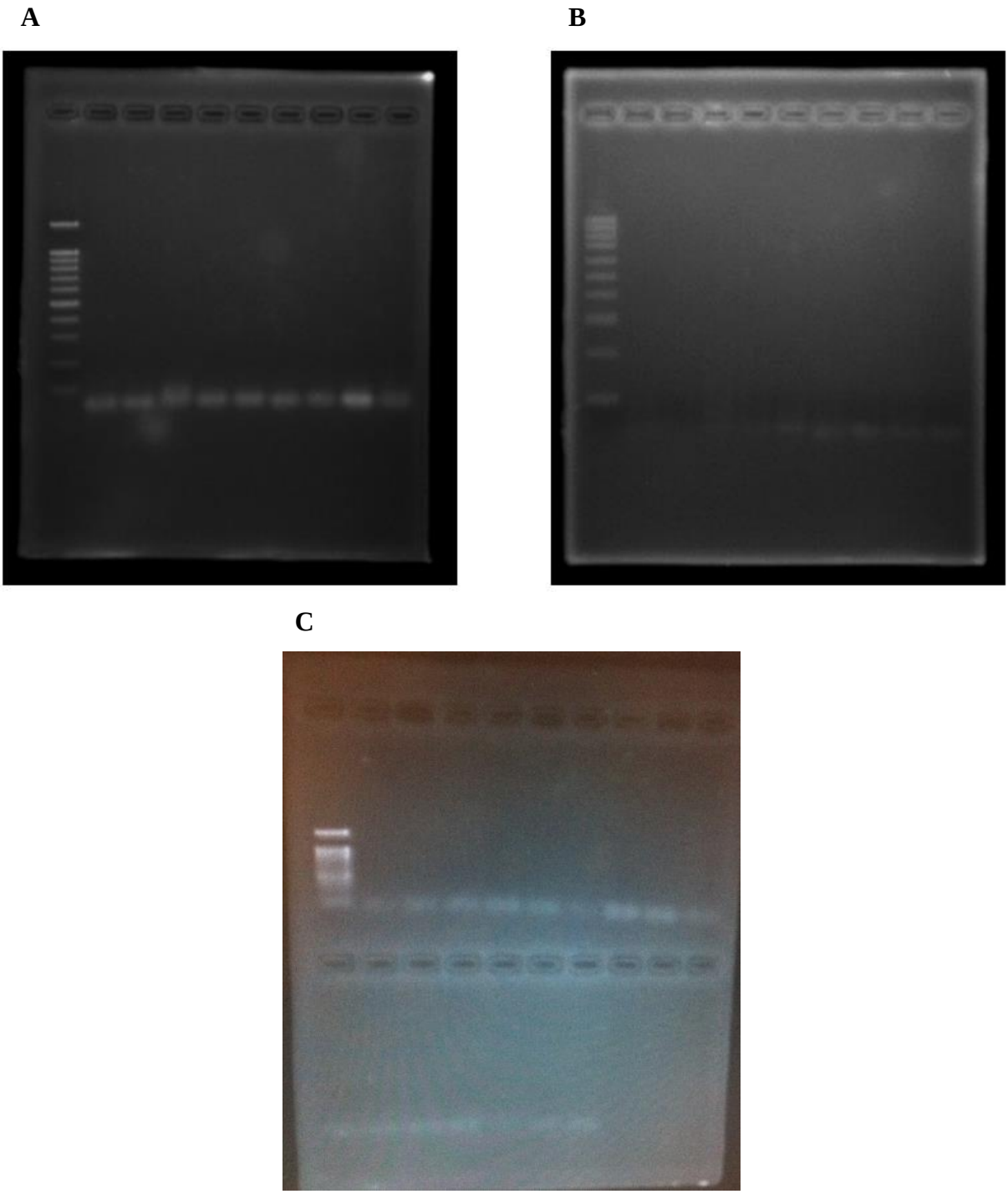


Figure 4.21. Gel images from gradient PCR experiments performed with 5 different primers for glomalin transcript. (A) Gradient PCR products of glexjuc23-2 primer on 3% agarose gel. From left to right; 100 bp ladder; 3rd week AM+1 cDNA, T_a: 60.3 °C; 8th week AM+3 cDNA, T_a: 60.3 °C; 12th week AM-1 cDNA, T_a: 60.3 °C; 3rd week AM+1 cDNA, T_a: 61.6 °C; 8th week AM+3 cDNA, T_a: 61.6 °C; 12th week AM-1 cDNA, T_a: 61.6 °C; 8th week AM+3 cDNA, T_a: 62.9 °C; 3rd week AM+1 cDNA, T_a: 62.9 °C; 12th week AM-1 cDNA, T_a: 62.9 °C. (B) Gradient PCR products of glexjuc23-1 (2nd to 6th well from left) and glex3-1 (7th to 10th well from left) primers on 3% agarose gel. From left to right; 100 bp ladder; 3rd week AM+1 cDNA, T_a: 57 °C; 3rd week AM+1 cDNA, T_a: 57.9 °C; 3rd week AM+1 cDNA, T_a: 60.3 °C; 3rd week AM+1 cDNA, T_a: 61.6 °C; 3rd week AM+1 cDNA, T_a: 62.9 °C;

8th week AM+3 cDNA, T_a: 57 °C; 8th week AM+3 cDNA, T_a: 57.9 °C; 8th week AM+3 cDNA, T_a: 60.3 °C; 8th week AM+3 cDNA, T_a: 61.6 °C. (C) Gradient PCR products of glex3-2 primer on 1% agarose gel. 100 bp ladder is in the first well of the upper part of the gel. From left to right: first five wells after the ladder; 5th week AM+3 cDNA, T_a from 56 to 60 °C and the next five wells are the negative controls of them. From the 2nd well of the lower part of the gel: 8th week AM+3 cDNA, T_a from 61 to 63 °C and the next five wells are the negative controls.

To verify the presence of mycorrhizal transcripts other than the glomalin transcript gradient PCRs were performed with qGint and AML primers. The expected 800 bp products of AML primer were demonstrated on the gel image. However, the expected 60 bp products of qGint primer were not seen. Also, primer dimer formation was observed for both AML and qGint primers. Two gel images of these PCR experiments are shown in Figure 4.22.

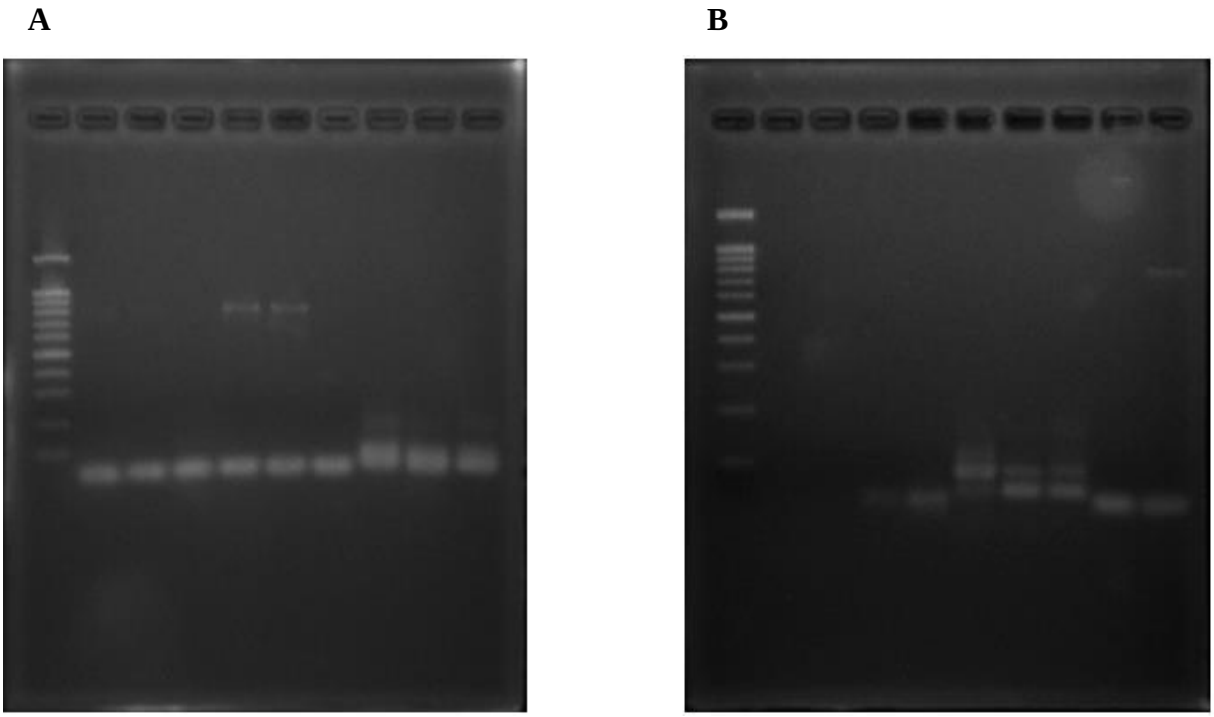


Figure 4.22. Electrophoresis of PCR products of qGint, AML, glexjunc-12 and glexjunc-23-2 primers on 3% agorose gel. (A) First 6 wells after the ladder are the products of AML primer and the next 3 wells are the products of qGint primer. From left to right: 100 bp ladder; 12th week AM-1 cDNA, T_a: 57 °C; negative control, T_a: 57 °C; 8th week AM+3 cDNA, T_a: 57 °C; 8th week AM+3 cDNA, T_a: 56 °C; 3rd week AM+1 cDNA, T_a: 57 °C; 3rd week AM+1 cDNA, T_a: 56 °C; 12th week AM-1 cDNA, T_a: 60 °C; 8th week AM+3 cDNA, T_a: 60 °C; 3rd week AM+1 cDNA, T_a: 60 °C. (B) 2nd and 3rd wells after the ladder are the products of glexjunc-12 primer, 4th and 5th wells are the products of glexjunc-23-2 primer, 6th, 7th and 8th wells are the products of qGint primer, 9th and 10th wells are the products of AML primer. From left to right: 100 bp ladder; 8th week AM+3 cDNA, T_a: 57 °C; 8th week AM+3

cDNA, T_a: 58.3 °C; negative control of glexjunc-23-2 at 61.6 °C; 8th week AM+3 cDNA, T_a: 61.6 °C; negative control of qGint at 60 °C ; 8th week AM+3 cDNA, T_a: 60 °C; 12th week AM-1 cDNA, T_a: 60 °C; negative control of AML at 56 °C; 3th week AM+1 cDNA, T_a: 56 °C.

The presence of 800 bp products of PCR using the AML primer showed that the mycorrhizal gene was expressed and mycorrhiza in the roots was alive and able to make gene transcription. However, no product of glomalin transcript was seen with the 5 primers used for glomalin. Moreover, there was no band in any of the extracts in SDS-PAGE gels, and the protein content in roots and soil were not obviously changed between AM+ and AM- groups. These three experiments points out the possibility of no glomalin production in these samples. On the other hand, since the protein amount in AM+ roots was higher than in AM- roots at each week and AM+ soil had 1725.04 mg more protein per kg soil than the AM- soil had, the presence of mycorrhiza somehow contributed to the soil fertility.

4.6. Soil pH Measurements

pH of the soil samples were measured at each sampling week to assess the availability of iron and aluminium which were the metals present at highest concentrations among the twelve measured metals in this mine tailing soil for the plant. Measured pH values are shown in Table 4.7.

Table 4.7. pH values of soil samples measured at each sampling week.

Sample Name	Soil pH	Sample Name	Soil pH
1st week AM- soil	5.7	8th week AM- soil-2	5.86
1st week AM+ soil	6.08	8th week AM- soil-3	6.49
2nd week AM- soil-1	5.96	8th week AM+ soil-1	6.5
2nd week AM+ soil-2	5.74	8th week AM+ soil-2	6.49
3rd week AM- soil-1	6.23	8th week AM+ soil-3	6.49
3rd week AM- soil-2	5.83	10th week AM- soil-1	6.34
3rd week AM- soil-3	6.31	10th week AM- soil-2	6.22
3rd week AM+ soil-1	6.38	10th week AM- soil-3	6.24
3rd week AM+ soil-2	6.42	10th week AM+ soil-1	6.12
3rd week AM+ soil-3	6.08	10th week AM+ soil-2	6.35
5th week AM- soil-1	6.73	10th week AM+ soil-3	6.47
5th week AM- soil-2	6.63	12th week AM- soil-1	6.69
5th week AM- soil-3	6.59	12th week AM- soil-2	6.71
3rd week AM+ soil-1	6.38	10th week AM- soil-3	6.24
3rd week AM+ soil-1	6.38	10th week AM- soil-3	6.24
3rd week AM+ soil-1	6.38	10th week AM- soil-3	6.24

Table 4.7. Continued.

3rd week AM+ soil-1	**6.38**	**10th week AM- soil-3**	**6.24**
3rd week AM+ soil-2	**6.42**	**10th week AM+ soil-1**	**6.12**
3rd week AM+ soil-3	**6.08**	**10th week AM+ soil-2**	**6.35**
5th week AM- soil-1	**6.73**	**10th week AM+ soil-3**	**6.47**
5th week AM- soil-2	**6.63**	**12th week AM- soil-1**	**6.69**
5th week AM- soil-3	**6.59**	**12th week AM- soil-2**	**6.71**
5th week AM+ soil-1	**6.67**	**12th week AM- soil-3**	**6.71**
5th week AM+ soil-2	**6.54**	**12th week AM+ soil-1**	**6.51**
5th week AM+ soil-3	**6.7**	**12th week AM+ soil-2**	**6.75**
8th week AM- soil-1	**6.43**	**12th week AM+ soil-3**	**6.59**

As seen in Table 4.14, the pH of the soil samples were around 6. In other words, the pH of the soil is close to neutral pH values. The pH around 6 means that $Fe(OH)_3$ and $Al(OH)_2^+$, which are not very soluble at this pH, were the dominant species in the soil.

4.7. Metal Analyses

Metal analyses of root, shoot and soil samples obtained at each sampling week were performed via ICP-OES. Concentrations of twelve metals were analysed by further calculation. Metals contents at each sample were calculated by basing on the volume of digestion solution. Since the amount of Cd was nearly 0 at each sample, it is not considered in these analyses.

Fe, Al, Si, Cr, Mn, Ni, Cu, Zn, Pb, Co and Mo amounts in root versus week graphs are shown in Figure 4.23 to Figure 4.33.

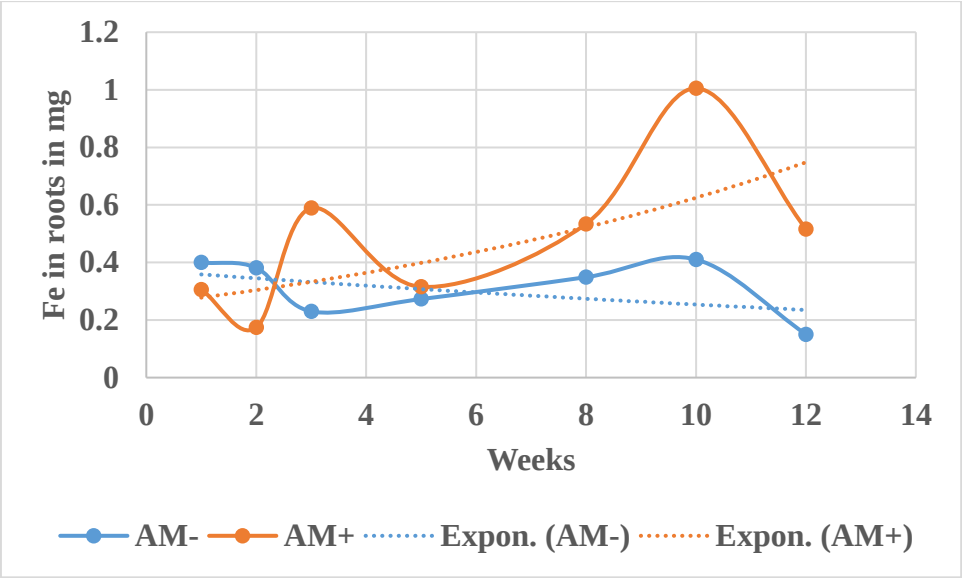


Figure 4.23. Amount of Fe in AM+ and AM- roots at each sampling week.

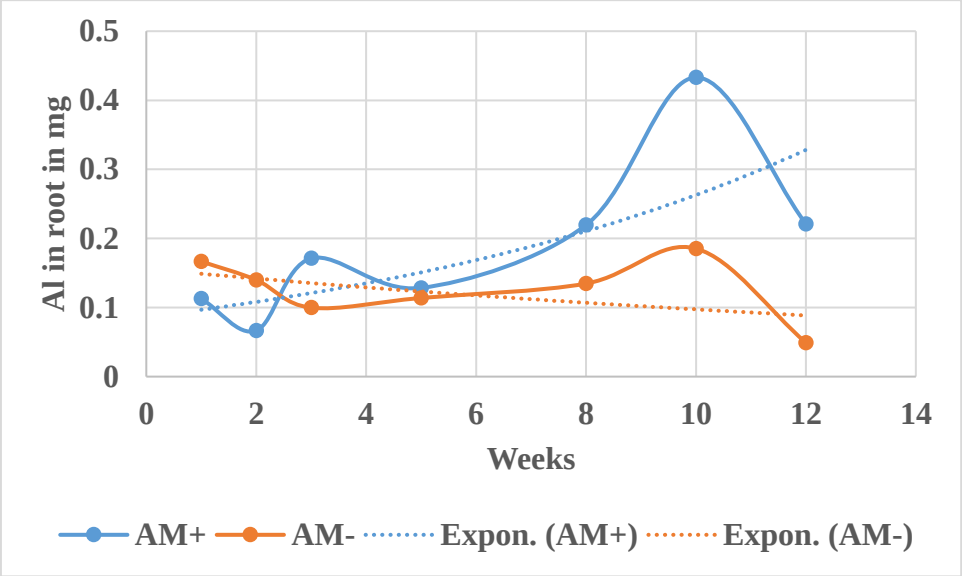


Figure 4.24. Amount of Al in AM+ and AM- roots at each sampling week.

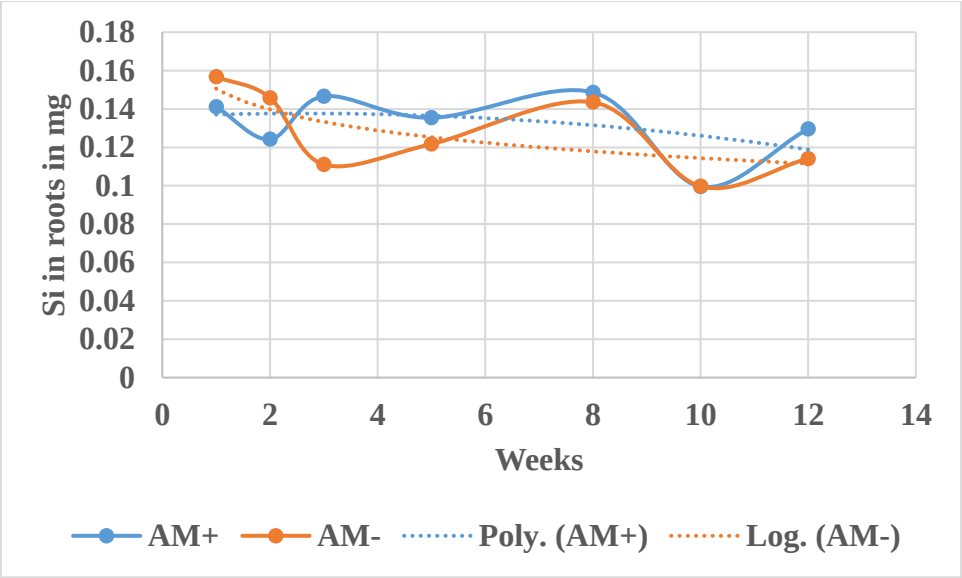


Figure 4.25. Amount of Si in AM+ and AM- roots at each sampling week.

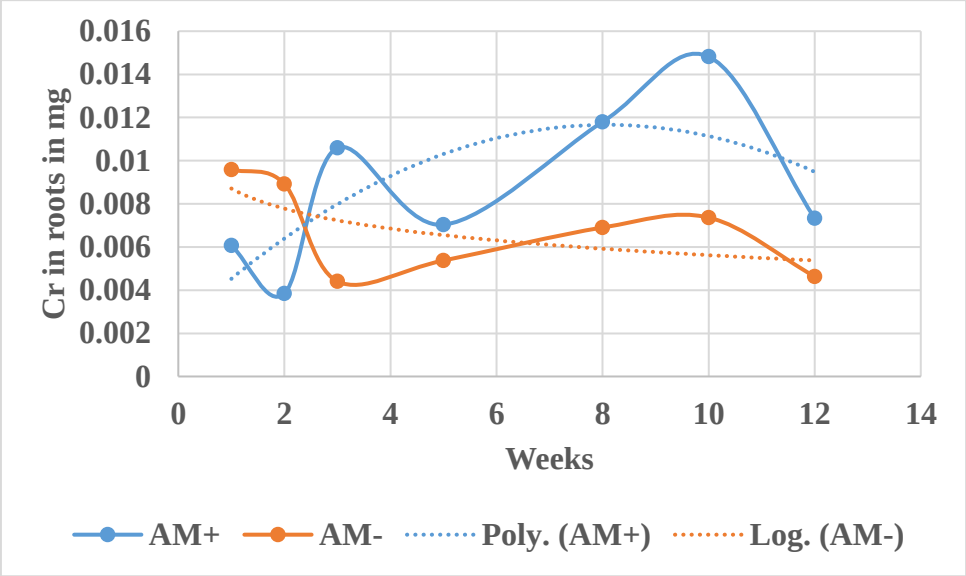


Figure 4.26. Amount of Cr in AM+ and AM- roots at each sampling week.

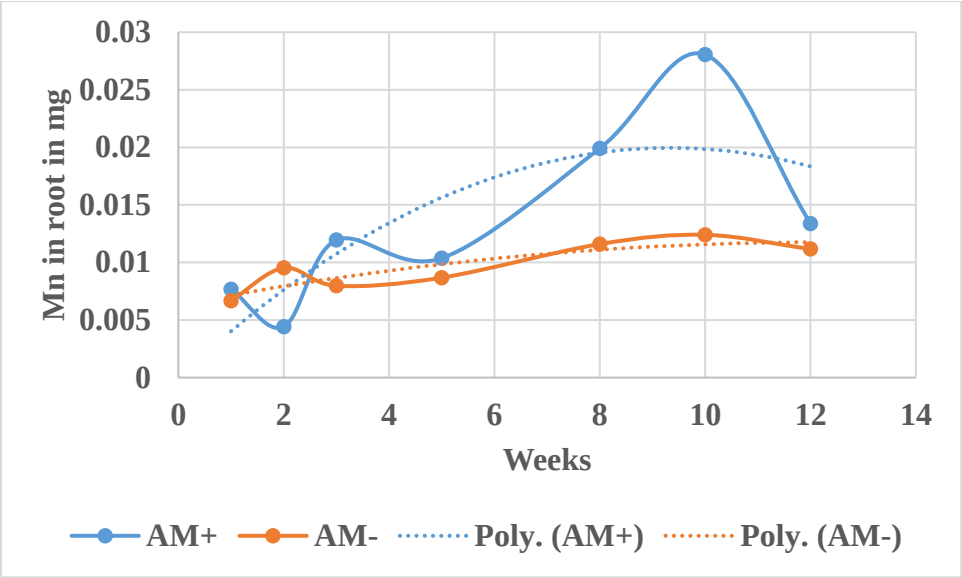


Figure 4.27. Amount of Mn in AM+ and AM- roots at each sampling week.

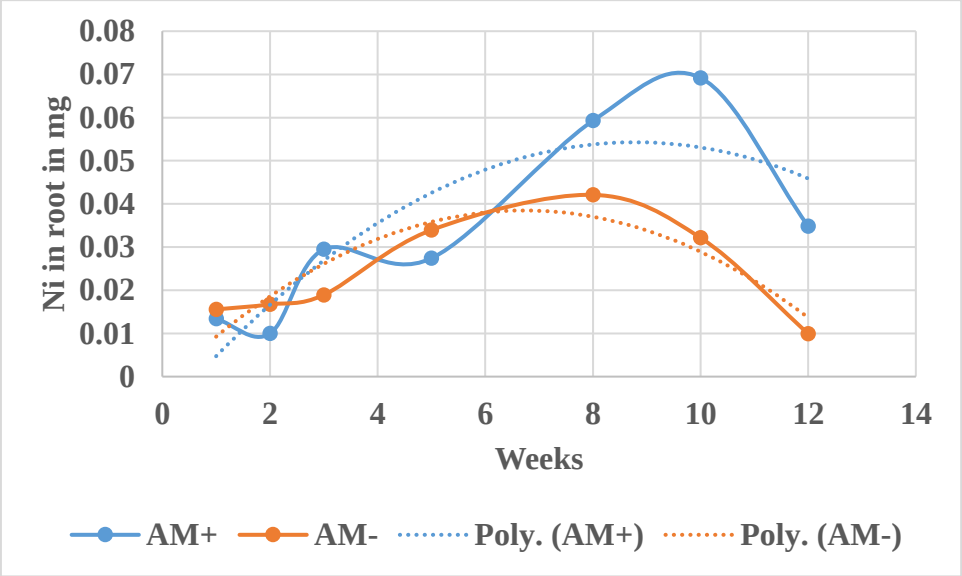


Figure 4.28. Amount of Ni in AM+ and AM- roots at each sampling week.

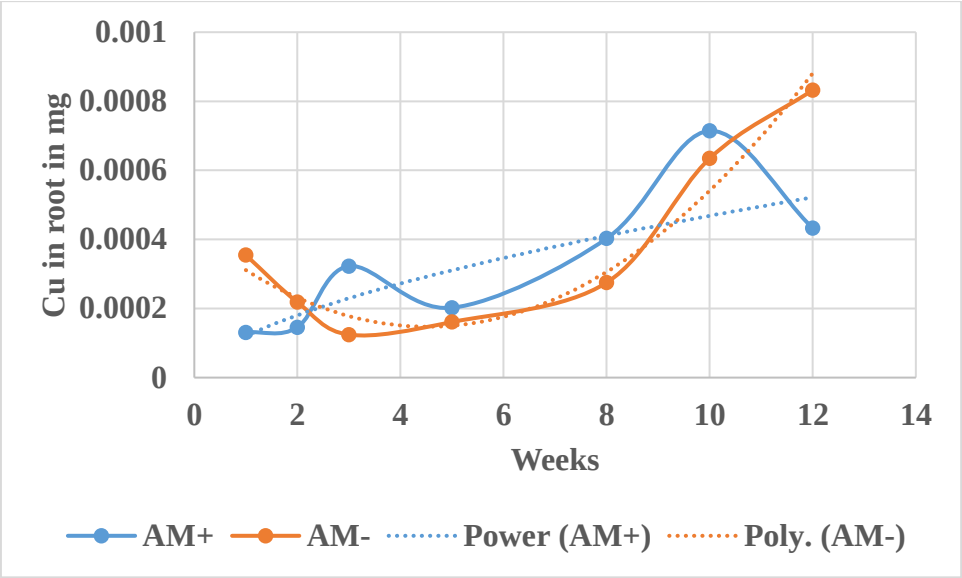


Figure 4.29. Amount of Cu in AM+ and AM- roots at each sampling week.

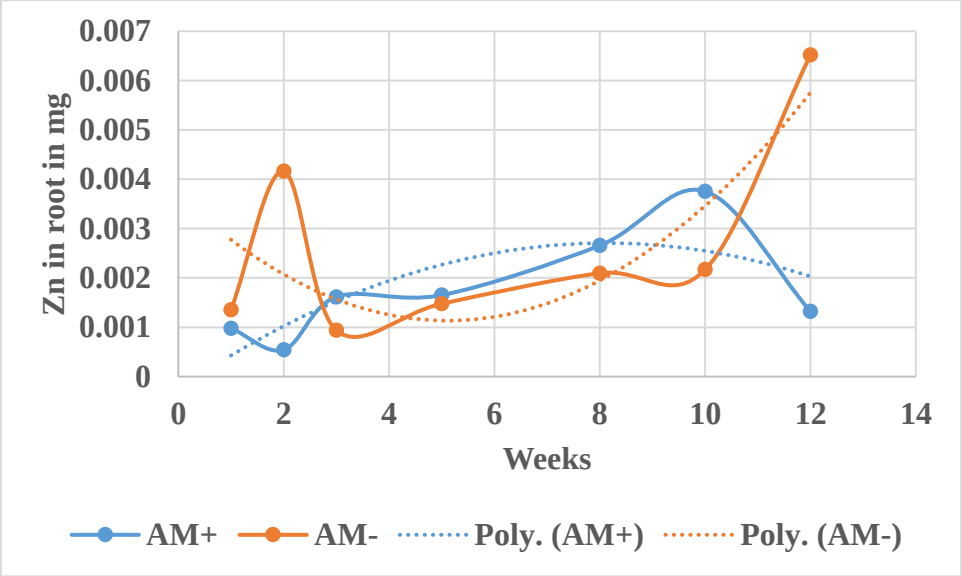


Figure 4.30. Amount of Zn in AM+ and AM- roots at each sampling week.

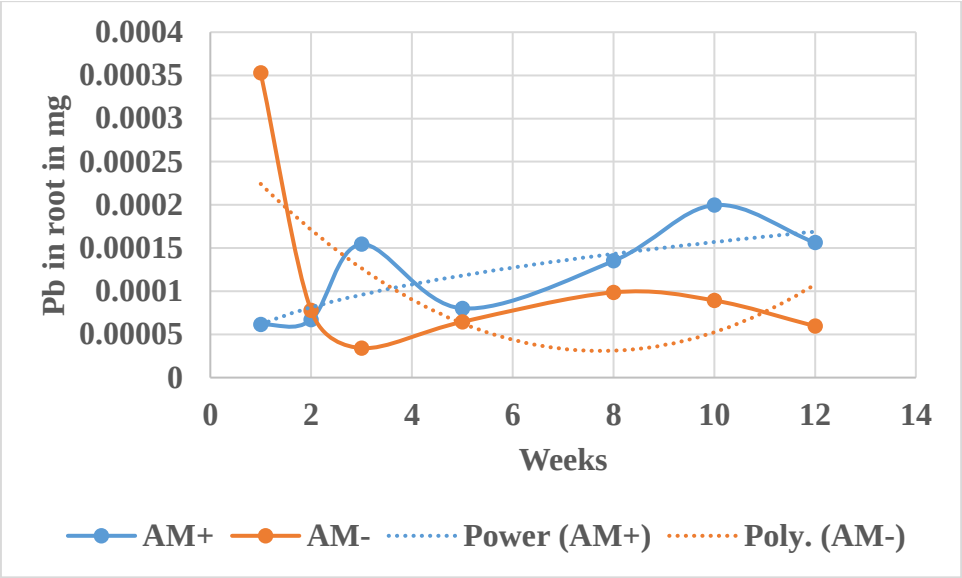


Figure 4.31. Amount of Pb in AM+ and AM- roots at each sampling week.

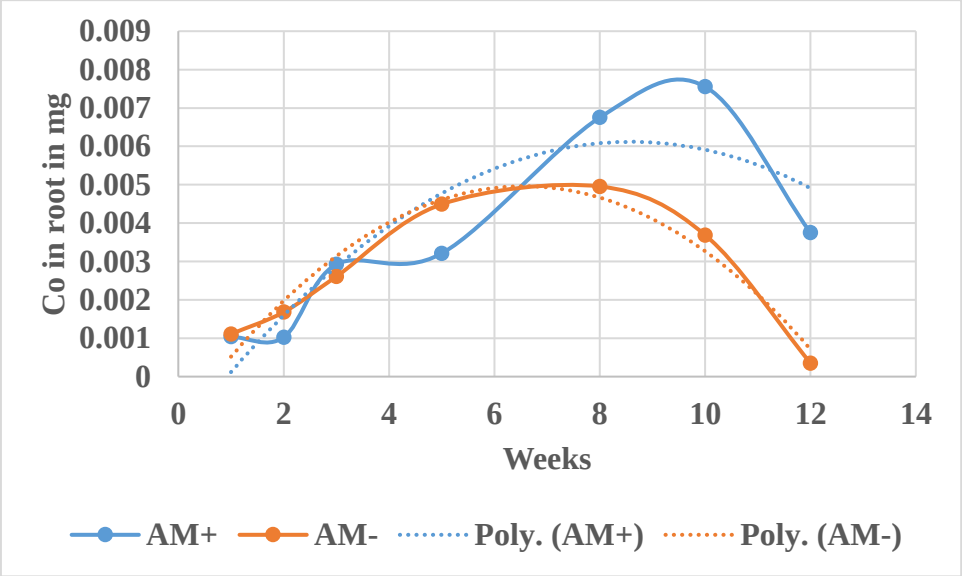


Figure 4.32. Amount of Co in AM+ and AM- roots at each sampling week.

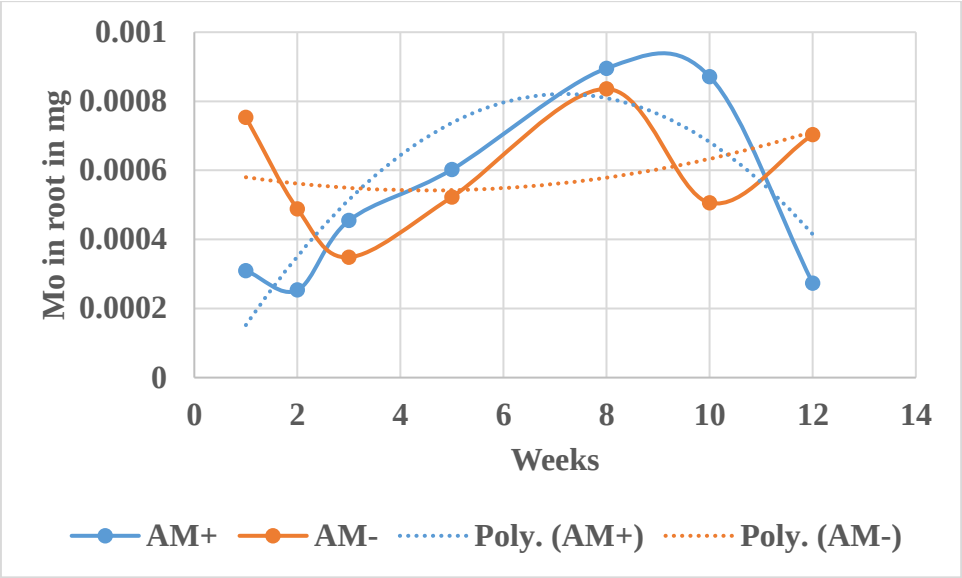


Figure 4.33. Amount of Mo in AM+ and AM- roots at each sampling week.

Two peaks of AM+ group are seen as common in all metal in root versus week graphs. One peak is seen at 3rd week as a sharp increase between 2nd and 3rd weeks. The other peak is seen at 10th week. The highest amount of metals exist in the roots are Fe and Al in descending order. Also, the Al and Fe graphs are too similar.

Fe, Al, Si, Cr, Mn, Ni, Cu, Zn, Pb, Co and Mo amounts in shoot versus week graphs are shown in Figure 4.34 to Figure 4.44.

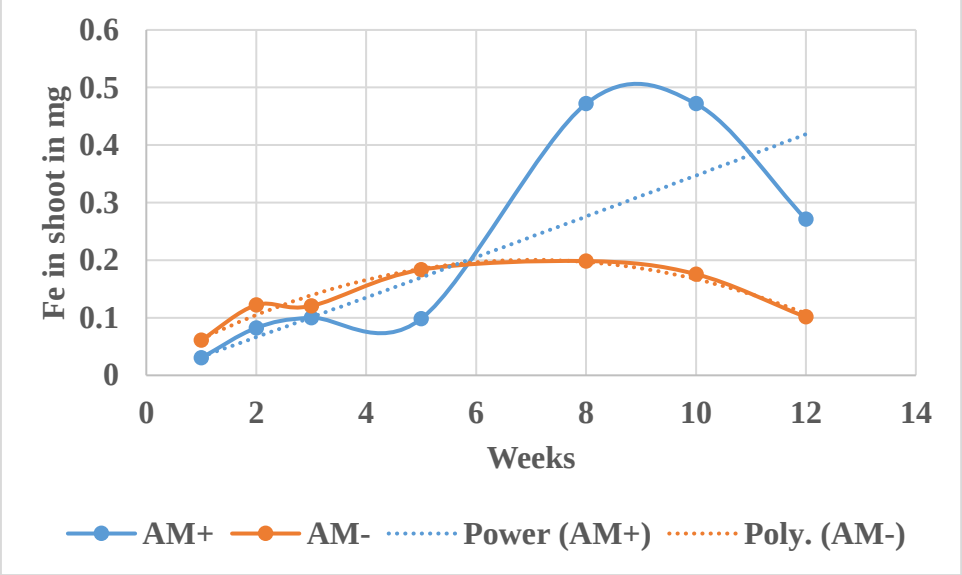


Figure 4.34. Amount of Fe in AM+ and AM- shoots at each sampling week.

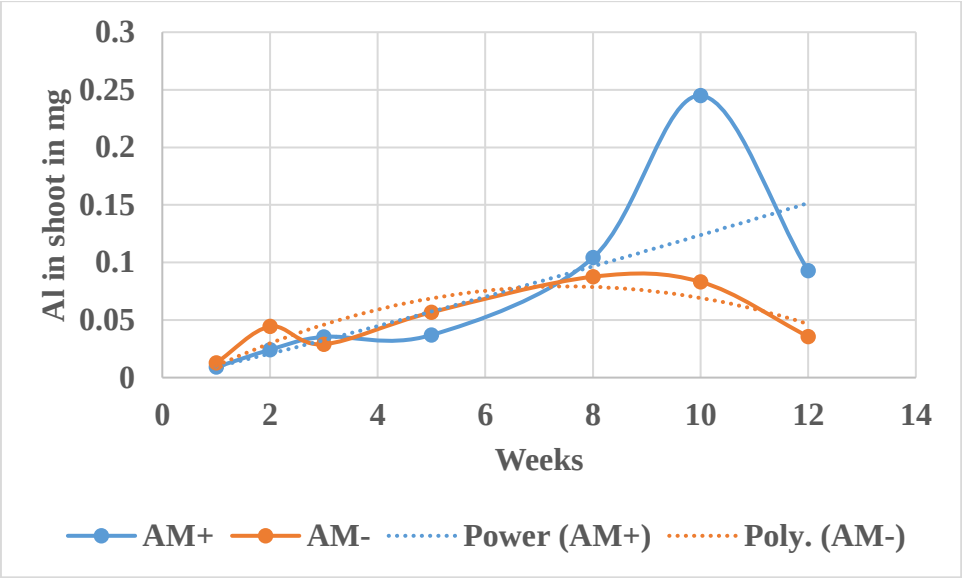


Figure 4.35. Amount of Al in AM+ and AM- shoots at each sampling week.

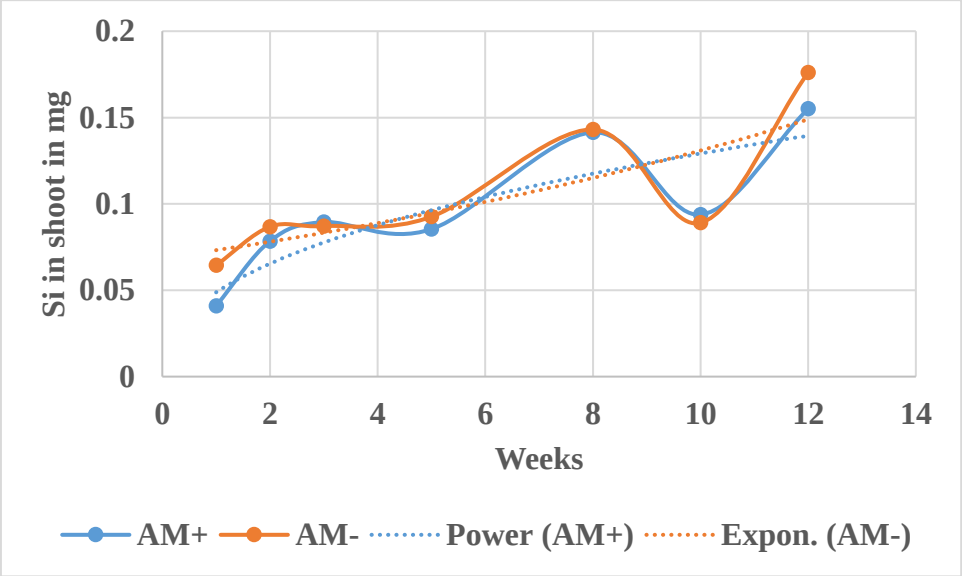


Figure 4.36. Amount of Si in AM+ and AM- shoots at each sampling week.

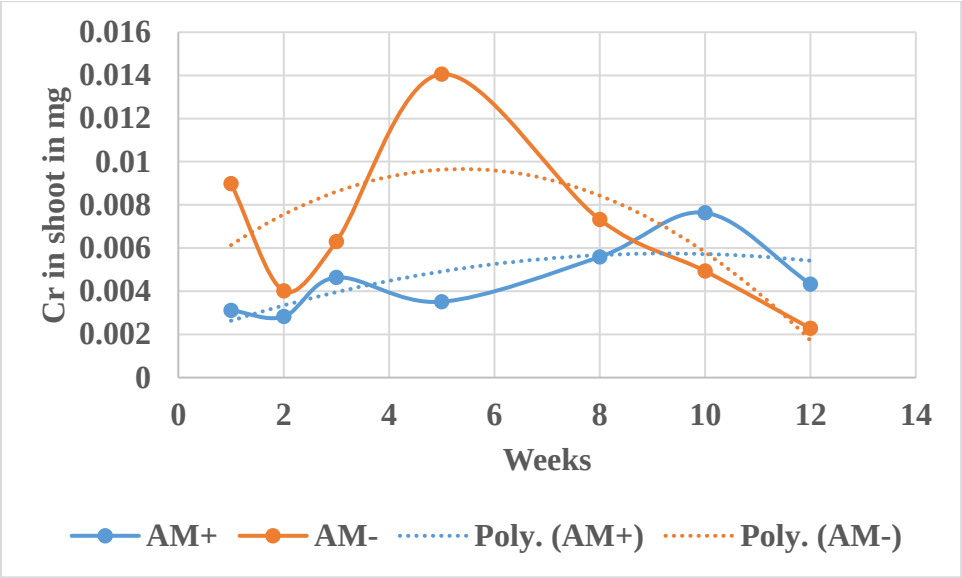


Figure 4.37. Amount of Cr in AM+ and AM- shoots at each sampling week.

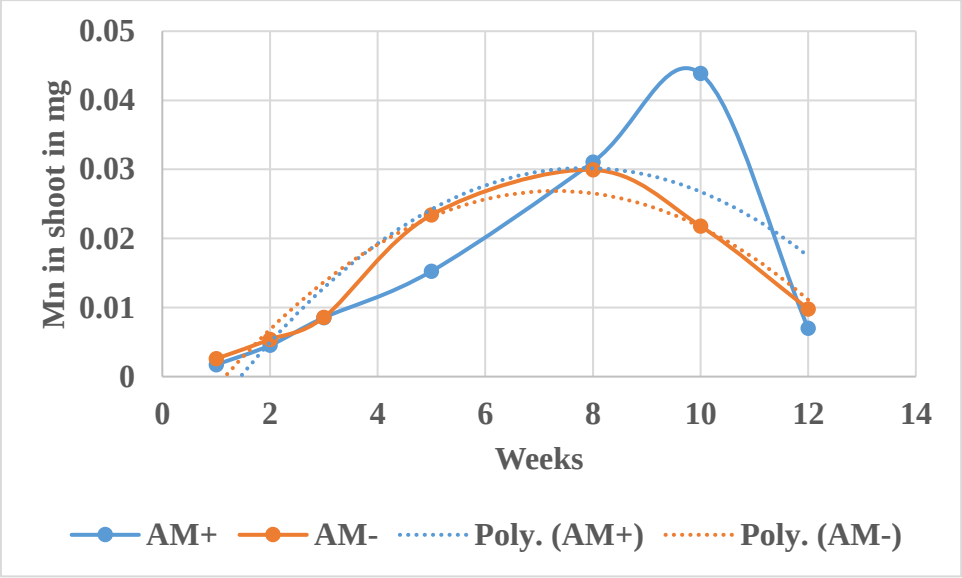


Figure 4.38. Amount of Mn in AM+ and AM- shoots at each sampling week.

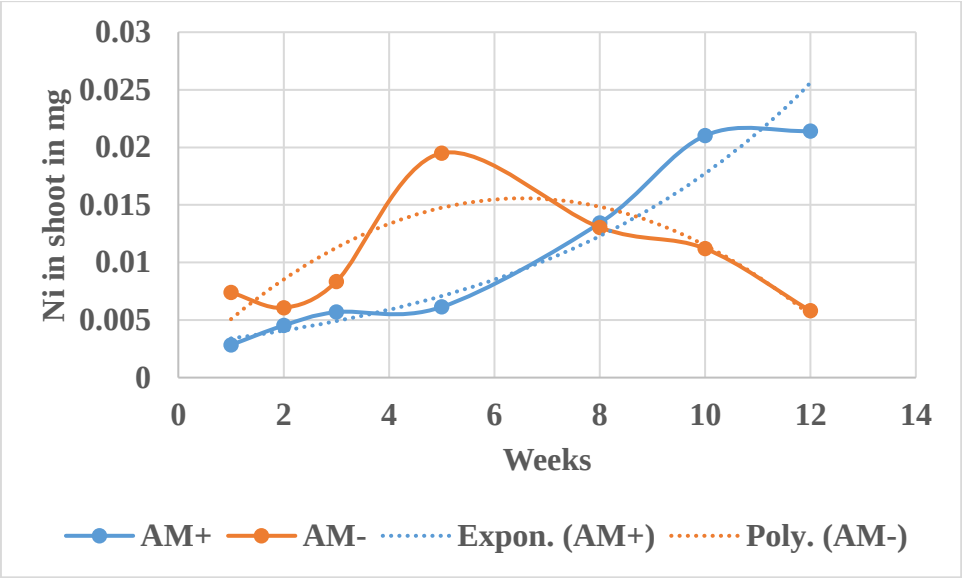


Figure 4.39. Amount of Ni in AM+ and AM- shoots at each sampling week.

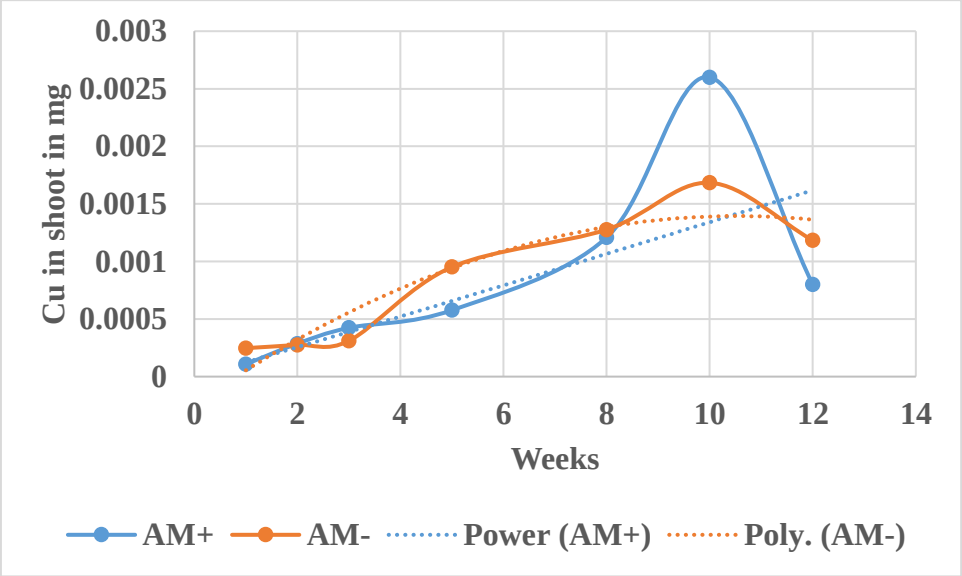


Figure 4.40. Amount of Cu in AM+ and AM- shoots at each sampling week.

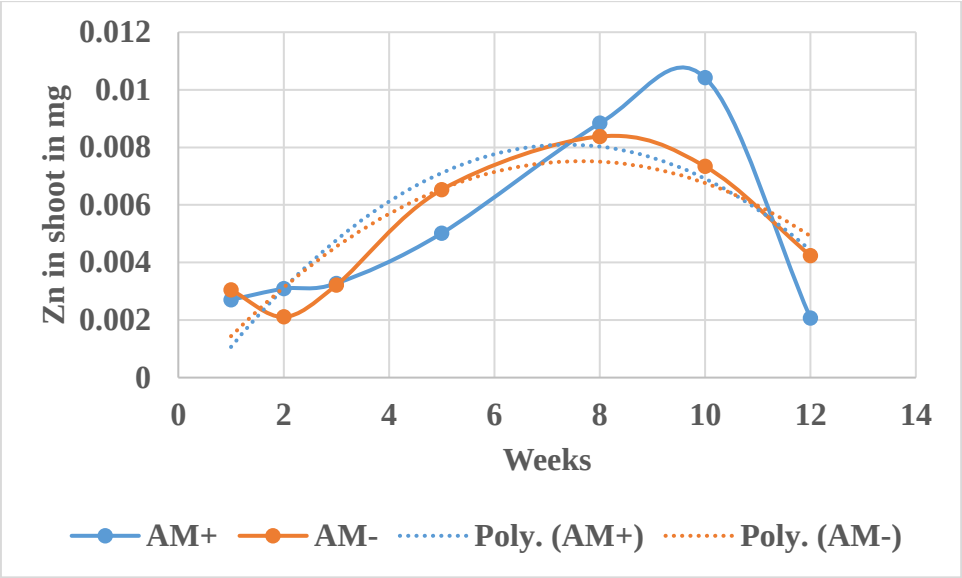


Figure 4.41. Amount of Zn in AM+ and AM- shoots at each sampling week.

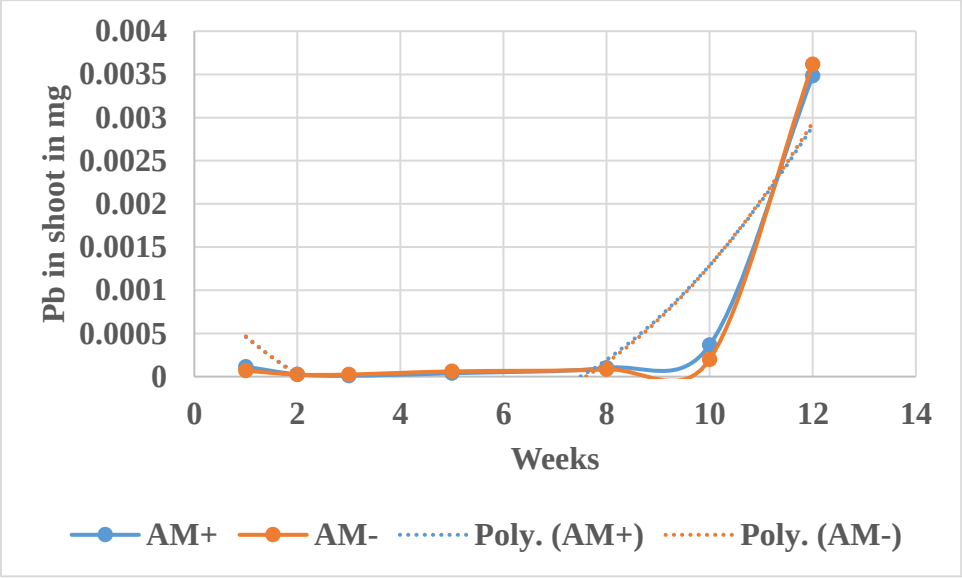


Figure 4.42. Amount of Pb in AM+ and AM- shoots at each sampling week.

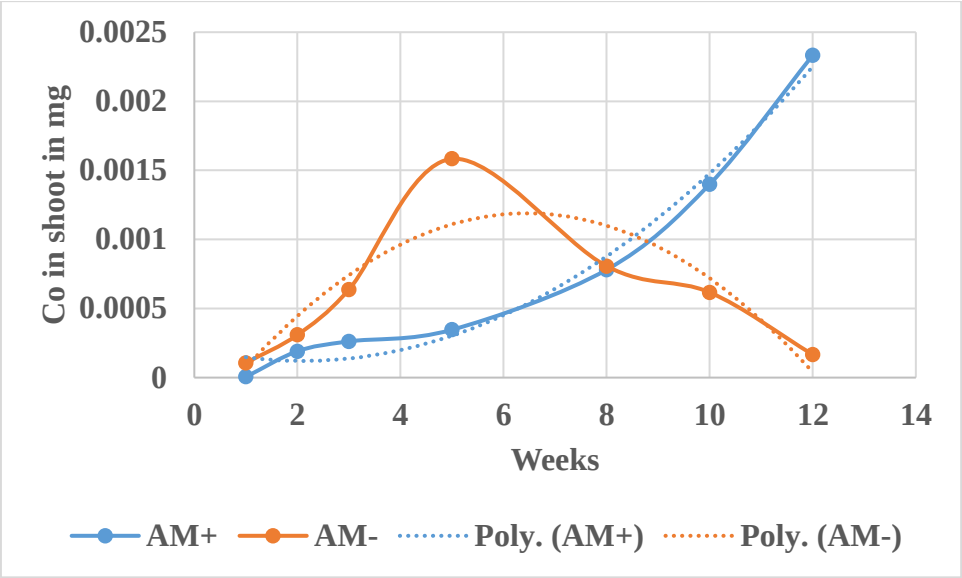


Figure 4.43. Amount of Co in AM+ and AM- shoots at each sampling week.

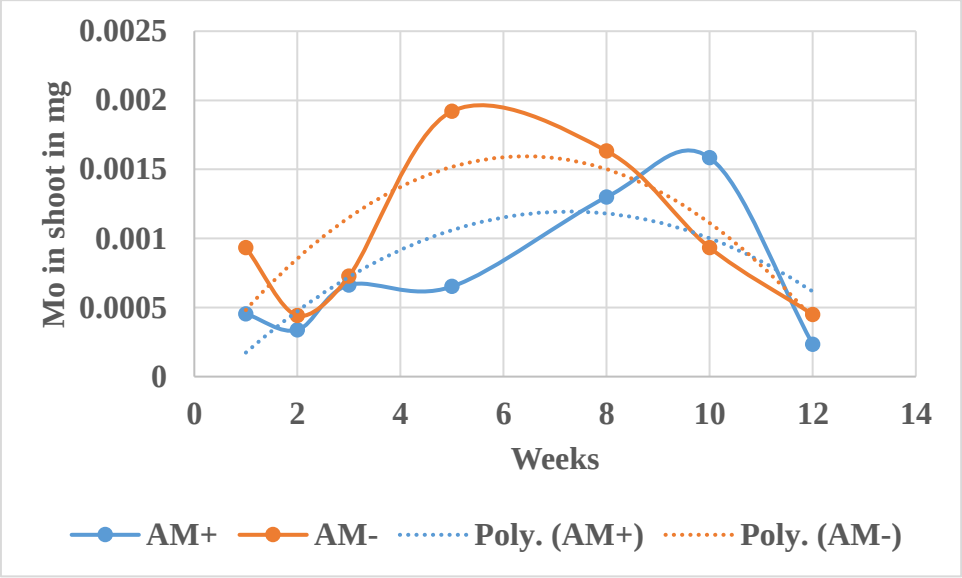


Figure 4.44. Amount of Mo in AM+ and AM- shoots at each sampling week.

The highest amount of metals exist in the shoots are found for Fe and Al in descending order. An increase in Fe and Al amounts in AM+ shoots are seen as from the 8th week. Also, Al and Fe graphs are too similar.

Fe amount in root versus week and amount of Fe per kg root versus week graphs are shown as bar plots in Figure 4.45 and Figure 4.46 respectively. Tracking the iron content in root week by week gives information about the absorbed iron from the soil.

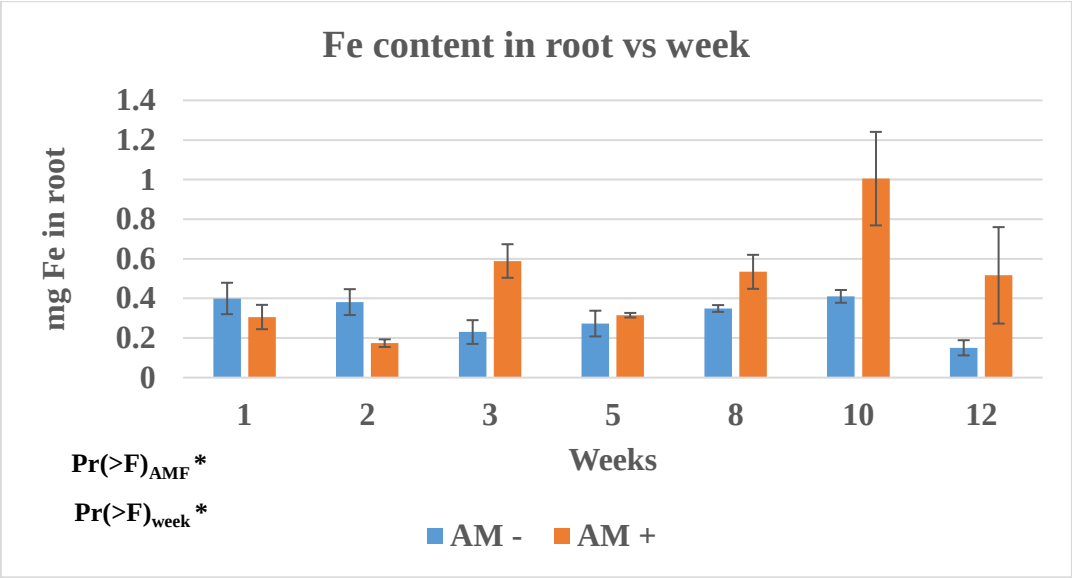


Figure 4.45. Amounts of Fe in AM+ and AM- roots at each sampling week are indicated as columns with ±SE. Two-way ANOVA was used to test the effect of AMF and week on Fe content in roots. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

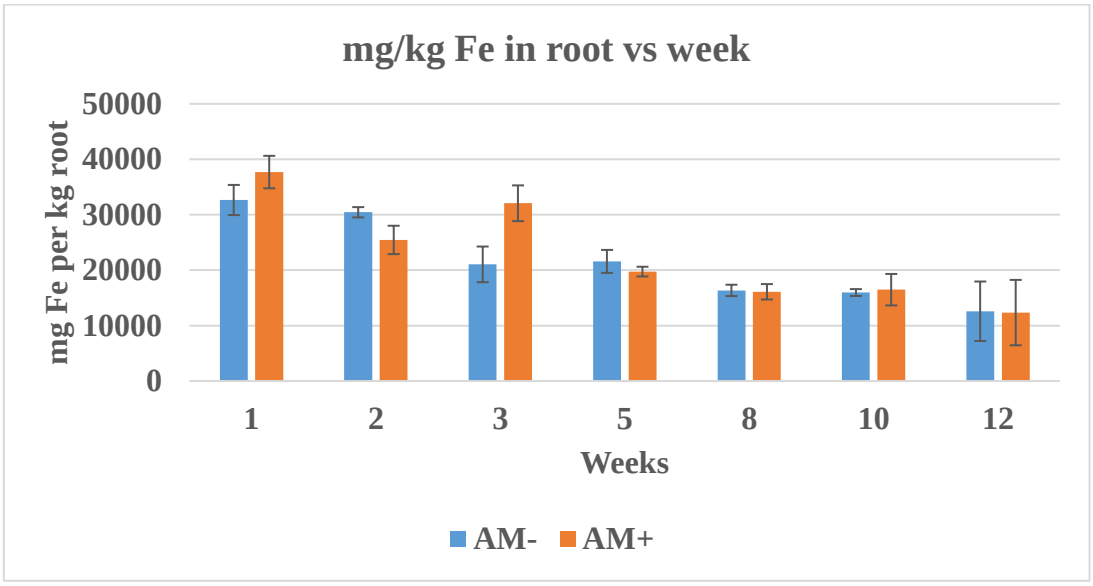


Figure 4.46. Amounts of Fe per kg AM+ and AM- roots at each sampling week are indicated as columns with ±SE.

As seen in Figure 4.45, both AMF and time had statistically significant effects on Fe content in root. Besides the two-way ANOVA test, multifactorial ANOVA was also performed to observe the effect of AMF, time and root weight on Fe content in roots. Root weight factor can be considered as a part of AMF effect or time effect as well since root weight increases with time and presence of mycorrhiza. Result of this multifactorial ANOVA analysis showed that only the root weight had a significant effect on Fe content in roots. This does not mean that AMF and/or time had no effect,

instead, root weight was the dominant factor on Fe amount in root. However, the changes in root weight might still depend on time and/or AMF as well.

It is clearly understood that Fe content in AM+ roots were higher than the Fe content in AM- roots from the 3rd week, however, this is not the case for mg/kg Fe in root.

Al amount in root versus week and amount of Al per kg root versus week graphs are shown as bar plots in Figure 4.47 and Figure 4.48 respectively. Tracking the aluminium content in root week by week gives information about the absorbed aluminium from the soil.

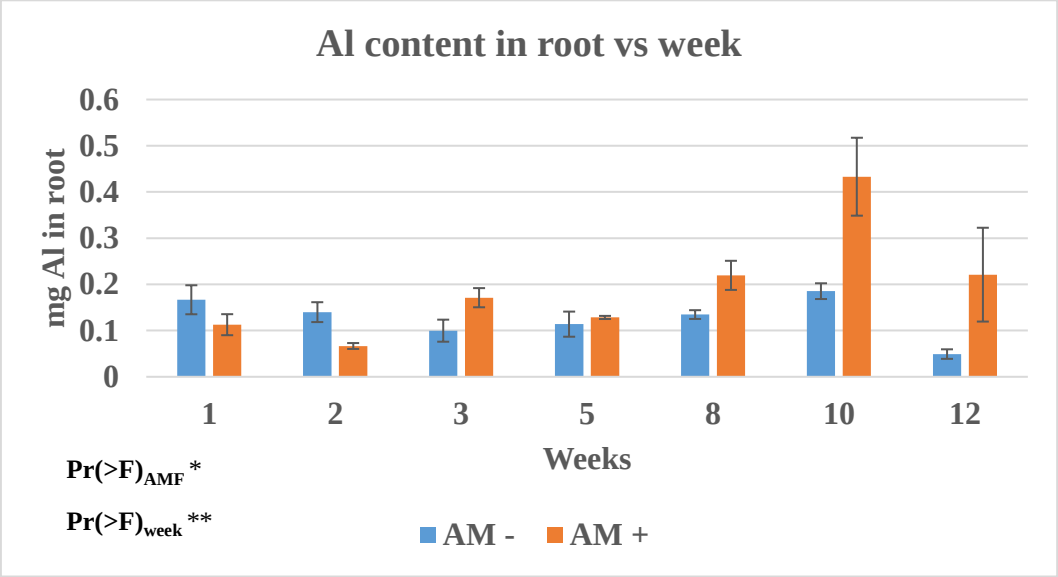


Figure 4.47. Amounts of Al in AM+ and AM- roots at each sampling week are indicated as columns with ±SE. Two-way ANOVA was used to test the effect of AMF and week on Al content in roots. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

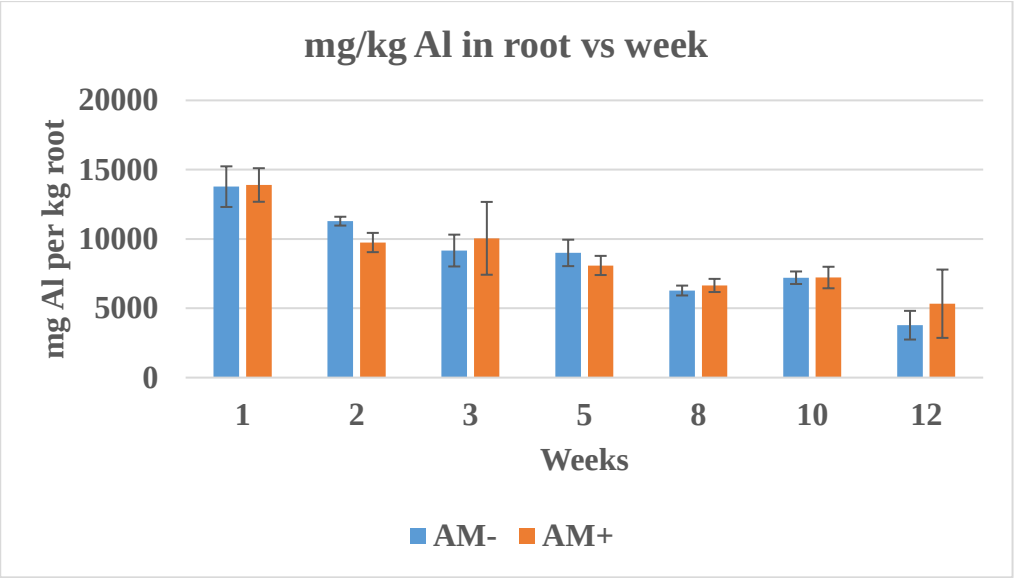


Figure 4.48. Amounts of Al per kg AM+ and AM- roots at each sampling week are indicated as columns with ±SE.

As seen in Figure 4.47, both AMF and time showed statistically significant effects on Al content in root. The significance level of time factor was more than the significance level of AMF factor. Besides the two-way ANOVA test, multifactorial ANOVA was also performed to observe the effect of AMF, time and root weight on Al content in roots. Root weight factor can be considered as a part of AMF effect or time effect as well since root weight increases with time and presence of mycorrhiza. Result of this multifactorial ANOVA analysis indicated that only the root weight had a significant effect on Al content in roots. This does not mean that AMF and/or time had no effect, instead, root weight was the dominant factor on Al amount in root. However, the changes in root weight might still depend on time and/or AMF as well.

As in the Fe graph in Fgure 4.41, Al content in AM+ roots were higher than the Al content in AM- roots from the 3rd week, however, this is not the case for mg/kg Al in root.

Fe amount in shoot versus week and amount of Fe per kg shoot versus week graphs are shown as bar plots in Figure 4.49 and Figure 4.50 respectively. Tracking the iron content in shoots gives information about the translocation of absorbed iron from the soil.

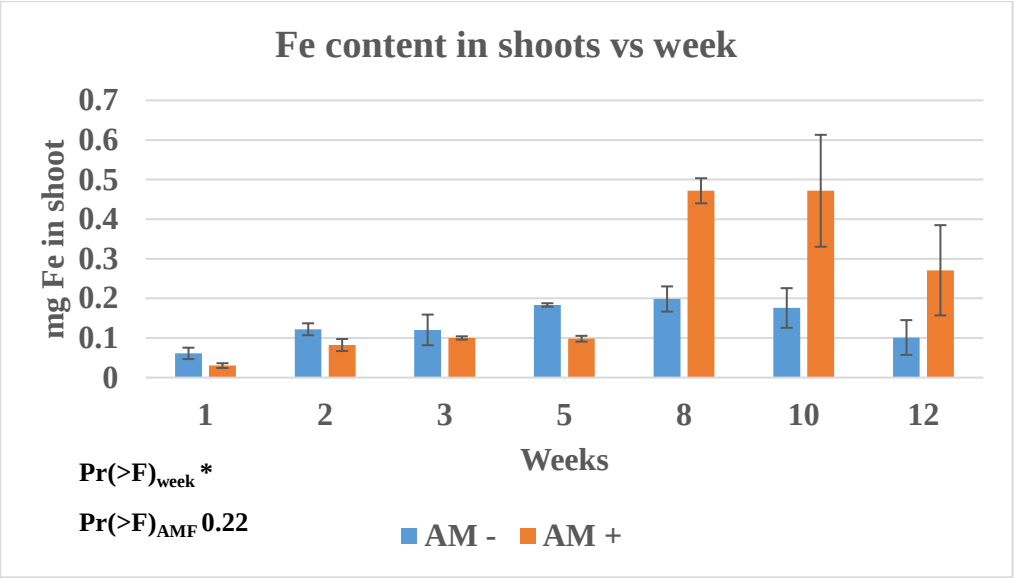


Figure 4.49. Amounts of Fe in AM+ and AM- shoots at each sampling week are indicated as columns with ±SE. Two-way ANOVA was used to test the effect of AMF and week on Fe content in shoots. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

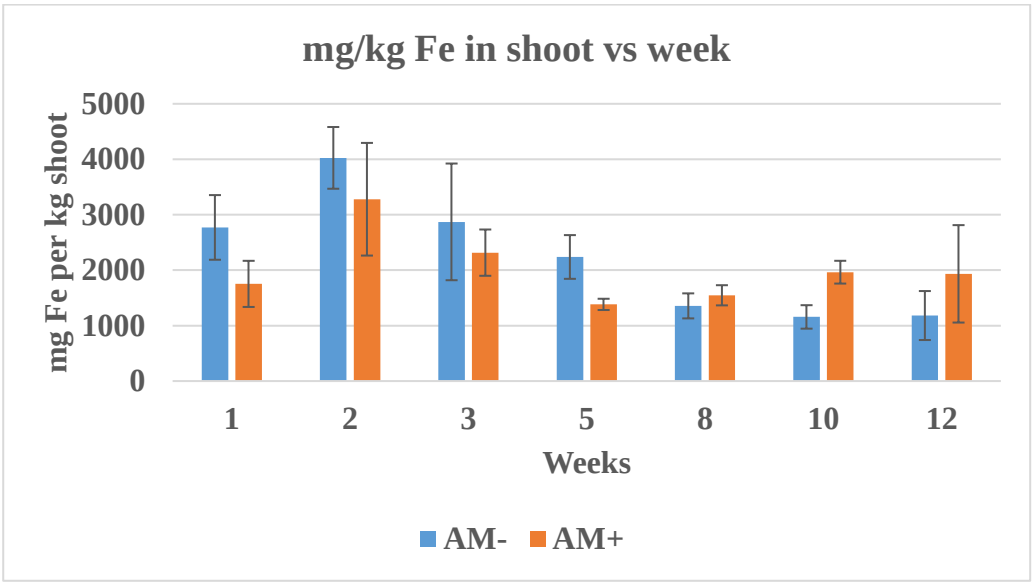


Figure 4.50. Amounts of Fe per kg AM+ and AM- shoots at each sampling week are indicated as columns with ±SE.

Two-way ANOVA results indicated that only the time had statistically significant effect on Fe content in shoot. Particularly, an increase in iron in shoots is seen from the 8th week and this increase is seen mostly in AM+ roots. On the other hand, mg Fe per kg shoot graph is too different from the Fe content in shoot graph which indicates the variation in shoot weights depending on the group, AM+ or AM-, and the time.

Al amount in shoot versus week and amount of Al per kg shoot versus week graphs are shown as bar plots in Figure 4.51 and Figure 4.52 respectively. Tracking the aluminium content in shoots gives information about the translocation of absorbed aluminium from the soil.

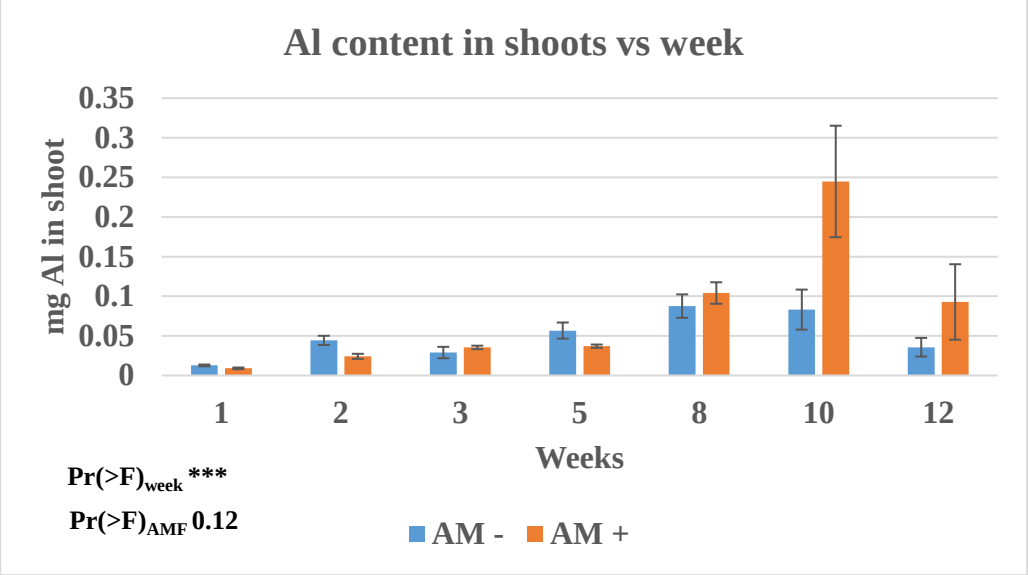


Figure 4.51. Amounts of Al in AM+ and AM- shoots at each sampling week are indicated as columns with ±SE. Two-way ANOVA was used to test the effect of AMF and week on Al content in shoots. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

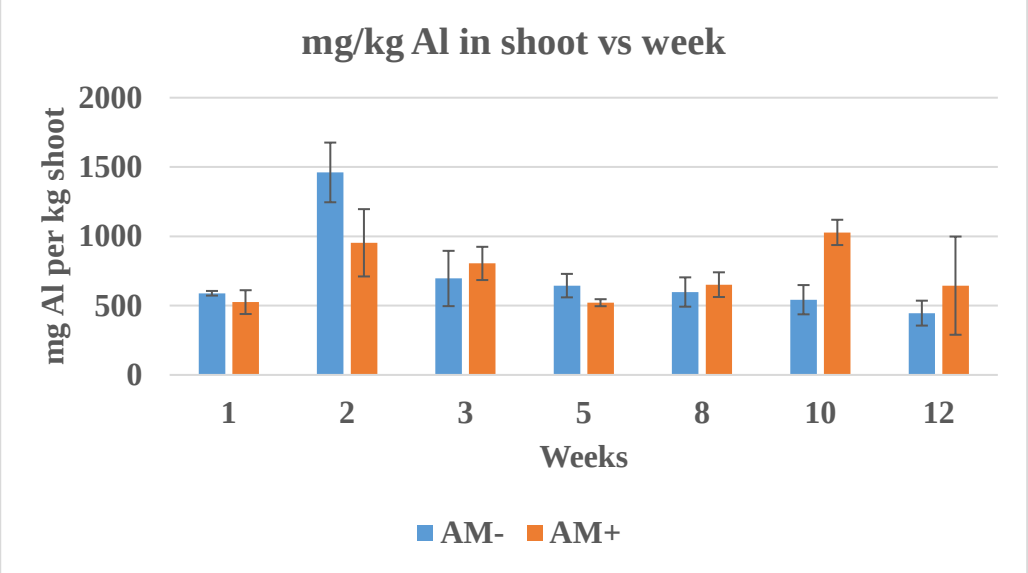


Figure 4.52. Amounts of Al per kg AM+ and AM- shoots at each sampling week are indicated as columns with ±SE.

Results of Al in shoots are very similar with the results for Fe as in the Al and Fe in roots results. Two-way ANOVA test indicated that only the time had statistically significant effect on Fe content in shoot. Particularly, an increase in aluminium in shoots is seen from the 8th week and this increase

is seen mostly in AM+ shoots. On the other hand, mg Al per kg shoot graph is too different from the Al content in shoot graph which indicates the variation in shoot weights depending on the group, AM+ or AM-, and the time.

Fe, Al, Si, Cr, Mn, Ni, Cu, Zn, Pb, Co and Mo amounts in soil versus week graphs are shown in Figure 4.53 to Figure 4.63.

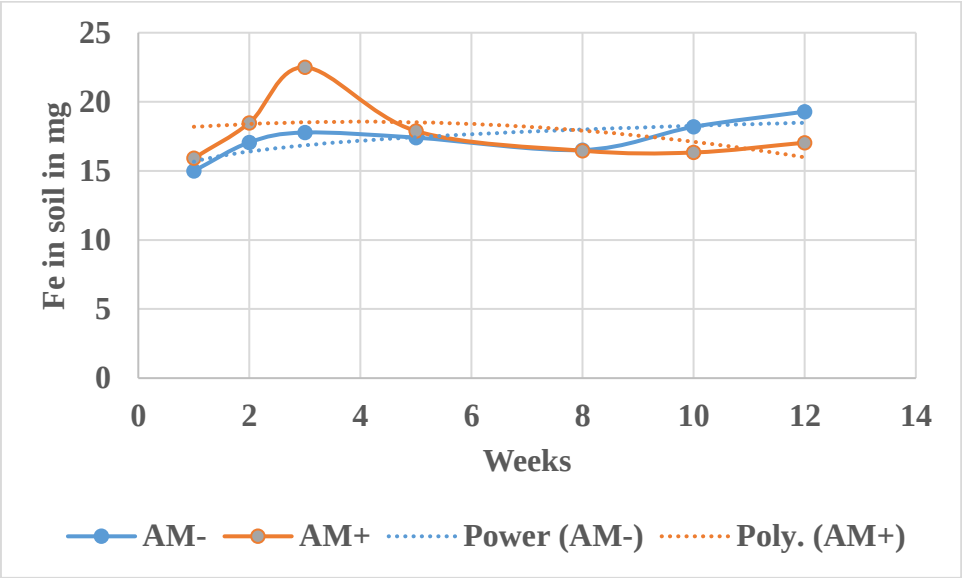


Figure 4.53. Amount of Fe in AM+ and AM- soil samples at each sampling week.

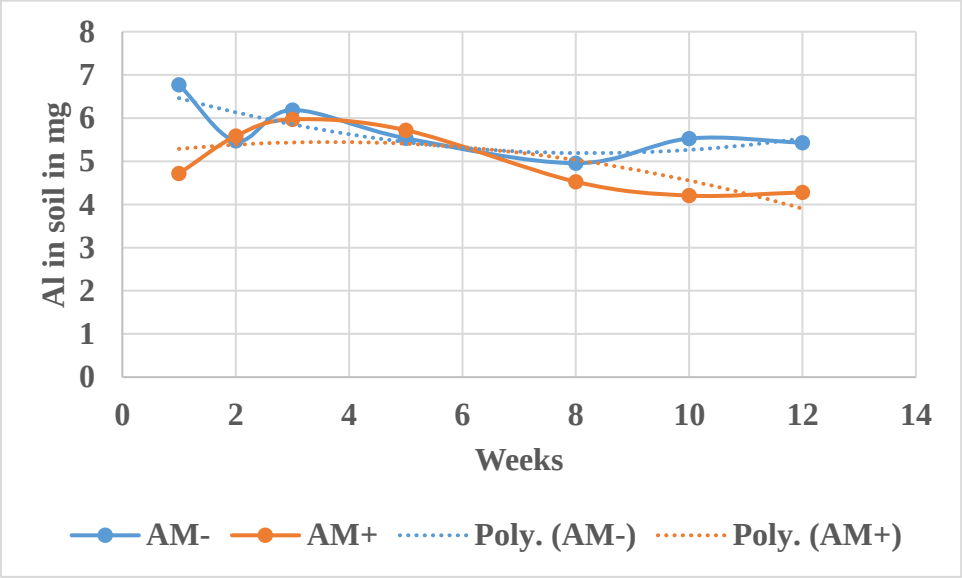


Figure 4.54. Amount of Al in AM+ and AM- soil samples at each sampling week.

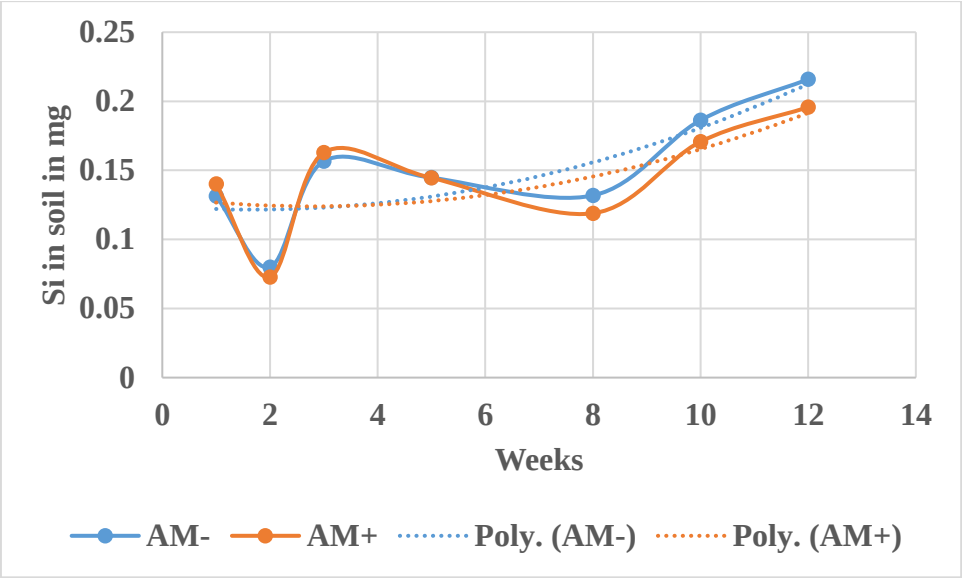


Figure 4.55. Amount of Si in AM+ and AM- soil samples at each sampling week.

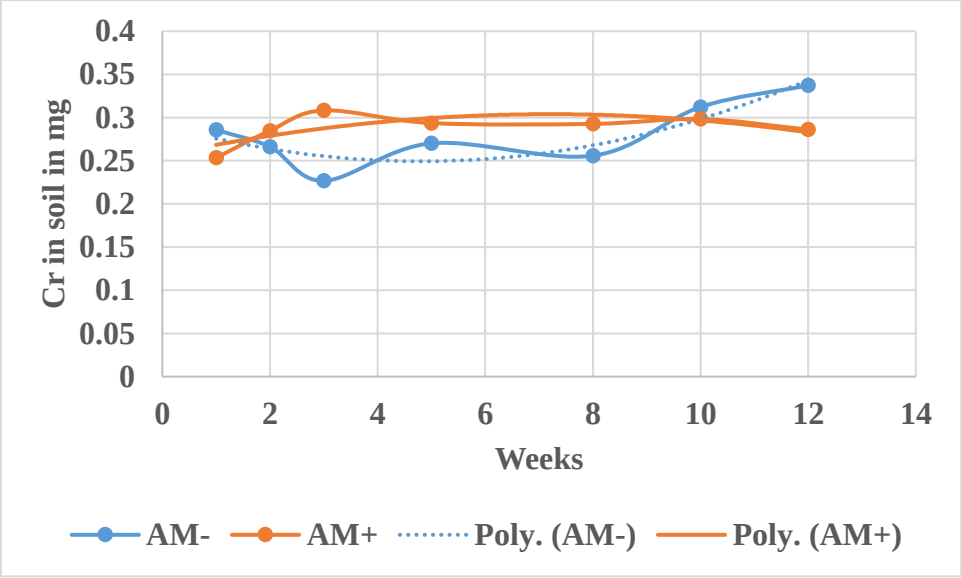


Figure 4.56. Amount of Cr in AM+ and AM- soil samples at each sampling week.

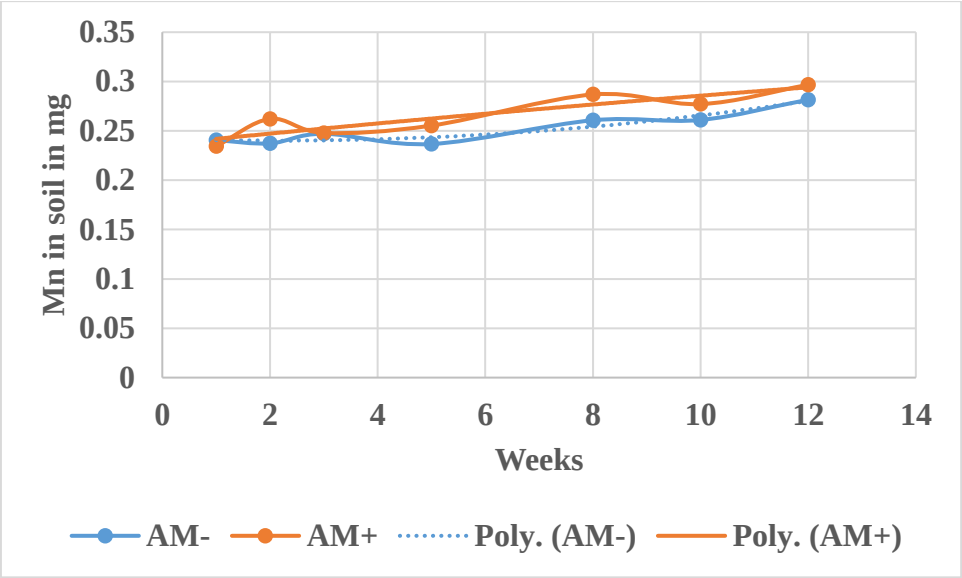


Figure 4.57. Amount of Mn in AM+ and AM- soil samples at each sampling week.

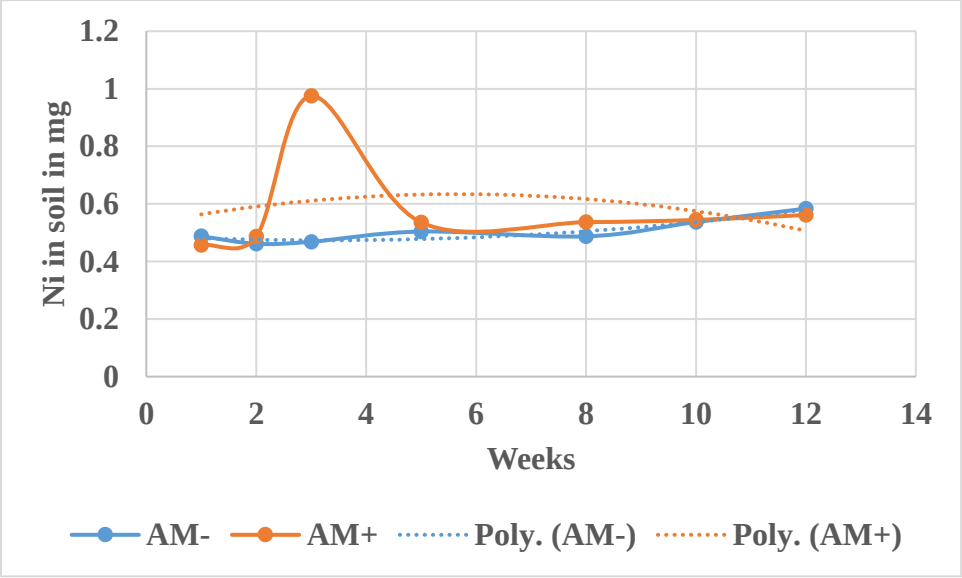


Figure 4.58. Amount of Ni in AM+ and AM- soil samples at each sampling week.

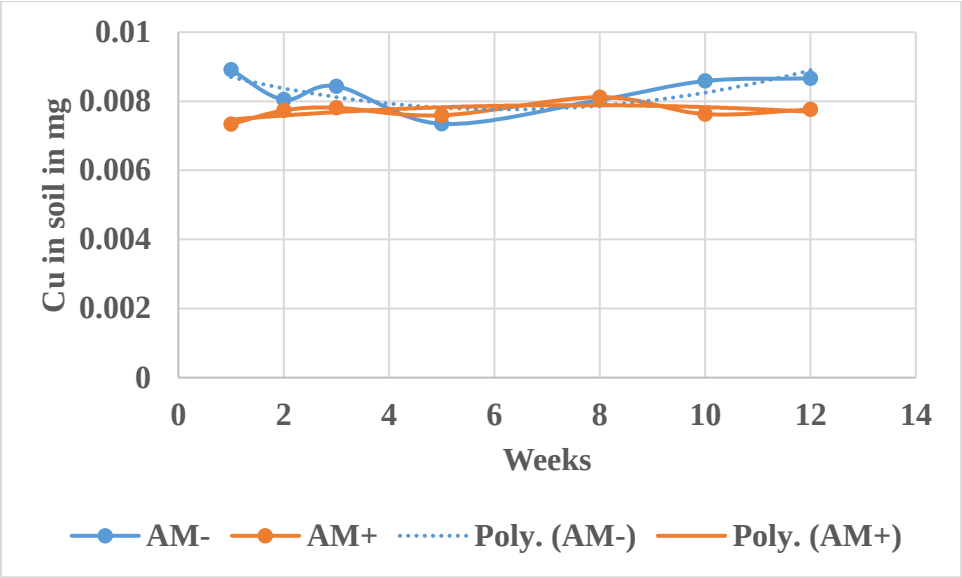


Figure 4.59. Amount of Cu in AM+ and AM- soil samples at each sampling week.

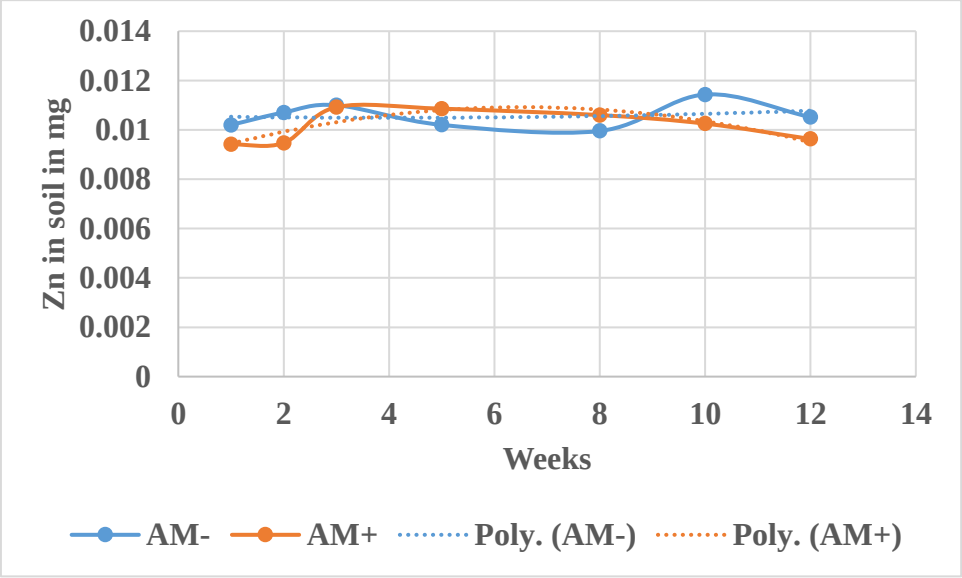


Figure 4.60. Amount of Zn in AM+ and AM- soil samples at each sampling week.

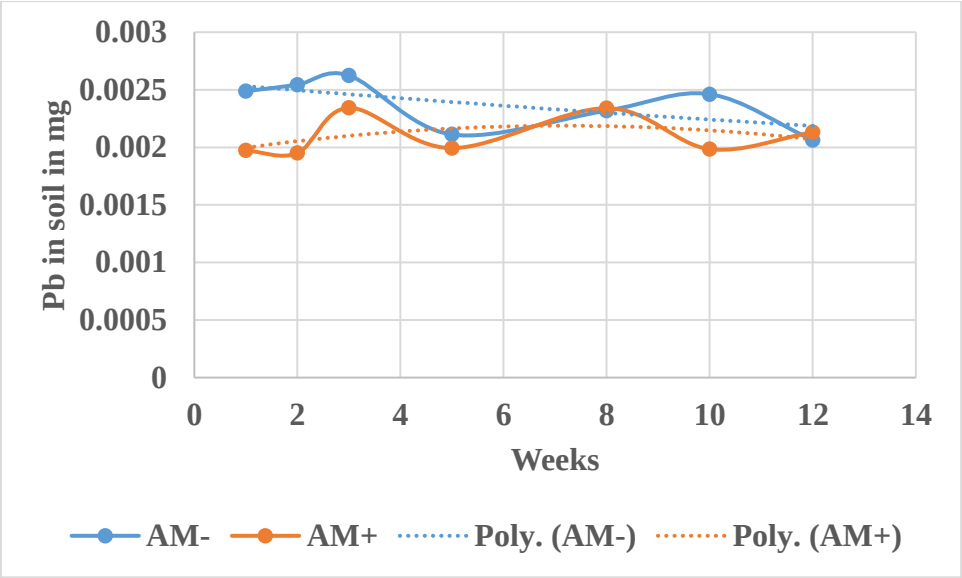


Figure 4.61. Amount of Pb in AM+ and AM- soil samples at each sampling week.

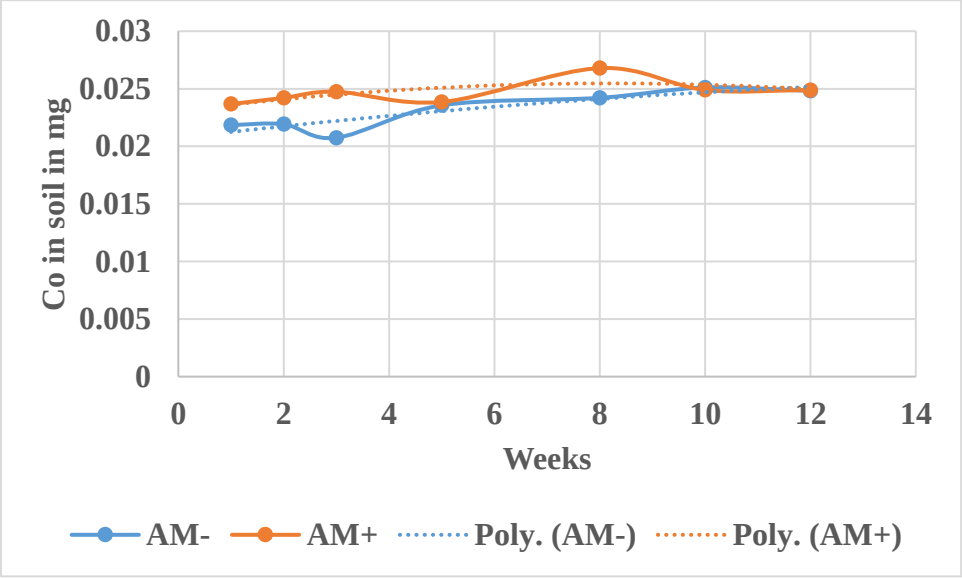


Figure 4.62. Amount of Co in AM+ and AM- soil samples at each sampling week.

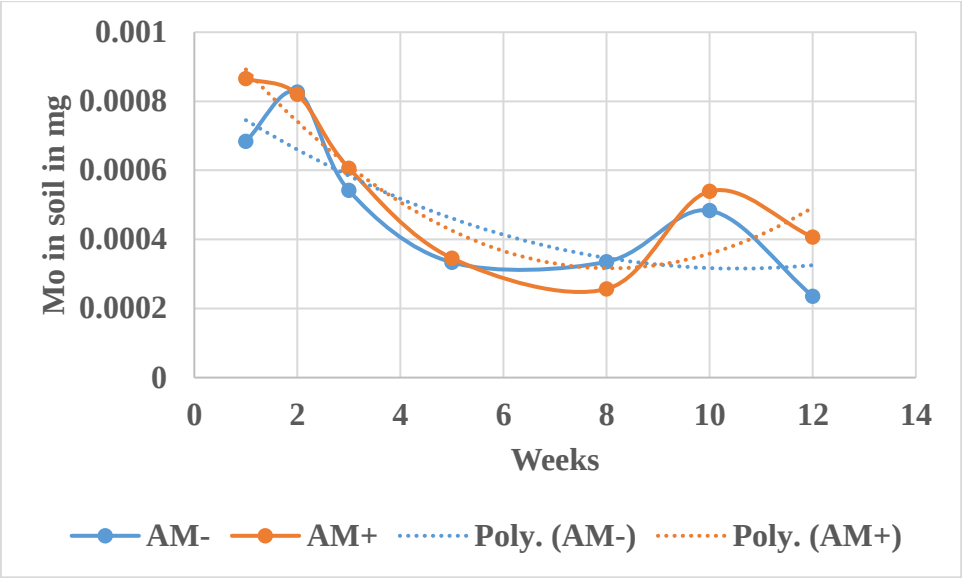


Figure 4.63. Amount of Mo in AM+ and AM- soil samples at each sampling week.

In AM+ soil samples a peak at 3rd week is seen in nearly all metal in soil versus week graphs whereas this is not observed in AM- group. It is interesting that AM+ roots had also a peak at 3rd week in all metal in root versus week graphs.

Since the amount of taken soil was equal for each soil sample in metal analyses, mg metal per kg soil graphs were the same as the amount (in mg) of metal in soil sample graphs in terms of the trends of the curves.

Amount of Fe per kg soil samples versus week and amount of Al per kg soil samples versus week graphs are shown in Figure 4.64 and Figure 4.65 respectively.

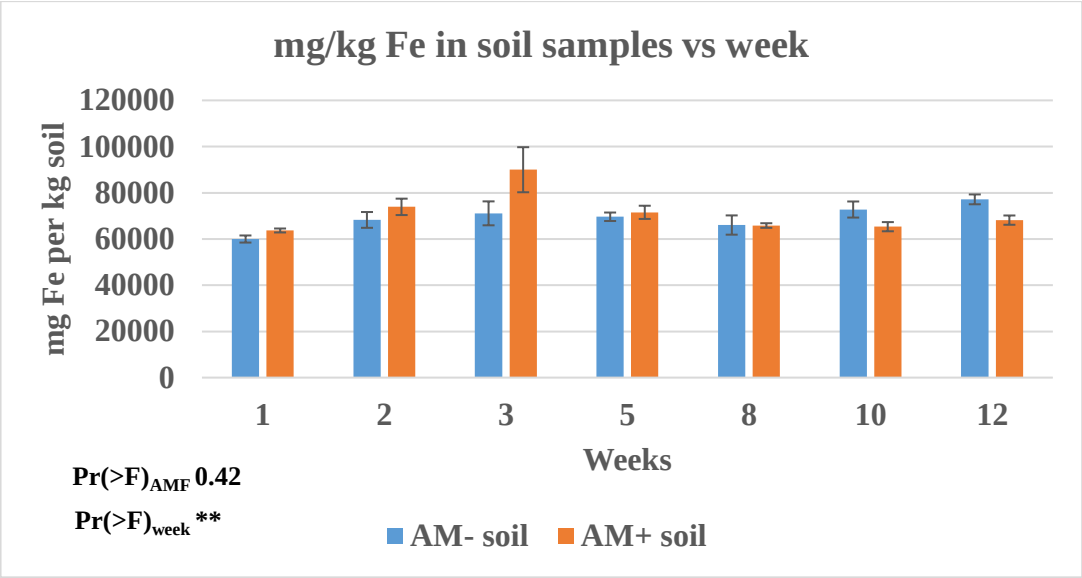


Figure 4.64. Amounts of Fe per kg AM+ and AM- soil samples at each sampling week are indicated as columns with ±SE. Two-way ANOVA was used to test the effect of AMF and week on Fe content in soil samples. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

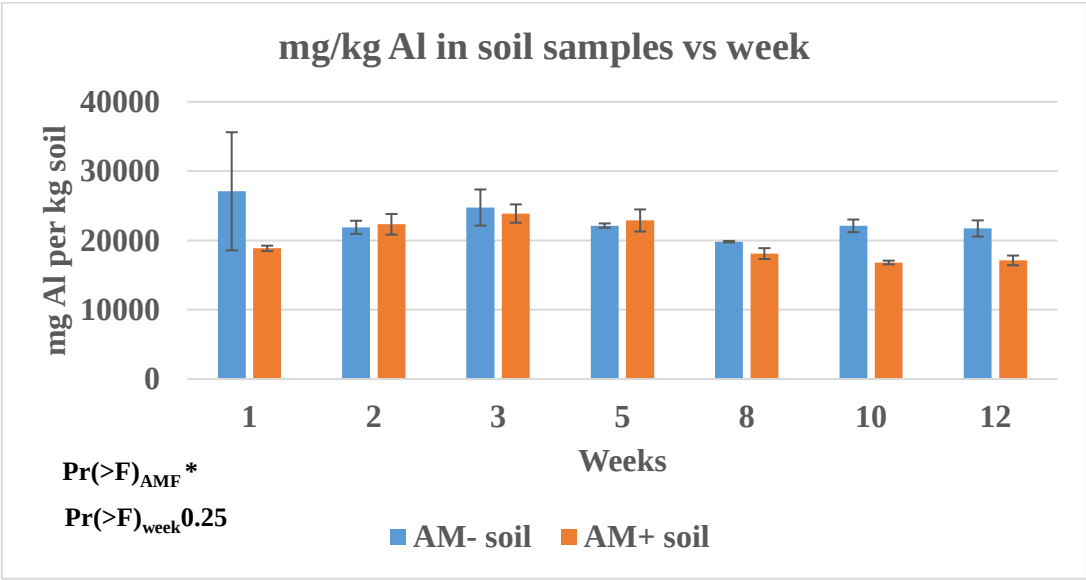


Figure 4.65. Amounts of Al per kg AM+ and AM- soil samples at each sampling week are indicated as columns with ±SE. Two-way ANOVA was used to test the effect of AMF and week on Al content in soil samples. Significance codes: *** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05.

According to the statistical analyses, only the time had statistically significant effect on iron in soil whereas only the AMF had statistically significant effect on aluminium in soil. Also, the iron and aluminium amounts per kg AM+ and AM- soil samples were too close to each generally.

4.8. Gene Expression Analyses

Before the gene expresion analysis, quality of RNA and cDNA samples and the integrity of cDNA were checked.

Some of the RNA and cDNA samples were run on agorose gel to check the quality. 28S and 18S eukaryotic rRNA bands were seen in RNA samples and no band was seen in cDNA samples as expected. The gel images are shown in Figure 4.66 and Figure 4.67.

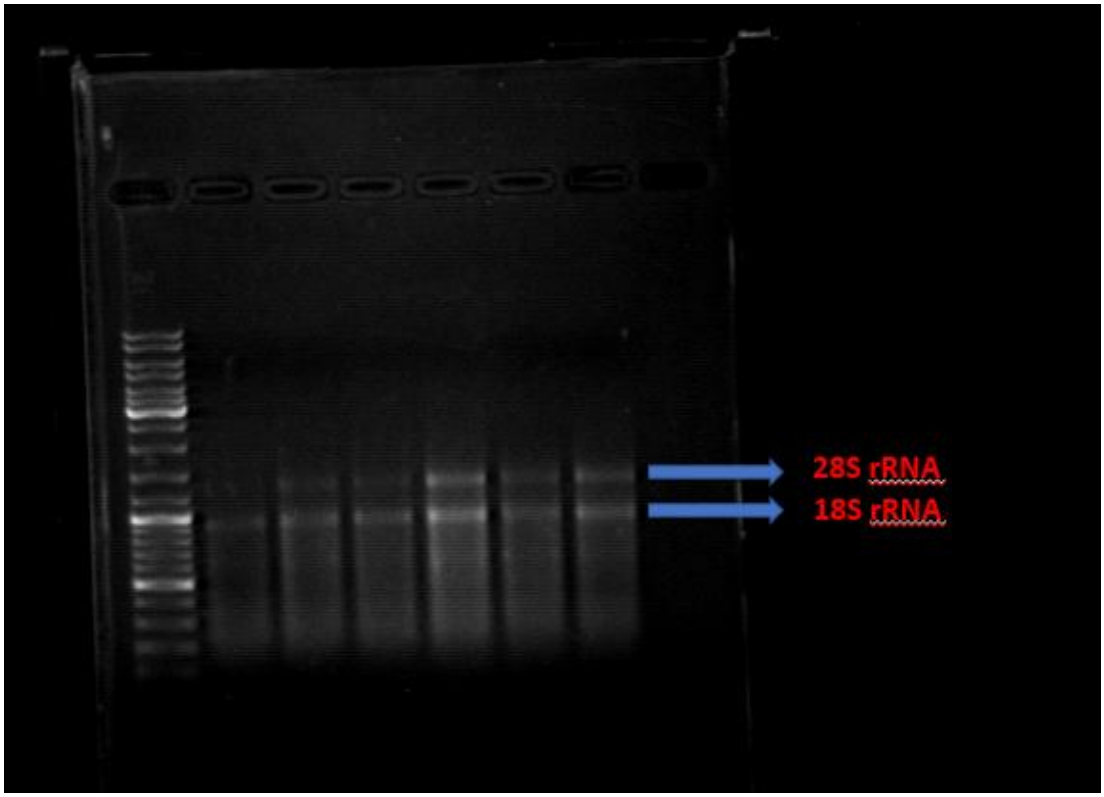


Figure 4.66. 5th week RNA samples on 1% agarose gel. From left to right; peqGOLD DNA ladder from 100 bp to 10000 bp fragments, RNA AM+1, RNA AM+2, RNA AM+3, RNA AM-1, RNA AM-2, RNA AM-3. 28S and 18S eukaryotic rRNA bands are shown with blue arrows.

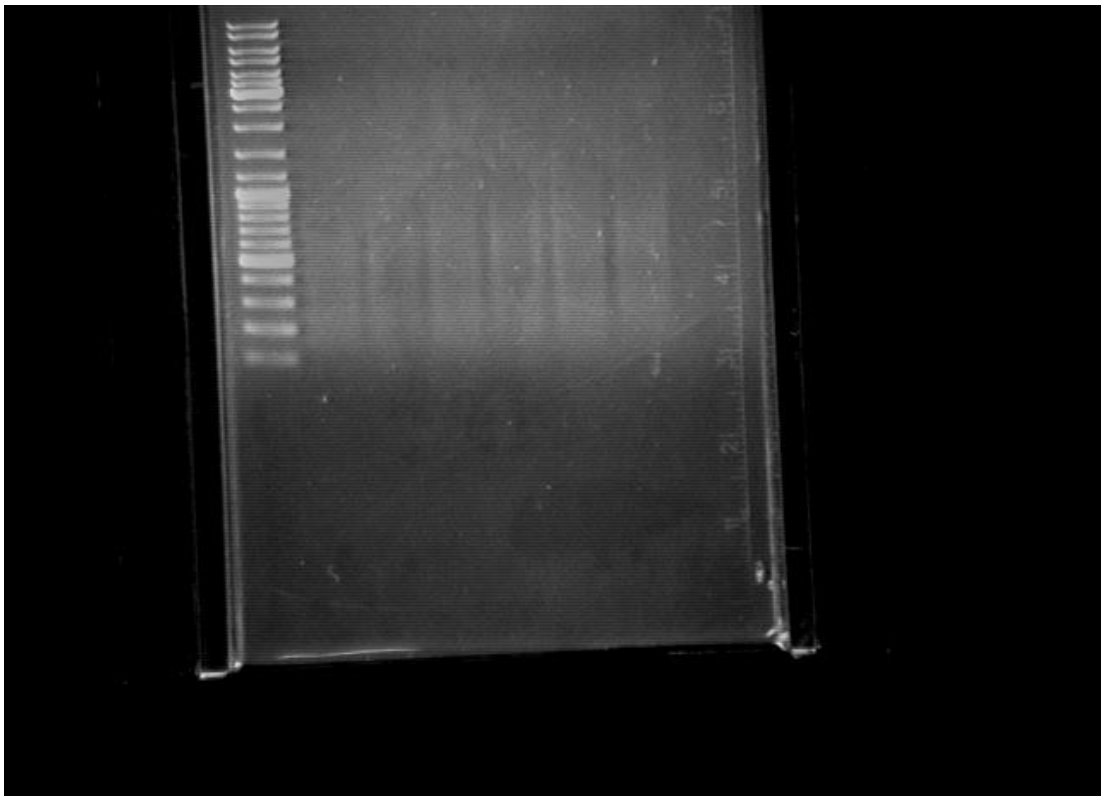


Figure 4.67. 5th week cDNA samples on 1% agarose gel. From left to right; peqGOLD DNA ladder from 100 bp to 10000 bp fragments, cDNA AM+1, cDNA AM+2, cDNA AM+3, cDNA AM-1, cDNA AM-2, cDNA AM-3.

Integrity of cDNA samples was assessed by PCR experiment with the primer targeting the sorghum bicolor mitochondrial gene. PCR products were run on the agorese gel. 110 bp product bands were obtained as expected, however, there were also some smear in 5th, 8th and 10th week samples. Gel images are shown in Figure 4.68.

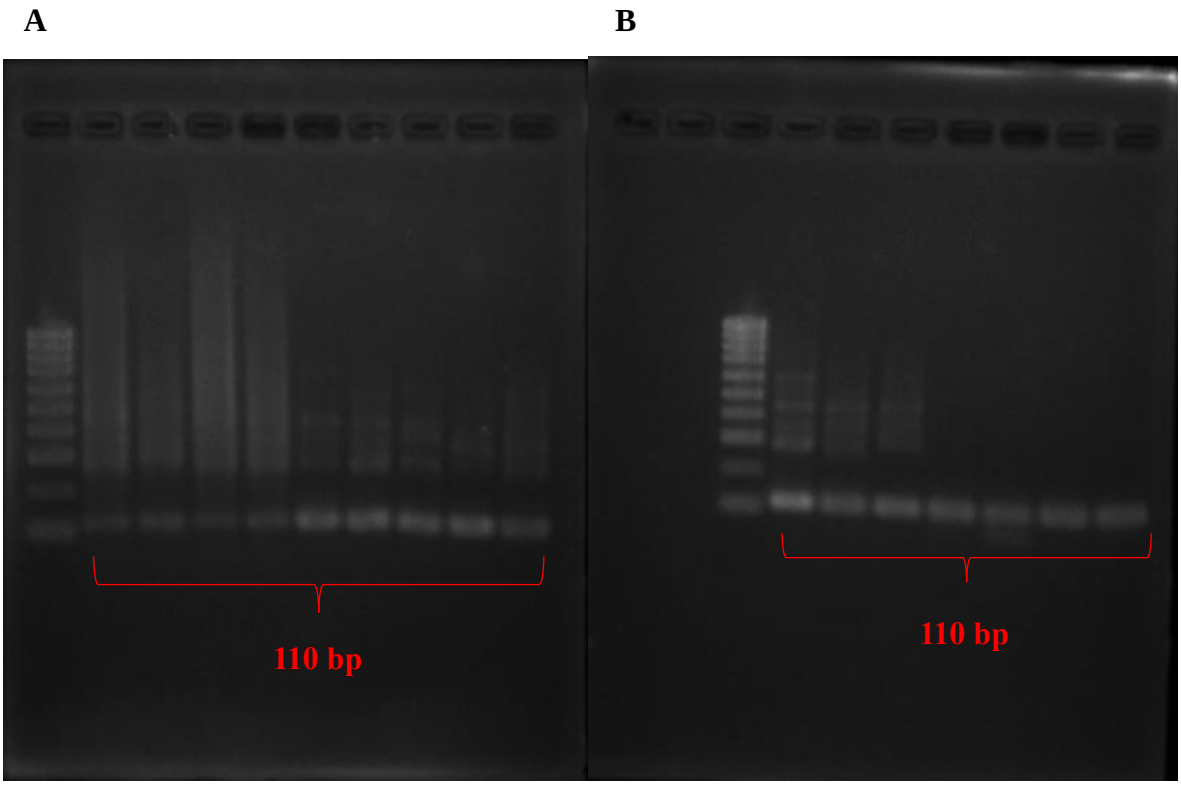


Figure 4.68. Electrophoresis of PCR products on 2% agorose gel. (A) From left to right; 100 bp ladder, 5th week cDNA AM+1, 5th week cDNA AM+2, 5th week cDNA AM-1, 5th week cDNA AM-2, 8th week cDNA AM+1, 8th week cDNA AM+2, 8th week cDNA AM-1, 8th week cDNA AM-2, 10th week cDNA AM+1. (B) From left to right; first two wells are empty, 100 bp ladder, 10th week cDNA AM+2, 10th week cDNA AM-1, 10th week cDNA AM-2, 12th week cDNA AM+1, 12th week cDNA AM+2, 12th week cDNA AM-1, 12th week cDNA AM-2.

For the gene expression analysis, 3 candidate primers (PP2A, EIF4α and CYP) which target different genes for reference gene, 2 candidate primers (Yellow stripe 1-242 and Yellow stripe 1-218) which target yellow stripe 1 gene, 2 candidate primers (YSL 15-116 and YSL 15-146) which target yellow stripe-like-15 (YSL 15) gene and one candidate primer (Alt_{SB}) which targets the Alt_{SB} gene were selected. Gradient PCRs were performed with the cDNA sample of 10th week AM+2 to determine the suitable primers and the genes for the expression analysis. Two images from the agarose gel electrophoresis of PCR products of these 8 primers are shown in Figure 4.69 and Figure 4.70.

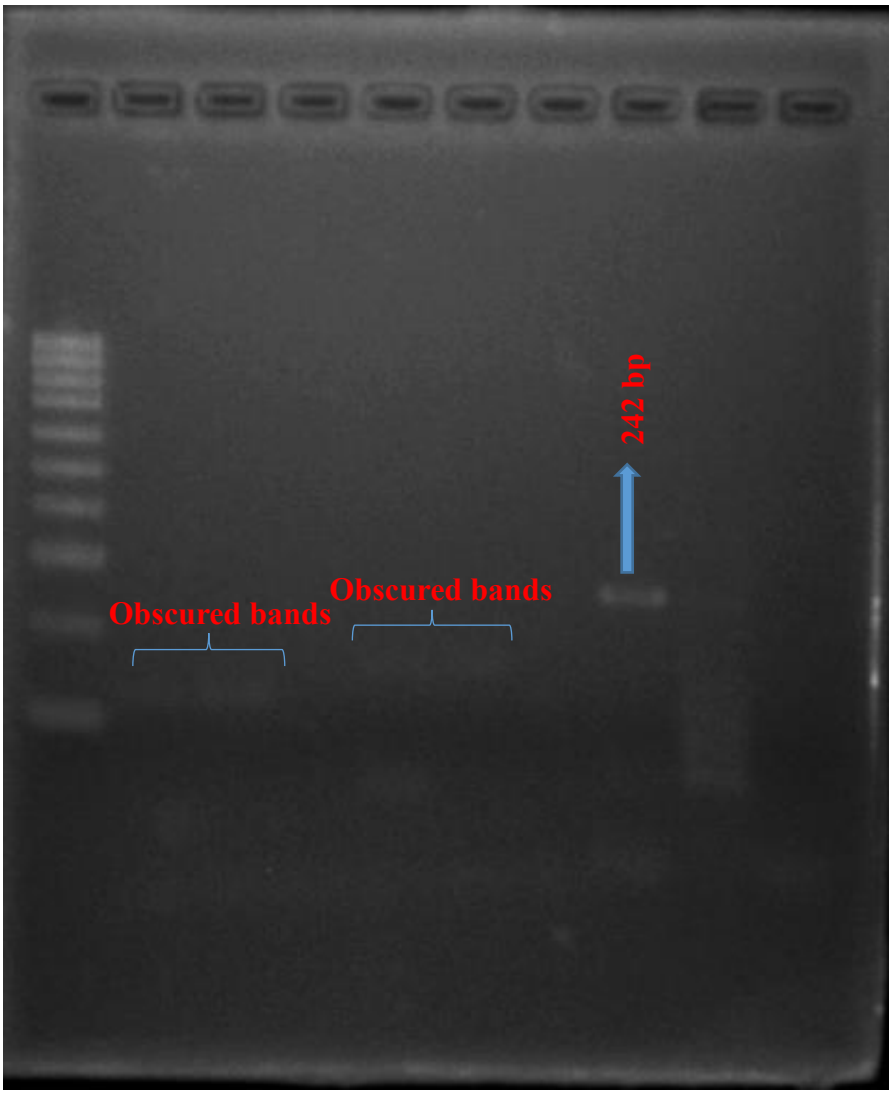


Figure 4.69. Electrophoresis of PCR products on 3% agorose gel. From left to right; 100 bp ladder, product of YSL 15-116 primer at 57.5 °C, product of YSL 15-116 primer at 58.2 °C, negative control of YSL 15-116 primer at 57.5 °C, product of YSL 15-146 primer at 58.2 °C, product of YSL 15-146 primer at 59.1 °C, negative control of YSL 15-146 primer at 58.2 °C, product of Yellow stripe 1-242 primer at 59.1 °C, product of Yellow stripe 1-242 primer at 60.2 °C, negative control of Yellow stripe 1-242 primer at 59.1 °C.

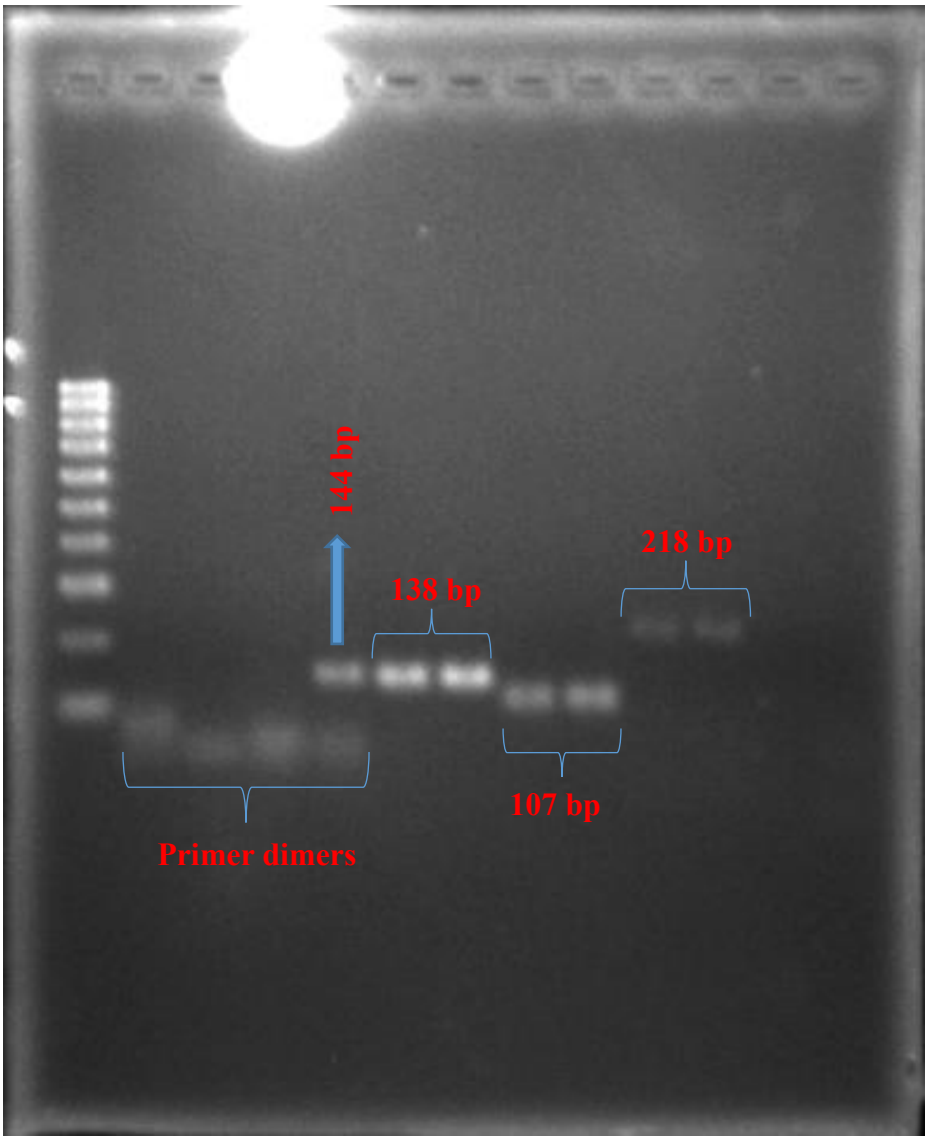


Figure 4.70. Electrophoresis of PCR products on 3% agorose gel. From left to right; 100 bp ladder, product of Alt_{SB} primer at 57.8 °C, product of Alt_{SB} primer at 58.2 °C, product of EIF4α at 58.2 °C, product of EIF4α at 59.1 °C, product of PP2A at 59.1 °C, product of PP2A at 60.2 °C, product of CYP at 60.2 °C, product of PP2A at 61.5 °C, product of Yellow stripe 1-218 at 59.1 °C, product of Yellow stripe 1-218 at 60.2 °C, negative control of Yellow stripe 1-218 at 59.1 °C, negative control of Alt_{SB} at 57.8 °C.

After these two gel results, PP2A and CYP primers were selected for the reference genes as worked efficiently at different annealing temperatures and they did not form primer dimers. Since there were obscure bands in the gel in Figure 4.65, it was decided to perform more gradient PCRs with YSL 15-116, YSL 15-146, Yellow stripe 1-242 and Yellow stripe 1-218 primers to test more annealing temperatures. Two gel images of products of these gradient PCRs are shown in Figure 4.71 and Figure 4.72.

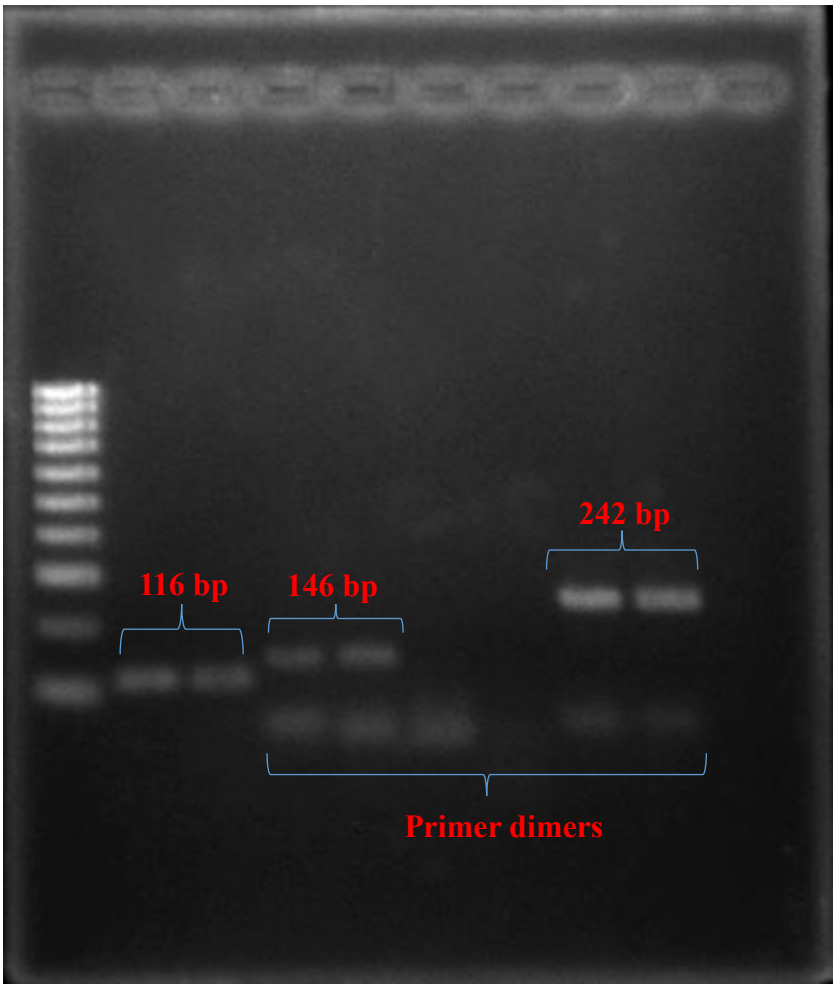


Figure 4.71. Electrophoresis of PCR products on 3% agorose gel. From left to right; 100 bp ladder, product of YSL 15-116 primer at 56.1 °C, product of YSL 15-116 primer at 61.1 °C, product of YSL 15-146 primer at 56.1 °C, product of YSL 15-146 primer at 61.1 °C, product of Alt_{SB} primer at 56.1 °C, product of Alt_{SB} primer at 59.8 °C, product of Yellow stripe 1-242 primer at 57.4 °C, product of Yellow stripe 1-242 primer at 58.5 °C.

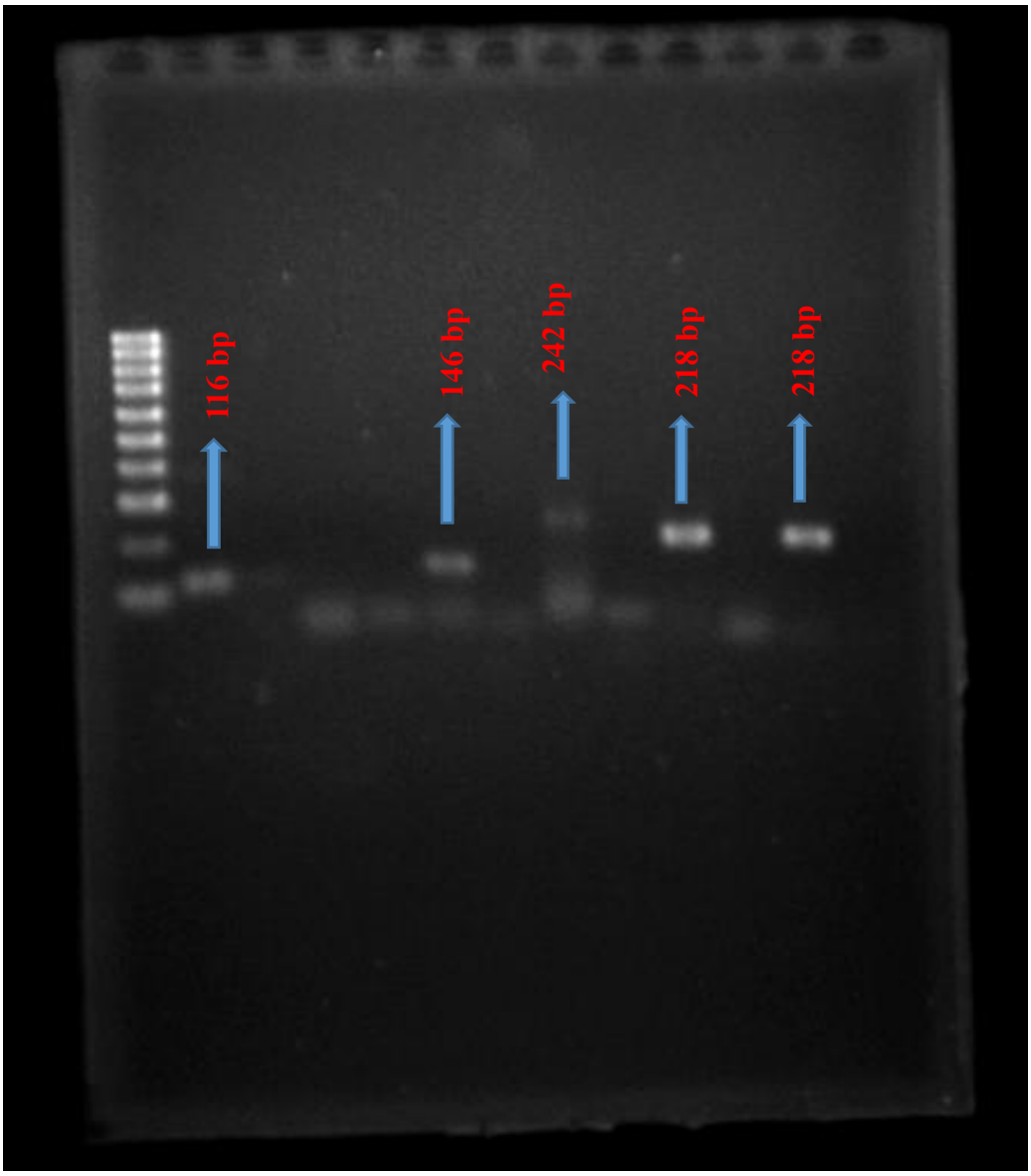


Figure 4.72. Electrophoresis of PCR products on 3% agorose gel. From left to right; 100 bp ladder, product of YSL 15-116 primer at 55 °C, negative control of YSL 15-116 primer at 55 °C, product of YSL 15-146 primer at 55 °C, negative control of YSL 15-146 primer at 55 °C, product of YSL 15-146 primer at 62.9 °C, negative control of YSL 15-146 primer at 62.9 °C, product of Yellow stripe 1-242 primer at 56 °C, negative control of Yellow stripe 1-242 primer at 56 °C, product of Yellow stripe 1-218 primer at 57.3 °C, negative control of Yellow stripe 1-218 primer at 57.3 °C, product of Yellow stripe 1-218 primer at 62 °C, negative control of Yellow stripe 1-218 primer at 62 °C.

After all these gradient PCRs, YSL 15-116 and Yellow stripe 1-218 primers were selected to be used in qRT-PCR analysis as the primers of target genes. 62 °C was determined as the best annealing temperature for Yellow stripe 1-218 primer and 56.1 °C was determine as the best annealing temperature for YSL 15-116 primer among the tested temperatures. First qRT-PCR analysis was performed with the cDNA samples of 5th week AM-3 and 8th week AM+3, and with the primers CYP, PP2A, Yellow stripe 1-218, YSL 15-116 as a pre-test. Annealing temperature was 61 °C. In the

melting curves, it was seen that CYP products formed one main peak and one shoulder besides the main peak. On the other hand, PP2A had a clear main peak. For this reason, PP2A primer was chosen to be used for the housekeeping gene and CYP primer was eliminated. YSL 15-116 primer did not work well as expected since its best annealing temperature was determined as 56.1 °C by gradient PCRs. Products of Yellow stripe 1-218 primer for some samples constructed a clear main peak in melting curves, however, in some samples there was another peak besides the main peak and/or the peak was not well-shaped. Melting curves of this pre-test qRT-PCR analysis are shown in Figure 4.73.

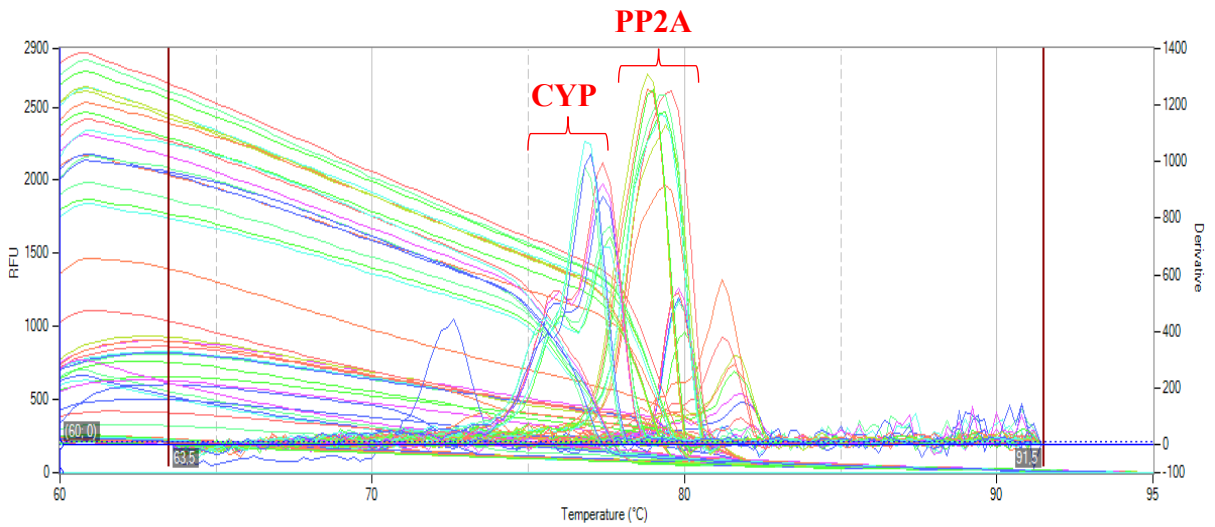


Figure 4.73. Melting curves of the products of CYP, PP2A, YSL 15-116 and Yellow stripe 1-218 primers. Melting curves of the products of PP2A and CYP primers are indicated with red brackets.

Since the melting curves of the products of Yellow stripe 1-218 primer were clear and well-shaped for some samples but not for all samples, another qRT-PCR analysis was performed with more samples and with Yellow stripe 1-218 and PP2A primers at the annealing temperature of 61 °C. However, melting curves were still not well-shaped and/or not single for some of the samples. The melting curves are shown in Figure 4.74. Moreover, the products of Yellow stripe 1-218 primer with well-shaped and single melting curves were run on agorose gel and no bands were observed. Therefore, Yellow stripe 1-218 primer was eliminated from the next gene expression analyses.

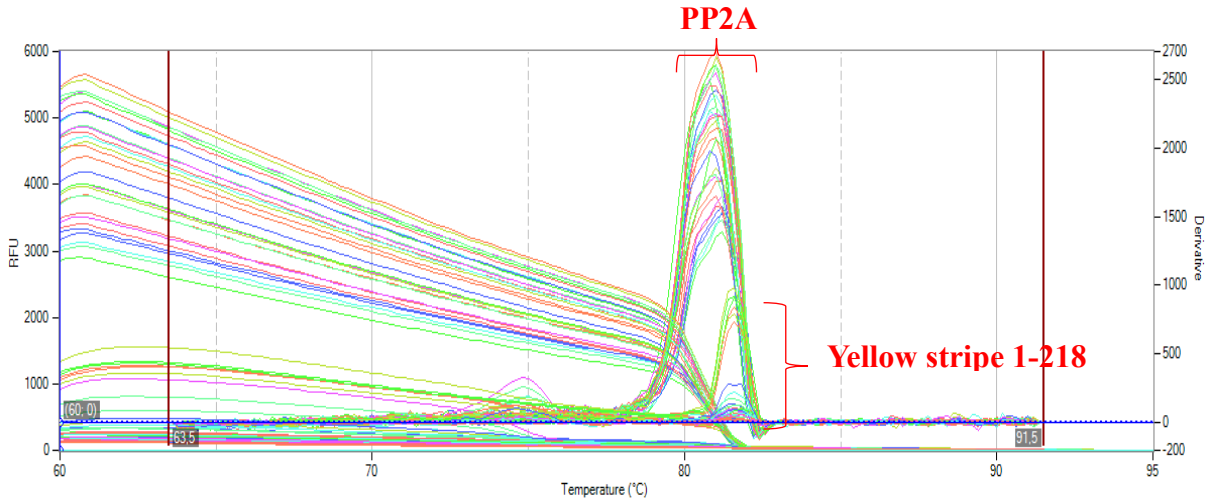


Figure 4.74. Melting curves of the products of PP2A and Yellow stripe 1-218 primers. Melting curves of the products of PP2A and Yellow stripe 1-218 primers are indicated with red brackets.

One more qRT-PCR analysis was performed with two cDNA samples and with Yellow stripe 1-218 and PP2A primers at the annealing temperature of 62 °C which had been determined as the best annealing temperature for Yellow stripe 1-218 primer by gradient PCRs. qRT-PCR mixes with PP2A primer were not prepared as triplicates since this was a testing for Yellow stripe 1-218 primer. However, qRT-PCR mixes with Yellow stripe 1-218 primer were prepared as triplicates. There were no melting curves for Yellow stripe 1-218 primer products which means that there was no product. The melting curve results are shown in Figure 4.75.

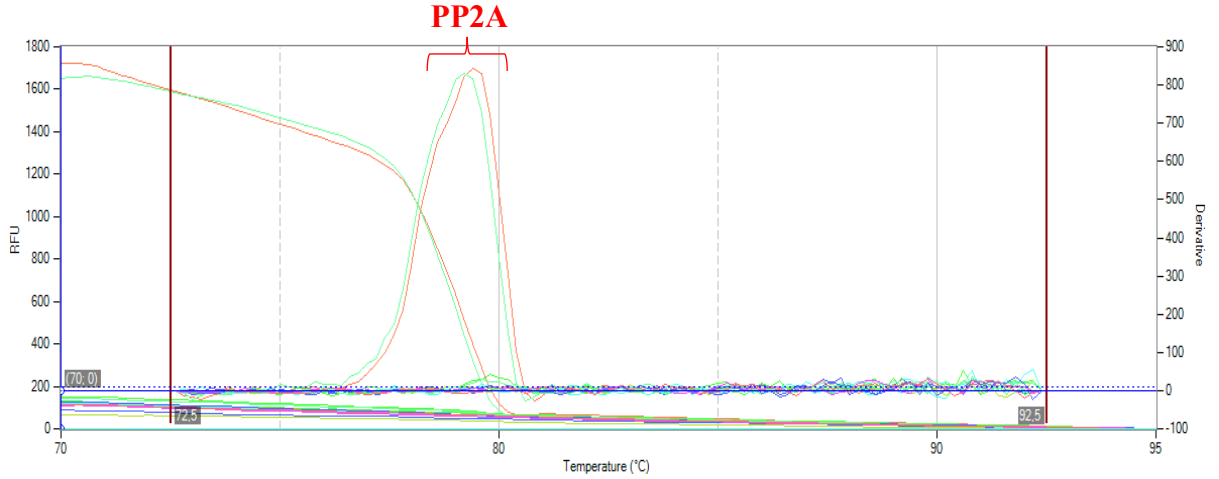


Figure 4.75. Melting curves of the products of PP2A and Yellow stripe 1-218 primers. Melting curves of the products of PP2A primer is indicated with the red bracket.

Gene expression analysis was done with YSL 15-116 and PP2A primers for target and housekeeping genes respectively. qRT-PCR analyses were performed with these primers at the annealing temperature of 56 °C. Melting curves were well-shaped and single for both primers. Melting curves of one of these qRT-PCR analyses are shown in Figure 4.76. Moreover, some of the

products of YSL 15-116 and PP2A primers in this qRT-PCR were run on the agarose gel and the presence of 116 bp and 138 bp products was confirmed. Two products of Yellow stripe 1-218 primer from the previous qRT-PCR at the annealing temperature of 61 °C were also run on this gel. As mentioned before, no band was observed for Yellow stripe 1-218 products. The gel image is shown in Figure 4.77.

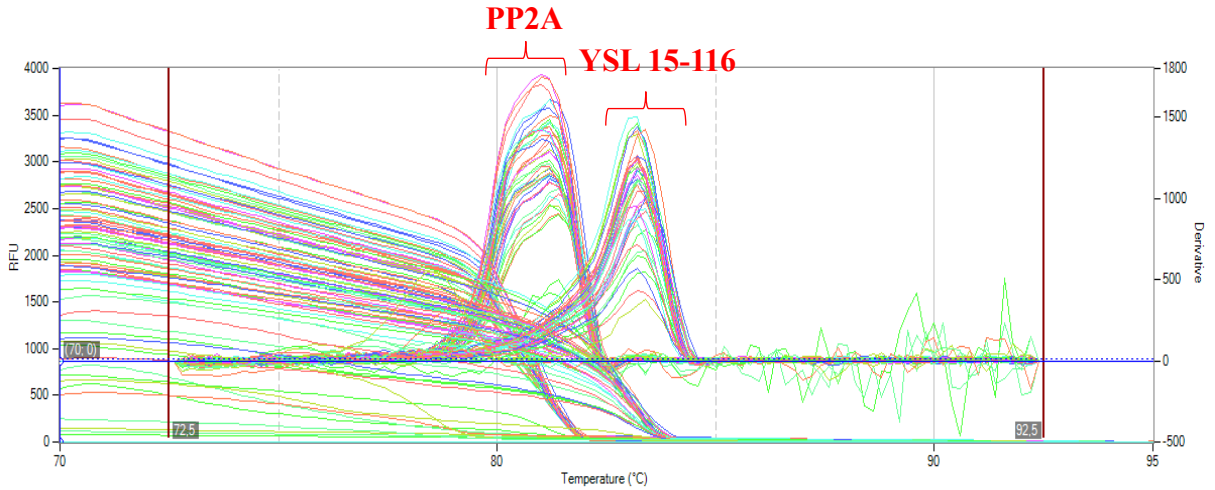


Figure 4.76. Melting curves of the products of PP2A and YSL 15-116 primers. Melting curves of the products of PP2A and YSL 15-116 primers are indicated with red brackets.

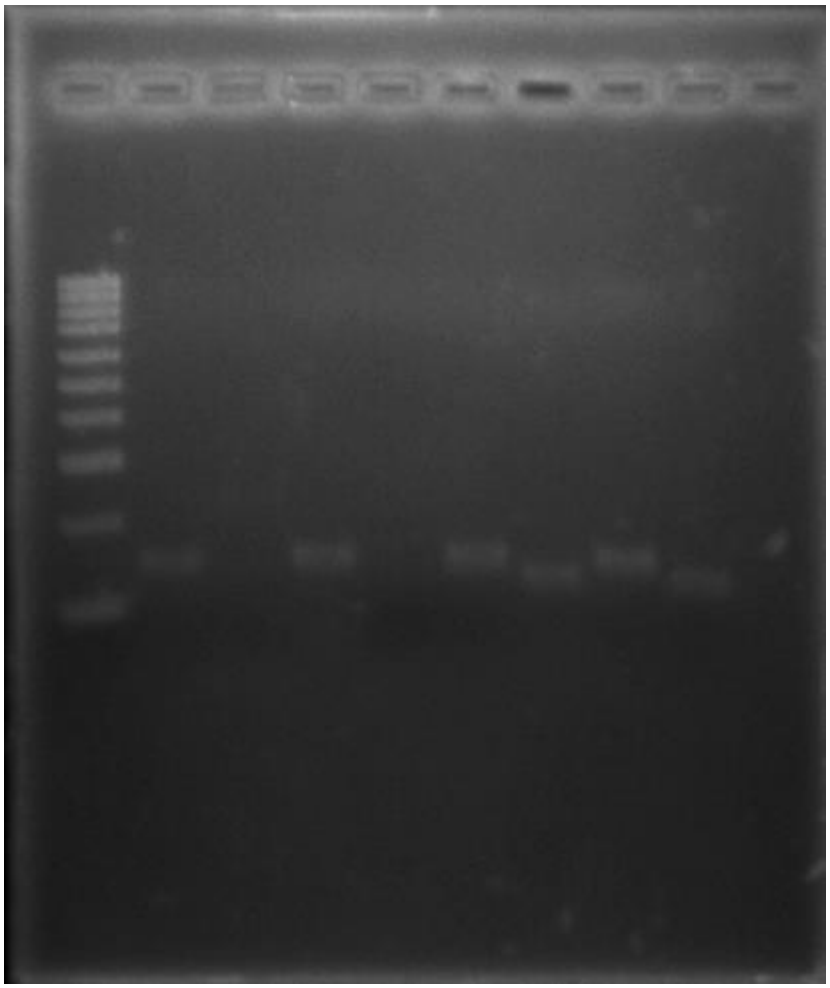


Figure 4.77. Electrophoresis of qRT-PCR products on 3% agorose gel. From left to right, 100 bp ladder, products of PP2A, Yellow stripe 1-218, PP2A, Yellow stripe 1-218, PP2A, YSL 15-116, PP2A, YSL 15-116 in order.

C_q results of YSL 15-116 products were normalized to the C_q results of PP2A products. To compare the YSL 15 expression levels in AM+ and AM- roots, the expression level of AM+ group were recalculated as relative to the expression level of AM- group for each sampling week. The relative expression levels of AM+ and AM- roots at 3^{rd}, 5^{th}, 8^{th}, 10^{th} and 12^{th} weeks are shown as bar plots in Figure 4.78 to Figure 4.82.

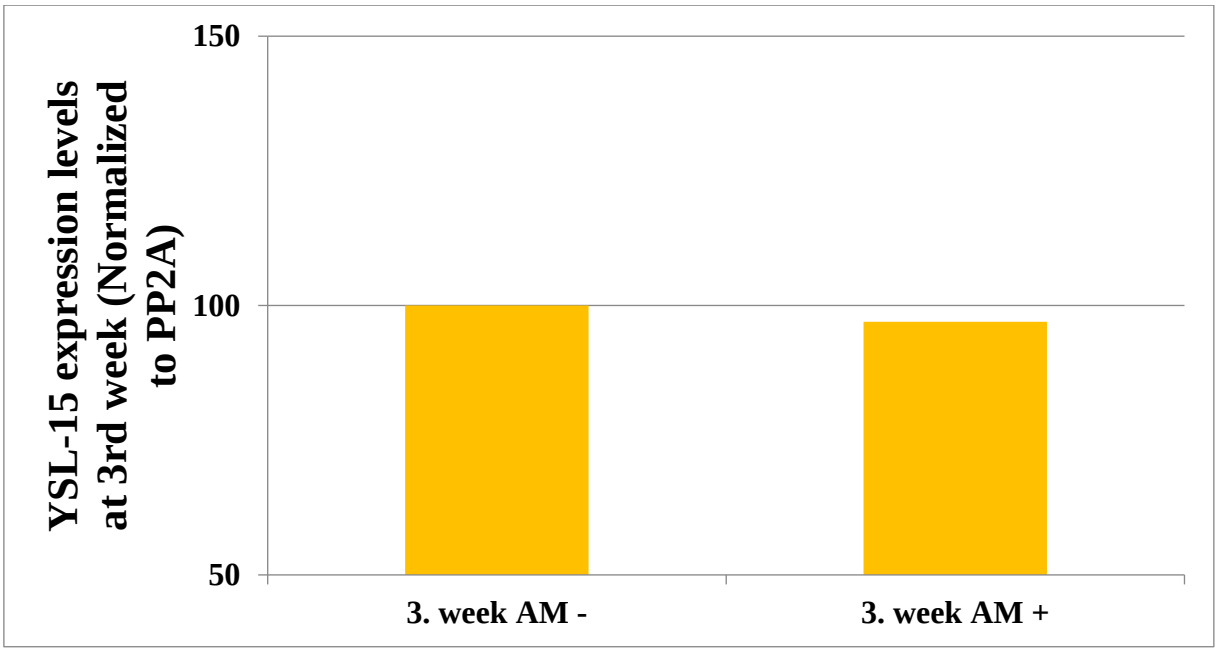


Figure 4.78. Relative YSL 15 expression levels of AM- and AM+ roots at 3rd week. YSL 15 gene expression of the samples were normalized to the expression of PP2A gene.

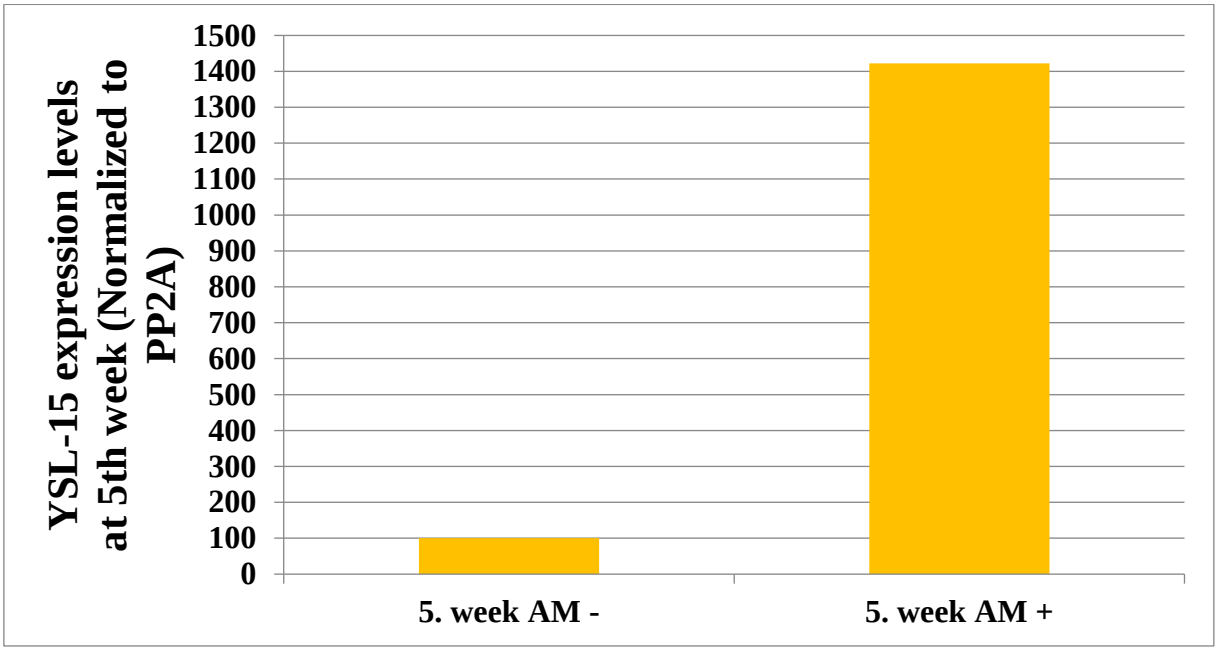


Figure 4.79. Relative YSL 15 expression levels of AM- and AM+ roots at 5th week. YSL 15 gene expression of the samples were normalized to the expression of PP2A gene.

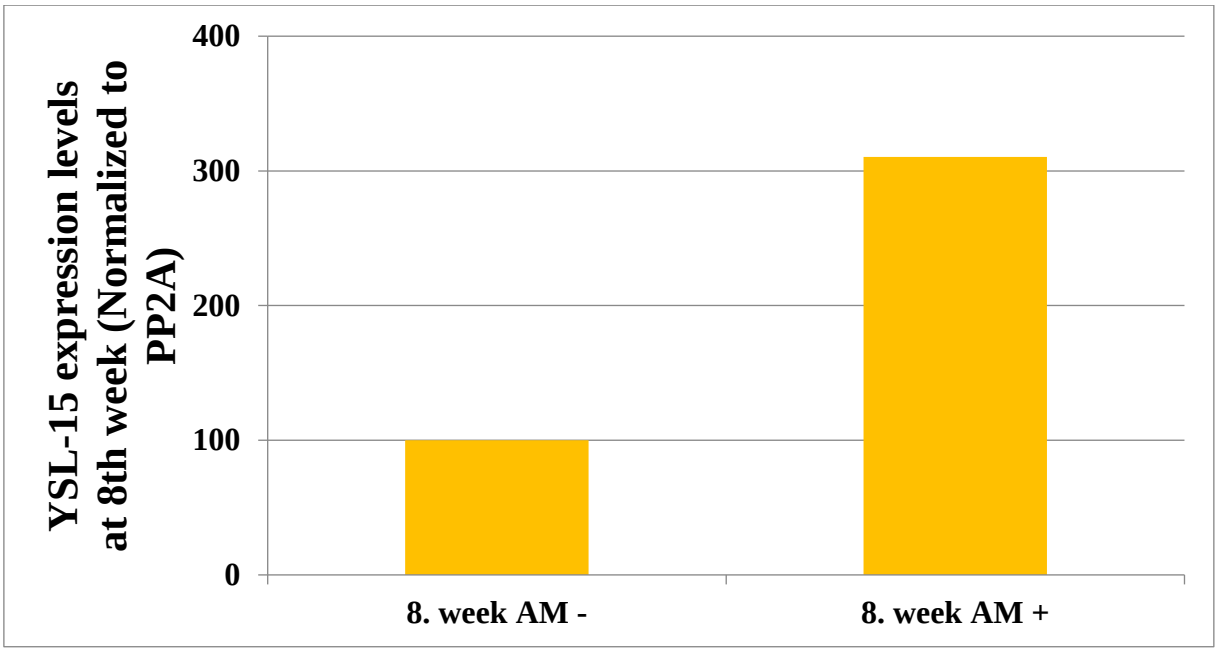


Figure 4.80. Relative YSL 15 expression levels of AM- and AM+ roots at 8th week. YSL 15 gene expression of the samples were normalized to the expression of PP2A gene.

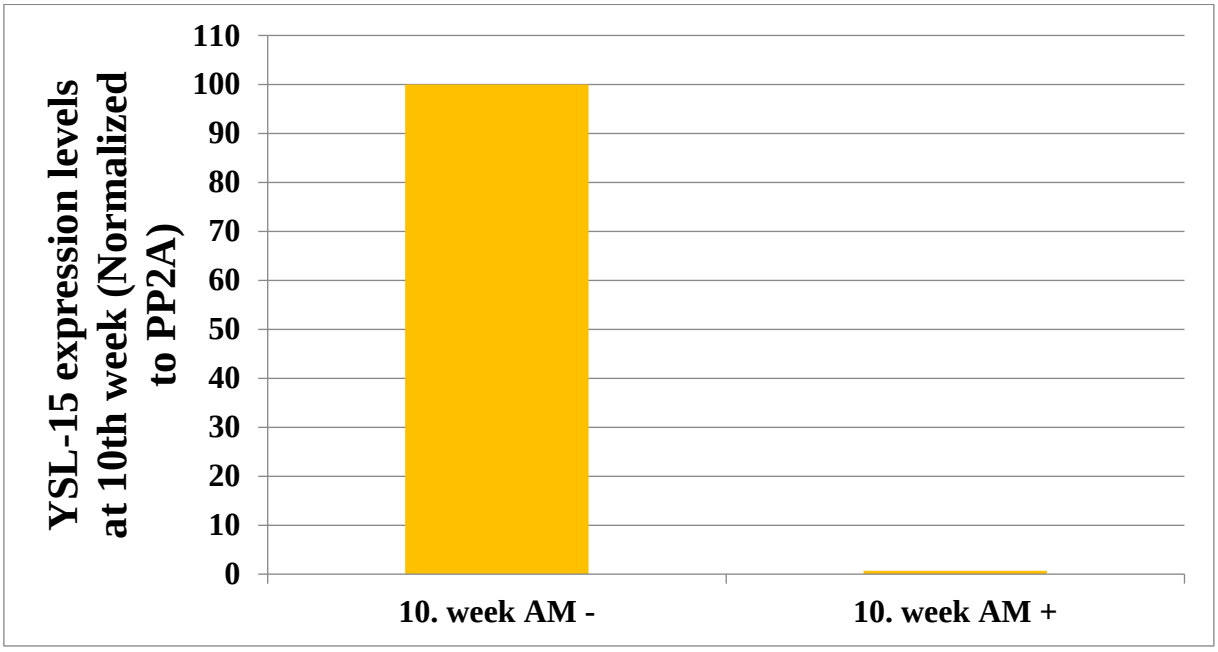


Figure 4.81. Relative YSL 15 expression levels of AM- and AM+ roots at 10th week. YSL 15 gene expression of the samples were normalized to the expression of PP2A gene.

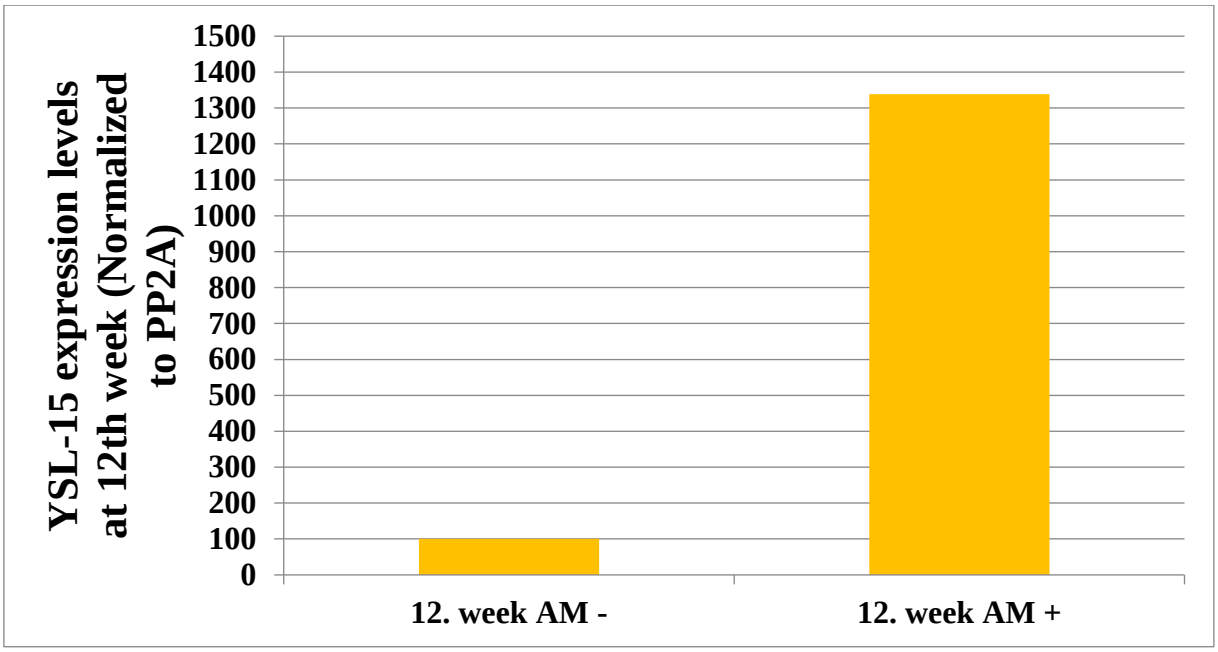


Figure 4.82. Relative YSL 15 expression levels of AM- and AM+ roots at 12th week. YSL 15 gene expression of the samples were normalized to the expression of PP2A gene.

The expression level of YSL 15 gene of AM+ roots was higher than that in AM- roots at 5th, 8th and 12th weeks and was lower than the AM- roots at 10th week. The expression levels AM+ and AM- roots were too close to each other at 3rd week. In addition to these, the biggest difference between AM+ and AM- roots in terms of the expression level was observed at 5th week.

To assess the effect of mycorrhization on the YSL 15 expression in sorghum roots, mycorrhization rate values and relative YSL 15 expression levels of AM+ roots were normalized and put into the same graph. The normalized values were between 0 to 1. The graph is shown in Figure 4.83.

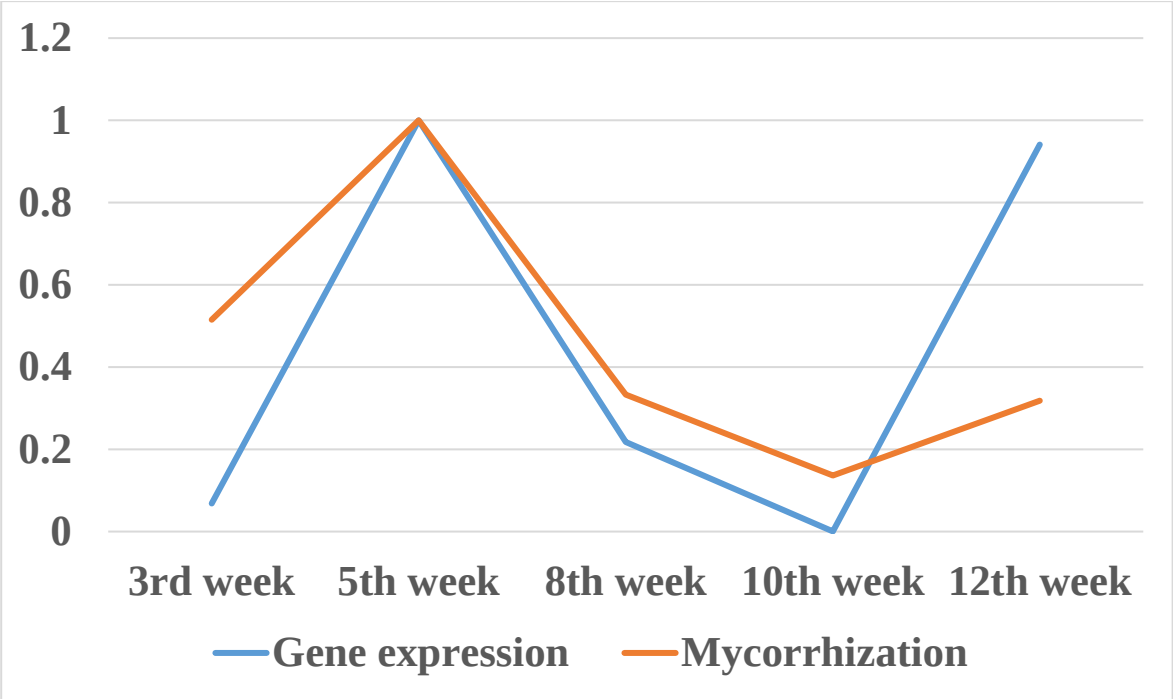


Figure 4.83. Normalized relative YSL 15 expression levels and mycorrhization rates of AM+ roots versus week graph.

Mycorrhization and the gene expression trends are very similar which indicates a possible close relationship between mycorrhization and the YSL 15 expression in AM+ roots.

5. DISCUSSION

The mine tailing soil that has been worked with in this study is a low fertility soil since no agricultural plant could be healthy grown. The metal analysis indicated that Al and particularly Fe concentrations are higher than the threshold levels in terms of world health organization warning in the experimental soil and this is one of the reasons for being with low fertility. Increasing the quality of the mine tailing soil by plant-mycorrhiza interactions and highlighting the physiological and genetic mechanisms behind this remediation were the main objectives of this research. In order to remove excess Fe and Al from this soil by plant and mycorrhiza, a greenhouse experiment was set up with *Sorghum bicolor* L. and *Rhizophagus irregularis.* Analyses of plant growth, symbiosis level of mycorrhiza in the root, pH of soil, metal and protein content of plant tissues and the soil, plant gene expression of metal transporters were conducted for 12 weeks following planting. *Sorghum bicolor* L. and *Rhizophagus irregularis* species were selected for this research in terms of the originality as Turkish indigenous species of Anatolia and their adaptive features to the native circumstance. Also, *Sorghum bicolor* L. is resistant to harsh conditions like drought, salinity, heavy metal pollution and it has high phytoextraction capacity of metals as a hyperaccumulator plant.

Before the greenhouse experimental unit was setup, germination assay experiments were performed in the lab as a preliminary test to confirm the ability of *Sorghum bicolor* L. seeds to germinate under conditions of the mine tailing soil, in terms of the Fe and Al concentrations. Germination assays proved the ability of sorghum seeds to germinate and grow in high Fe and Al concentrations.

The symbiosis of AMF with sorghum roots was observed week by week by microscopic counts. Results of the microscopic assay show that mycorrhization rate was an indication of the symbiosis level in mycorrhizal inoculated plants. The symbiosis was seen intensely in the AM+ roots which were inoculated with AMF. However, some AMF also appeared in the AM- roots which were not inoculated with AMF. This points out the naturally found mycorrhiza in this mine tailing soil. The soil was not sterilized by autoclaving before the greenhouse setup, therefore, there was a probability of pre-existing wild mycorrhizal fungi at non-inoculated roots. I should emphasize that the aim of using natural mine tailing soil without any sterilization was to stimulate the research with the actual field conditions in this research.

Based on our initial root observations mycorrhization started newly at the 2nd week in inoculated plants and at the 1st week in non-inoculated plants. It is generally known from mycorrhizal studies that root mycorrhization initiates at the 3rd week following inoculation, however, our observations apparently remarks the early formation of symbiosis phenomena that mycorrhization can initiate before the 3rd week. It could also be considered that the apparent initiation of mycorrhization was observed at 3rd week under certain circumstances. At the 2nd week, mycorrhizal structures occured only in a few fields of view that the object occupies under the microscope whereas there were hyphae approximately in the half of the fields. That's why the 3rd week might be significant as the apparent initiation of mycorrhization.

The mycorrhization rate in AM+ roots could achieve to its higher level at the 5th week. Hyphal collonization appeared almost in every fractions of the roots at the 5th week. The sudden decrease in the number of AMF spores around the roots between 3rd and 5th weeks could be due to the sudden increase in mycorrhization until the 5th week. This means that probably many spores at 3rd week germinated until the 5th week. Besides the high rate of the presence of AMF at the root at 5th week, the intensity of mycorrhization was the highest at 5th week. This remarkable result might be occured due to a high amount of transporting water and nutrients via AMF and plant roots at the 5th week. However, both the mycorrhization rate and the intensity were decreased after 5th week and dropped to the lowest levels at 10th week. This was probably because of the decrease in temperatures after the 5th week which was the last week of September. Moreover, a sudden and dramatic decrease in both daytime and night temperatures in İstanbul between 8th and 10th weeks may explain the lowest mycorrhization at 10th week. However, a slight increase in mycorrhization rate was observed until 12th week since we mounted the hydrogen lamps to provide optimal greenhouse conditions for plants after the 10th week. By the increase in daytime and night temperatures because of the hydrogen lamps might boost the mycorrhization again.

Root mycorrhization rate decreased by the dropping temperatures as contrary to the 5th week which nominated as highest root mycorrhization performance during the average daytime and night temperatures were 30 and 21 °C. This shows that the AMF-sorghum symbiosis strongly depends on temperature in this soil. This result is consistent with the previous researches which have been stated that AMF colonization is temperature sensitive and decreases by the low temperatures (Gavito et. al., 2005). Moreover, the decrease in mycorrhization when the temperatures decreased points out a possible entrance of dormancy phase by mycorrhiza.

In the 12th week, trypan blue stained root images, dark blue round shape figures and tiny filaments around them were observed. These figures were probably the newly formed spores of the AMF because they were round shaped like spores, small like unmatured spores and they had the tiny filaments like hyphae fragments. When a spore of AMF germinates, it forms the hyphae first, thus, AMF reaches the roots and forms the arbuscules and vesicles. When this cycle is completed, mycorrhiza forms new spores which are distributed around the root since these newly formed spores are carried on the tips of extraradical hyphae. Therefore, the tiny filaments in the image might be the hyphae that carry the newly formed small round shaped spores.

In plant growth assays, root, shoot weights and heights of the plants were measured week by week. It was revealed that AMF showed statistically extremely significant positive impact on root weight. Moreover, weights of the mycorrhizal roots were higher than the non-mycorrhizal roots from the 3rd week which was the week of apparent initiation of mycorrhization. In other words, there was a strong correlation between mycorrhization and root growth. It is clear that AMF increased the root growth in our experimental mine tailing soil, however, mycorrhizal biomass had also a contribution on the increased root weights. Also, the high water holding capacity of mycorrhizal roots thanks to the symbiosis might provide more water mass to the AM+ roots. According to the two-way ANOVA results, the time showed an extremely significant effect on root weight besides the AMF. This is an already expected result since root growth increases by time until maturation. It is also interesting that the standard errors in the weights of mycorrhizal roots were generally higher than in the weights of non-mycorrhizal roots. This shows a high variability in weights of mycorrhizal roots which is probably because of the higher number of variable factors in AM+ plants. For the plants in association with AMF, there are plant-plant, plant-mycorrhiza and mycorrhiza-mycorrhiza interactions which affect the root growth together. The variable levels of these interactions at different mycorrhizal roots might induce a higher standard error in root weights.

It was seen that AMF revealed no statistically significant effect on the shoot weight whereas time had a significant effect. The positive effect of time on shoot weight is an already expected result as in the root weight results because plant stem grows by time after plantation. The AMF was seen as a statistically significant factor for root weight whereas it was not for shoot weight. This might because the AMF mostly encourages the growth of the root of the plant; also mycorrhizal biomass and probable high water content in mycorrhizal roots have a contribution on this.

In the results of plant height measurements, it was seen that both AMF and time had statistically significant effects on height. AMF induced the increase in heights of the plants. Therefore, the

mycorrhiza did not only enhanced the root growth but also the plant growth. However, the significance level of AMF effect on root weight was higher than the significance level of AMF effect on height. This means that AMF mostly increased the root weight. On the other hand, the level of positive effect of time was the same for the root and shoot weights, and height as expected because time is independent of the mycorrhization.

The photo from the greenhouse at the 10th day after plantation shows an obvious difference between AM+ and AM- groups in terms of the number of plants per pot. AM+ pots had higher number of plants than the AM- pots even though the same number of seeds planted in each pot. This points out a positive effect of AMF on germination besides the growth. Thus, AMF showed positive effects on plant even at germination stage. In other words, AMF may encourage seed germination and root growth immediately after the germination eventhough the symbiosis has not been established or it is not at an observable stage.

In protein measurements, the contribution of AMF to soil fertility and the production of mycorrhizal protein glomalin were assessed. The method used in protein extraction from root and soil samples is known as the method specific to the extraction of glomalin related soil protein (GRSP). GRSP includes mostly the glomalin protein but also includes some other soil proteins which are resistant to the harsh extraction conditions of this method. High pressure and temperature conditions during the extraction induce especially the free small proteins to be degraded. However, glomalin and some other resistant proteins like the ones in complexes with humic and fulvic acids are extracted as GRSP. During the spectrophotometric measurements after the extraction, the absorbance values of the samples were observed during 1 hour after adding the Bradford Reagent. The highest absorbance values were recorded at the 30th minute and all the absorbance values that were taken into account were the values recorded at 30th minute. This late peak of absorbance might be due to the too low concentrations of proteins in the samples. Rarity of the proteins might reinforced the binding of Coomassie Blue dye to the N terminal of the proteins within the samples. The same situation was observed for the BSA standard with the lowest concentration. The absorbance value was increasing slowly and decreasing slowly after it reached its highest value whereas the absorbance values of the BSA standards with higher concentrations were increasing and decreasing suddenly. Therefore, the optimum timing for the absorbance measurement for the samples in this research was determined to be 30 minutes after the addition of Bradford Reagent.

It was expected to see an obvious difference between the GRSP content of AM- and AM+ roots because of the presence of AMF in AM+ roots, however, protein amounts were similar to each other.

According to the statistical analysis, AMF had a significant effect on GRSP content in roots at the lowest significance level among the determined significance levels in this study. However, this might be due to the weight difference between AM+ and AM- roots; AM+ roots were heavier which means more extracted proteins from AM+ roots. At 1st and 2nd weeks, protein content was higher in AM+ roots even though weight of AM+ roots were lower than the AM- roots. Therefore, AMF might increased the amount of protein in root by enhancing the root growth or by inducing the production of proteins by root. Although the protein content of AM+ roots was higher at each week, this is not the case when mg protein per kg root is calculated for each week and each group. Since the weights of AM+ roots were too much higher than the AM- roots, mg/kg proteins in AM+ roots were lower than in AM- roots even though AM+ roots contained more protein in mg. However, at 1st and 2nd weeks, weights of AM+ roots were lower than the AM- roots, therefore, mg proteins per kg AM+ roots were higher than the AM- roots. AM+ roots had higher protein content in mg and lower root weight at 1st and 2nd weeks, thus, even a bigger difference was seen between AM+ and AM- group in mg/kg protein in root graph. As in the root weight graph, the standard errors in the protein content in AM+ roots were higher than the standard errors in protein content in AM- roots. This shows a high variability in the protein amount of mycorrhizal roots which is probably because of the higher number of variable factors in AM+ plants. For the plants in symbiosis with AMF, there are plant-plant, plant-mycorrhiza and mycorrhiza-mycorrhiza interactions which affect the protein production together. The variable levels of these interactions at different mycorrhizal roots might induce a higher standard error in the protein amount in AM+ roots. Time also had a significant effect on GRSP content in roots which was the result of increasing root growth over time.

The trends of protein content in soil in mg and mg protein per kg soil graphs were the same because of the equal amounts of soil samples that had been taken for the extraction. Only the time had a statistically significant effect on protein amount in the soil, however, this might be due to the random sampling of soil from the pots. Because it was seen from the figures that protein content kept decreasing as plants were growing. Similarly, the statistical non-significance of AMF on protein in soil might be due to the random sampling and the littleness of the amount of taken soil sample because only 0.25 grams of soil was taken from each of the 1.7-liter pots. Although there was no difference between AM+ and AM- soil samples in terms of the protein, AM+ group had 1725.04 mg more protein per kg soil than the AM- soil at an average total. This means that AMF induced an increase in the soil fertility.

In SDS-PAGE experiments, it was expected to see closer bands to the BSA standards' bands since molecular weights of BSA and glomalin are so close to each other, 66.5 and 63.1 kDa

respectively. As mentioned before, most of the extracted proteins in GRSP mix are expected to be glomalin; therefore, the closer band to BSA standard might have been used as an estimation of the presence of glomalin protein. However, no band was observed in any of the loaded extract samples. This concludes that the glomalin protein might not have existed or its concentration was too low to be detectable on the gel. This result coincides with the Bradford Assay results since there was no obvious difference between AM+ and AM – roots and soil samples in terms of the protein. To ensure the lack of glomalin, gradient PCR experiments were performed with 5 different primers to check the existence of glomalin transcripts in cDNA samples. However, no band was observed in any of the experiments. On the other hand, the presence of 800 bp product of AML primer indicated the existence of mycorrhizal transcripts in the cDNA samples obtained from AM+ roots. This means that mycorrhizal genes were expressed but the glomalin gene was not expressed or its expression level was too low to be observable on the gel. Bradford Assay, SDS-PAGE and gradient PCR experiments have suggested that glomalin protein was not produced by the AMF or its production was too low to be detectable. Mycorrhiza induced the soil fertility to increase by encouraging the root growth and/or the protein production by the roots but not by the glomalin production by itself. Glomalin production might be related to the concentration levels of metals in soil or the type of the metals because glomalin is a chelating agent of metals and produced as a defense mechanism against too high concentrations of metals in soil. It was seen from the previous research that glomalin production was geting increased by the increasing levels of Cu and at 1000 ppm, the production reached a significant level (Tunalı M. M., 2015). However, in this mine tailing soil here, the Fe concentration was 663 ppm and the Al concentration was 76.2 averagely.

Soil pH measurements were performed at each sampling week. pH values were nearly the same at each week and they were at a range between 5.70 - 6.75 which is slightly acidic. Macronutrients are more available at higher pH whereas micronutrients are more available at lower pH values. In the pH range between 5.70 - 6.75, the availability of nutrients are optimal for most of the plants. At this pH range, iron is found mostly in $Fe(OH)_3$ form which is nearly insoluble and aluminum is found mostly in $Al(OH)_2^+$ form which is nearly insoluble as well. Also, iron and aluminium complexes with the humic substances are the common species of organically bound iron and aluminium in the soil at the specified pH range (Gerke, 1997). Fe-citrate and Al-citrate complexes present in little amounts at this pH range because comlexes with citrate requires lower pH values (Gerke, 1997). In addition, since the sorghum roots produce phytosiderophores, such as mugineic acid to the rhizosphere, the present iron in the soil might have found mostly as Fe-mugineic acid complex. Plant root is able to make the Fe and Al soluble within the rhizosphere by releasing these exudates. AMF also helps the

plant to make the micronutrients like Fe and Al available. Metal analyses results have encouraged the mycorrhizal contribution on Fe and Al absorption by the roots.

In metal analyses part of the research, 12 metals were measured in roots, shoots and soil samples. Since the concentrations of Fe and Al were higher when compared with the other elements, Fe and Al absorption and translocation within the plant body were focused on. Statistical analysis results showed the significant effect of AMF and time on Fe and Al content in roots. Both Fe and Al amounts in mg were higher in AM+ roots than in AM- roots from the 3rd week which is the week of the initiation of mycorrhization. This showed that presence of AMF and the symbiosis increased the absorption of Fe and Al by roots in this mine tailing soil. As shown in plant growth assays, the weight of AM+ roots were higher than the AM- roots from the 3rd week as well. Therefore, AMF might induced high Fe and Al absorption by encouraging the root growth which is called as growth dilution effect. Other than that, AMF might increased the metal absorption by interfering the molecular and/or genetic mechanisms of Fe and Al absorption by the roots. Also, mycorrhizal uptake and storage of metals have a contribution on the increased levels of Fe and Al content in AM+ roots.

Although the data indicates that Fe and Al accumulation were higher in AM+ roots from the 3rd week, this is not the case in mg/kg Fe and Al in roots graphs. Because the weights of AM+ roots were too much higher than the AM- roots from the 3rd week and this induced a decrease in mg/kg Fe and Al values in AM+ group.

It was also interesting that two peaks of AM+ roots at 3rd and 10th weeks were common in all root graphs of 12 metals. This means that metal content in mycorrhizal roots increased suddenly at 3rd and 10th weeks.

It was seen from the plant shoot graphs that Fe and Al contents in AM+ shoots increased clearly from the 8th week. This is an indication of late but increased translocation of absorbed metals from root to shoot in AM+ group. Both Fe and Al contents in AM+ shoots dramatically increased specifically at 10th week. Moreover, this increase at 10th week was seen as a peak in nearly all 12 metals' shoot graphs. AMF might encouraged the translocation of absorbants from root to shoot. However, according to the statistical analysis AMF has no significant effect on Fe and Al content in shoots. This non-significance resulted from the first 5 week values since Fe and Al content in AM+ shoots increased suddenly from the 8th week. Also, the metal contents in both AM+ and AM- shoots were too much lower than in roots. Sorghum plant preferred to concentrate its absorbed metals in its roots rather than in shoots. As in the root graphs, the trends of metal content in plant shoot in mg

graph and metal content in mg per kg shoot graph were different from each other. This is again due to the differences in shoot weights.

In the metal content in soil graphs, a common peak at 3rd week was observed for nearly all 12 metals. This was the same for the root graphs as discussed before. High levels of metals in the soil at 3rd week might induced a proportional effect on the roots by the increase in metal absorption at 3rd week. Also, initiation of mycorrhization at 3rd week might increased metal absorption by the roots or newly germinated mycorrhiza might increased the metal content in roots by the absorption of metals by mycorrhizal structures.

Fe and Al contents in AM+ and AM- soil samples showed similar patterns . Also, Fe and Al contents did not show an obvious change week by week. The random sampling of the soil might have an effect on this result. However, there were significant differences between weeks and between AM+, AM- roots and shoots in terms of Fe and Al contents. Therefore, the metal content of the soil might change if more plants are planted to the field instead of a pot.

In qRT-PCR experiments, expression level of YSL-15 gene in AM+ and AM- roots were compared at each sampling week. YSL-15 is one of the yellow stripe like genes which are responsible for the metal uptake from the soil and transport of absorbed metals within the plant body. YSL-15 is specifically responsible for the uptake of iron phytosiderophores from the soil but also it has a role on long distance transport of this iron from root to shoot and leaves. In the gene expression analyses, the expression levels of AM+ and AM- roots were nearly at the same level at 3rd week which was the week of initiation of mycorrhization. At 5th, 8th and 12th weeks which were the weeks with high mycorrhization rates, the expression in AM+ roots was too much higher than in AM- roots. These mean that mycorrhization increased the expression of YSL-15 gene in roots and eventually increased the uptake of iron from the soil independent of increasing the root growth. Moreover, the biggest difference between AM+ and AM- roots among the 3rd, 5th, 8th and 12th weeks in terms of the expression was seen at the 5th week which had the top mycorrhization rate. However, at 10th week which had the lowest mycorrhization rate due to the sudden and dramatic decrease in daytime and night temperatures, the expression level in AM+ roots was too much lower than in AM- roots. Mycorrhizae might have started to entered to the dormancy phase at 10th week due to the decrease in temperatures. Therefore, mycorrhizae might transferred their iron storage as well as their all nutrient storage to the host which was the plant and specifically the root of the plant. Because of the high iron transfer to the roots, AM+ roots might not needed more iron absorption from the soil at the 10th week. That’s why the YSL-15 expression level in AM+ roots was lower than in AM- roots even though the

iron content in AM+ roots and shoots were too much higher than in AM- roots at the 10th week. Moreover, the biggest difference in Fe and Al content between AM+ and AM- roots and shoots were observed at the 10th week. In other words, the sudden increase in metal content both in AM+ roots and shoots at the 10th week might resulted from the transfer of mycorrhizal storage to the AM+ roots and this iron sufficiency might caused the decrease in the expression level of YSL-15 gene in mycorrhizal roots at the 10th week.

Besides the YSL-15 gene, expression of Yellow stripe 1 gene in sorghum was also assessed. Although there were single and clear melting curves for some of the products of Yellow stripe 1-218 primer, no band was observed when the qRT-PCR products were run on the agarose gel. Also, the products were detected by the qRT-PCR equipment after the 45th cycle approximately. All these mean that expression of Yellow stripe 1 gene was too low to be detectable. The roots preferred to express YSL-15 gene instead of Yellow stripe 1 gene for the iron uptake from the soil.

To prove whether there was a correlation between the YSL-15 gene expression and the mycorrhization, the relative expression and mycorrhization rate values were normalized as between 0 and 1. As shown in Figure 4.83 there is a strong positive correlation between the expression of YSL-15 gene in roots and the mycorrhization. The expression levels were decreased when the mycorrhization decreased and the expression increased when th mycorrhization increased as well. Therefore, mycorrhization encouraged the uptake of iron from the soil by increasing the expression of YSL-15 gene in roots.

6. CONCLUSION

For the remediation and quality increase of this mine tailing soil, the symbiosis of *Sorghum bicolor* L. and *Rhizophagus irregularis* was assessed in terms of the symbiosis efficiency, plant growth, phytoextraction of iron and aluminum, contribution to soil fertility. Also, the genetic mechanism behind this phytoextraction was examined.

It could be concluded that the *Sorghum bicolor* L. and *Rhizophagus irregularis* symbiosis was effective in the phytoextraction of Fe and Al from the soil and it contributed to the soil fertility. Therefore, they can be utilized for the field application of this mine tailing area. The AMF significantly increased the root growth by the efficient symbiosis, however, the symbiosis strongly depended on the temperature in this soil. The mycorrhiza increased the metal absorption by the plant. Mycorrhizal contribution to this increase resulted from the increase in the growth of mycorrhizal roots, which can be called a growth dilution effect, and from the increase in the expression of YSL-15 gene in the roots. Moreover, mycorrhizal absorption and storage had a contribution on this high level of phytoextraction of Fe and Al. The AMF also increased the soil fertility by increasing the root growth and eventually by increasing the protein protein production from the roots, however, not by the glomalin production in this soil and in this research. The glomalin production might depend on the metal type and concentration in soil, therefore, further research should be done to understand the glomalin production. Also, the Bradford Assay method for the determination of GRSP might be revised by depending on the concentration levels. In this research, GRSP determination was done according to the maximum absorbance values at 30^{th} minute after the addition of Bradford Reagent to the samples because the protein amount was too low in the samples. Further research should be done to optimize the method for various GRSP concentration levels.

The mine tailing area in Kütahya has a continental climate, therefore, the night temperatures decrease even below zero degree before the June and after the August. Since it was concluded that the AMF and the sorghum symbiosis depended on the temperature and requires hot climates in this soil, the field application in this mine area in Kütahya should be done between June and August.

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APPENDIX

Root and shoot weights of the plants at each sampling week are shown in Table A with the standard errors below.

Table A. Root and shoot weights of the plants, and the standard errors.

Pot Name	Sample Name	Weight of root in mg	Weight of shoot in mg	Root weight SE	Shoot weight SE
week 1 AM+ pot/1	1	9.4	54	1.40118997	6.190404761
	2	5.1	41.2		
	3	5.3	32.7		
week 1 AM+ pot/2	1	1.3	23.1	1.12398102	6.411708041
	2	4.2	34.8		
	3	5	45.3		
week 1 AM+ pot/3	1	3.6	34.1	0.617341973	12.51483564
	2	5.5	69.6		
	3	5.4	73.4		
week 1 AM- pot/1	-1	10.1	75	1.609347694	13.03870307
	-2	5	40.7		
	-3	9.5	83.3		
week 1 AM- pot/2	-1	9.4	70.2	1.434495653	5.422893856
	-2	9.2	86		
	-3	5	69.3		
week 1 AM- pot/3	-1	4.6	66.3	0.536449231	2.518156292
	-2	4.1	62.5		
	-3	2.8	71.2		
week 2 AM+ pot/1	1	4.3	79.8	0.48074017	16.00045138
	2	2.7	24.5		
	3	3.1	55.4		
week 2 AM+ pot/2	1	5	56.8	0.584047182	8.295447212
	2	5.1	43.7		
	3	3.3	28.1		
week 2 AM+ pot/3	1	4.3	86.5	1.442605667	10.20952714
	2	1	51.5		
	3	5.9	64.6		
week 2 AM- pot/1	-1	19	65	5.056019515	7.819278171
	-2	2.7	55.6		
	-3	5.3	82.3		

Table A. Continued.

week 2 AM- pot/2	**-1**	**8**	**81.4**	**0.602771377**	**6.367713701**
	-2	**9.9**	**62.5**		
	-3	**8.2**	**81.8**		
week 2 AM- pot/3	**-1**	**9.5**	**57.7**	**1.827870649**	**29.59481937**
	-2	**8.8**	**132.3**		
	-3	**3.7**	**34.1**		
week 3 AM+ pot/1	**1**	**27.5**	**167.4**	**4.73861208**	**6.583565397**
	2	**11.8**	**169.7**		
	3	**15.5**	**148.9**		
week 3 AM+ pot/2	**1**	**11.2**	**42.4**	**1.527525232**	**21.64842822**
	2	**10.2**	**112.4**		
	3	**15.2**	**100.7**		
week 3 AM+ pot/3	**1**	**8.2**	**66.8**	**0.956265886**	**19.56220846**
	2	**9.6**	**128**		
	3	**11.5**	**122.6**		
week 3 AM- pot/1	**-1**	**4.1**	**126**	**0.493288286**	**9.339759692**
	-2	**5.8**	**124.8**		
	-3	**5.1**	**97.4**		
week 3 AM- pot/2	**-1**	**9.6**	**116.8**	**1.637409879**	**18.27350845**
	-2	**9.2**	**145.3**		
	-3	**4.5**	**82.1**		
week 3 AM- pot/3	**-1**	**7.8**	**80**	**1.052510227**	**15.4970965**
	-2	**4.2**	**132.8**		
	-3	**5.5**	**114.8**		
week 5 AM+ pot/1	**1**	**9.8**	**132.6**	**2.532455988**	**20.58877796**
	2	**7**	**188**		
	3	**15.6**	**121.4**		
week 5 AM+ pot/2	**1**	**15.8**	**137.1**	**2.271563338**	**17.60236727**
	2	**8**	**155.3**		
	3	**12.8**	**95.8**		
week 5 AM+ pot/3	**1**	**10.5**	**150.8**	**0.450924975**	**13.191285**
	2	**10.6**	**194**		
	3	**9.2**	**159.5**		
week 5 AM- pot/1	**-1**	**4.3**	**165.3**	**1.049338416**	**23.16465219**
	-2	**6.9**	**226.4**		
	-3	**3.4**	**150.8**		
week 5 AM- pot/2	**-1**	**10.1**	**219.7**	**2.33404751**	**33.44449797**
	-2	**3**	**104.2**		
	-3	**3.2**	**169.8**		

Table A. Continued.

week 5 AM- pot/3	**-1**	**13.3**	**233.9**	**1.424000624**	**37.07152246**
	-2	**8.8**	**356.5**		
	-3	**9.3**	**328.3**		
week 8 AM+ pot/1	**1**	**19.2**	**237.7**	**2.173578718**	**42.10939457**
	2	**18.6**	**302.8**		
	3	**12.4**	**157.2**		
week 8 AM+ pot/2	**1**	**29.6**	**232.5**	**5.282781254**	**16.69823677**
	2	**20.4**	**242.1**		
	3	**11.3**	**187.9**		
week 8 AM+ pot/3	**1**	**11.8**	**115.5**	**1.553490693**	**29.09434844**
	2	**15.6**	**189.4**		
	3	**17**	**211.8**		
week 8 AM- pot/1	**-1**	**18.5**	**299**	**2.482606158**	**41.44362972**
	-2	**10.2**	**162.7**		
	-3	**12.4**	**191.8**		
week 8 AM- pot/2	**-1**	**14.1**	**314.3**	**1.848723283**	**42.28357969**
	-2	**11.1**	**223.3**		
	-3	**7.7**	**169.4**		
week 8 AM- pot/3	**-1**	**11.3**	**134**	**1.644181661**	**25.24891637**
	-2	**16.9**	**206.4**		
	-3	**13.2**	**212.7**		
week 10 AM+ pot/1	**1**	**15**	**173.3**	**6.458327957**	**15.72835373**
	2	**23.7**	**171.2**		
	3	**37.2**	**125.1**		
week 10 AM+ pot/2	**1**	**52**	**510.7**	**6.903863653**	**74.21842389**
	2	**31.7**	**293**		
	3	**30.9**	**283.4**		
week 10 AM+ pot/3	**1**	**8.8**	**179.4**	**11.4659399**	**38.96874816**
	2	**47.9**	**312.7**		
	3	**34.4**	**227.6**		
week 10 AM- pot/1	**-1**	**14.8**	**132.7**	**0.819213715**	**26.05577351**
	-2	**17.6**	**209.9**		
	-3	**15.8**	**130.8**		
week 10 AM- pot/2	**-1**	**12.1**	**172.9**	**0.202758751**	**18.81863733**
	-2	**11.7**	**166.7**		
	-3	**12.4**	**113.6**		
week 10 AM- pot/3	**-1**	**15.7**	**277.8**	**1.42243922**	**14.26561523**
	-2	**18.6**	**242.5**		
	-3	**13.7**	**230.2**		

Table A. Continued.

week 12 AM+ pot/1	**1**	**18.7**	**254.4**	**4.135617648**	**30.95109045**
	2	**32.3**	**346.8**		
	3	**21.6**	**347.7**		
week 12 AM+ pot/2	**1**	**15.1**	**234.5**	**6.898067362**	**41.20343837**
	2	**23.1**	**263.4**		
	3	**38.6**	**370**		
week 12 AM+ pot/3	**1**	**31**	**473.4**	**1.219744964**	**82.85811836**
	2	**27.2**	**337.4**		
	3	**30.7**	**186.5**		
week 12 AM- pot/1	**-1**	**15**	**291.1**	**5.134307267**	**38.70203842**
	-2	**9**	**179.7**		
	-3	**26.5**	**300**		
week 12 AM- pot/2	**-1**	**7.8**	**252**	**3.650114153**	**10.16141941**
	-2	**18.7**	**240.8**		
	-3	**7.7**	**217.5**		
week 12 AM- pot/3	**-1**	**5.1**	**184.6**	**2.282055604**	**21.72359189**
	-2	**8.8**	**170.4**		
	-3	**13**	**241.5**		