

MOLECULAR AND MORPHOLOGICAL IDENTIFICATION OF AMF IN
KUTAHYA MINE-TAILINGS: BIO-PHYTOREMEDIATING AT MINE SITE

by

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Dedicated to my grandfather Kadir Karaçay who passed away last year.

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ABSTRACT

MOLECULAR AND MORPHOLOGICAL IDENTIFICATION OF ARBUSCULAR MYCORRHIZAL FUNGI (AMF) IN KUTAHYA MINE-TAILINGS: BIO-PHYTOREMEDIATING AT MINE SITE

Tavsanlı is a coal mining site located in Kutahya, Turkey. The tailings of this mine site are contaminated with heavy metals. Heavy metal contamination possesses a threat to the biota, ecosystem function and structure. Thus, remediation of mine tailing is crucial for the removal and destruction of toxic elements. Bio-phytoremediation is the use of plants, microorganisms or both to remove, detoxify, sequester contaminants or convert them into non-toxic materials. Arbuscular Mycorrhizal Fungi (AMF) have the ability to form symbiotic relationship with plenty of plant species and help them cope with extreme environmental conditions such as heavy metal pollution, drought, acidity. Since Tavsanlı stands for a stressed environment due to its contamination, it is hypothesized that native plants have an interaction with Arbuscular Mycorrhizal Fungi in order to support plants to thrive in harsh conditions. This thesis examined how bio-phytoremediation methods can be implemented for the Tavsanlı mine tailings. Soil samples and native plants were obtained from the mine site and greenhouse experiments were carried out with *Sorghum bicolor* L. seeds as host plant. Then roots were examined using both molecular and morphological techniques to investigate the presence of AMF which are known to bioremediate heavy metal contaminated sites. Following the DNA extraction, specific primers that target the Internal Transcribed Spacer (ITS) region were used to identify the AMF in the soil samples, DNA isolates were sequenced and compared to the fungi database.

ÖZET

KÜTAHYA MADEN TOPRAĞINDAKİ ARBUSKÜLER MİKORHİZAL FUNGUSLARIN MOLEKÜLER VE MORFOLOJİK YÖNTEMLERLE BELİRLENMESİ: MADEN BÖLGESİNDE BİYO-FİTOREMEDİASYON

Tavşanlı, Türkiye'nin Kütahya şehrinde bulunan bir kömür maden ocağıdır. Maden ocağının etrafı ağır metallerle kontamine olmuştur. Ağır metal kontaminasyonu biyota, ekosistem işlevi ve yapısı için tehdit oluşturmaktadır. Bu nedenle, maden topraklarının remediasyonu toksik elementlerin giderimi ve tahribatı için büyük önem teşkil etmektedir. Biyo-fitoremediasyon; kirleticilerin giderimi, detoksifiye edilmesi veya toksik olmayan materyallere çevrilmesi amacıyla bitkilerin, mikroorganizmaların veya her ikisinin kullanılmasına denir. Arbusküler Mikorhizal Fungi (AMF) birçok bitki türüyle simbiyotik ilişki kurarak onların ağır metal kontaminasyonu, kuraklık, asitlik gibi ekstrem çevre koşullarında hayatta kalmasına yardım etmektedir. Tavşanlı ağır metal kontaminasyonu nedeniyle stresli bir çevre olduğu için, yerel bitkilerin zorlu koşullar altında hayatta kalmak için AMF ile ilişki içinde olduğu hipotezi kurulmuştur. Bu çalışmada biyo-fitoremediasyon yöntemlerinin Tavşanlı maden toprağı için nasıl uygulanabileceğı araştırılmıştır. Maden bölgesinden toprak ve yerel bitki örnekleri alınıp sera deneylerinde *Sorghum bicolor* L. tohumları konukçu bitki olarak kullanılmıştır. Sorgum bitki kökleri moleküler ve morfolojik yöntemler kullanılarak AMF varlığını araştırmak için incelenmiştir. Toprak örneklerindeki AMF'yi tespit etmek için DNA ekstraksiyonunu takiben, ITS bölgesini hedefleyen spesifik primerler kullanılmıştır. Elde edilen DNA izolatları dizilenmiştir ve fungi veritabanıyla karşılaştırılmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

Abbreviation	Explanation
AM	Arbuscular Mycorrhiza
AMF	Arbuscular Mycorrhizal Fungi
BC	Before Christ
Bp	Base pair
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
HM	Heavy Metal
HRM	High Resolution Melting
ICP-OES	Inductively Coupled Plasma - Optical Emission Spectrometry
ITS	Internal Transcribed Spacer
LSU rRNA	Large Subunit Ribosomal Ribonucleic Acid
NGS	Next Generation Sequencing
OD	Optical Density
OTU	Operational Taxonomic Unit
PAH	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
RDP	Ribosomal Database Project
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
SSU rRNA	Small Subunit Ribosomal Ribonucleic Acid
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA

1. INTRODUCTION

Coal is the primary fuel that used for generating electricity worldwide. Tavsanlı is one of the active coal mines in Turkey. The area is located in the west of Kutahya city, Anatolia as demonstrated in Figure 1.1. The mining activities in the area have started in 4000 BC based on the studies on history and archaeology (Arik and Yaldiz, 2010).



Figure 1.1. The location of Kutahya in the map of Turkey.

The process of mining includes the release of waste in a great extent (Mishra et al., 2008). Accordingly, mining is responsible for particular environmental damage due to the production of high quantity and diversity of residues. These residues adversely affect the quality of main parameters of ecosystems including water, soil, air and hence the living organisms (Lam et al., 2017).

Mine tailings are untreated by-products of mineral processing which are usually located in open-air tailing ponds. Sand, fine grained solid material, water, large amounts of metals and overall mixtures compose mine tailings. These kind of tailing ponds give rise to environmental pollution besides damaging local resources (Wang et al., 2017). Hence, it has been reported that mine tailings pose a serious threat to human health and the environment (Yang et al., 2012). For instance, due to the discharge of mine tailings with excessive high metals, biomass production of plants is suppressed (Jabeen et al., 2009). Plant growth is adversely affected due to the increased uptake of heavy metals by plants which may result in plant mortality (Schmidt, 2003). Because of the reasons above, a need for an eco-friendly soil revegetation process has risen. Accordingly, a wide range of studies have been

carried out especially during the last decade in order to improve soil remediation methods by using plant species, a process called bio-phytoremediation (Wei et al., 2016). The use of various plant species to sequester, extract, and detoxify contaminants is known as phytoremediation and it is an effective, non-intrusive, inexpensive and readily accepted technology to clean up contaminated soils (Alkorta et al., 2004). Due to its applicability in soil remediation and also for its ability to enhance the management of related wastes; phytoremediation has been drawing attention worldwide. This technology can be applied to all plant-influenced processes that help the treatment of contaminated vegetation (Cunningham et al., 1997). These plants are called hyperaccumulators (Cunningham and Ow, 1996). A hyperaccumulator is a plant capable of growing in soils with very high concentrations of metals, extracting these metals through its roots, and concentrating extremely high levels of metals in their tissues. Hyperaccumulators which live on contaminated sites have been shown to develop a tolerance for heavy metals, however, this tolerance could lie within the plant, the microorganisms that form symbiotic relationship with plant roots, or a combination of the two (Alkorta et al., 2004). Among all microbes that reside in the soil, Arbuscular Mycorrhizal Fungi (AMF) has been shown to enhance the ability of plants to tolerate contaminated soil (da Silva et al., 2005). The majority of terrestrial plant roots (at about 80%) live in a symbiosis with soil fungi and form structures called mycorrhiza. It has been shown that AMF can be affected by heavy metal toxicity, but in many cases mycotrophic plants growing in soils contaminated with heavy metals are colonized by AMF (Wei et al., 2014). Many reports concerning this have quantified spores and estimated root colonization in situ. In the last few years, research interest has focused on the diversity and tolerance of AMF in heavy metal contaminated soils trying to understand the basis underlying adaptation and tolerance of AMF to heavy metals in soils, since this could facilitate the management of these soil microorganisms, for phytoremediation/bioremediation programs (Zarei et al., 2010).

The aim of this study is to identify indigenous mycorrhizal fungus as soil microorganisms in the target mine site. This could be important for understanding the ecological complexities between native and diverse plants live in mine tailings and their symbioses with mycorrhizal fungi. Furthermore, our interests led us to investigate the role of plant-microbe interactions in phytoremediation of polluted soils. It is hypothesized that there is a well-adapted community of AMF that reside in the Tavsanlı area that enhance the ability of plants to survive heavy metal contamination in the tailings. The objective of this study is to investigate the soil biological factors that will help native plants cope in Tavsanlı.

2. BACKGROUND AND LITERATURE REVIEW

2.1. Heavy Metal Pollution

Heavy metal (HM) is a term used to describe a various group of elements consisting of metals and metalloids which are known to be detrimental to all living species when existing in high concentrations (Grandjean and Landrigan, 2006). Due to their persistence in the environment and the toxic nature regarding to the biota, heavy metals are considered to be dangerous (Cao et al., 2015).

Major sources of heavy metal derivation are mainly natural and anthropogenic, however, anthropogenic derivation is considered to be most dangerous due to its widely distribution and high toxicity (Chen et al., 2017). Human activities such as smelting, smoking cigarettes, sewage discharge processes, covering industrial tools and transportation vehicles with heavy metals i.e. cadmium are also anthropogenic sources of heavy metal pollution in the atmosphere (Kirkham, 2006). Moreover; anthropogenic activities such as industrialization, mining, the use of pesticides and fertilizers, generation of energy and especially mining processes lead to heavy metal pollution in the environment (Zhuang et al., 2009). The major sink for heavy metals which released to the environment by such activities is soils (dos Santos et al., 2017).

Heavy metal pollution in soil is crucial due to the immutable nature of metals because unlike organic pollutants, most metals do not undergo degradation by chemical or microbial agents (Silva et al., 2013). Due to their lower degradation rates, heavy metals may persist in soil for extended period of time (Ali et al., 2013). Despite the fact that certain metals are essential for biological systems in specific concentrations, if metals are present in higher concentrations, they may become harmful by inhibiting functional groups, dislocating other metal ions or altering the configuration of biological molecules (Alkorta et al., 2004). The toxic effect of heavy metal pollution is triggered by the consumption of heavy metals by primary producers. Afterwards, heavy metals may accumulate in the living tissues of primary and secondary consumers as a result of biomagnification through food chain (Abdu et al., 2017). Consequently, heavy metals may accumulate in reproductive organs, livers etc. of the mammals causing damage in the function of their bodies, cardiovascular diseases, neurodegenerative diseases and cancer. It has been shown that occurrence of upper gastrointestinal cancer highly correlated with the heavy metal pollution in Turkey (Türkdoğan et al. 2002). Järup (2003) also reported that Cd and Pb are associated with the various diseases in humans which damages

cardiovascular system, kidney, blood, nervous system and bone structure. Cellular materials including DNA and nuclear proteins have been proven to interact with metal ions leading to DNA damage and conformational alterations (Beyersmann and Hartwig, 2008). Arsenic, cadmium, chromium, lead and mercury are the most dangerous heavy metals among all due to their higher toxicity levels and should be taking into special consideration in order to protect the public health (Yedjou et al., 2006).

In conclusion, heavy metal pollution in soils possess a threat to biological systems including humans and animals when the concentration exceeds standard safety levels (Ordóñez et al., 2011). Accordingly, the removal of heavy metals from soil is essential for the safety of ecosystem.

2.2. Remediation of Heavy Metal Polluted Soils

Although overall degradation of metals does not occur, alterations in their speciation and bioavailability are possible (Aggangan et al., 2015). The sufficient protection and restoration of heavy metal polluted soils need their characterization and remediation. Microorganisms that are able to use metals as final electron acceptors or reduce metals are used for metal remediation (Garbisu and Alkorta, 1997). However, when it comes to remediate the metal polluted soil; such as mine tailings, metal-accumulating plants are one step ahead than microbial processes due to their ability to extract metal from contaminated soils directly (Garbisu and Alkorta 2001; Garbisu et al., 2002). Over the last decade, A wide range of studies have been performed to improve mine tailing remediation methods with the objective to sustain a better ecological environment for mining areas. Currently, the main treatment methods of mine tailings are physical remediation, chemical remediation and bioremediation.

Physical remediation includes soil replacement and thermal desorption of soil. Soil replacement is achieved by utterly or partly replacing the contaminated soil with a healthy soil in order to reduce the heavy metal content, therefore increase the soil capacity. This technique requires intensive manpower and only effective in small areas of heavily contaminated soil (Gong et al., 2018). Moreover, the removed contaminated soil becomes hazardous waste and needs to be handled and disposed afterwards. Consequently, soil replacement is a costly method which is not feasible for larger areas of soil (Shukla et al. 2017). Thermal desorption method is applied by heating the polluted soil through steam or microwave in order to volatilize the pollutant. The technique has been commonly used to remove volatile metals such as Hg from the soil due to their volatility (Yao et al.,

2012). Vacuum pressure is used to obtain volatile metals which are going to be removed afterwards. Thermal desorption method is less energy-consuming compared to incineration, however, the high energy consumption is still the greatest downside of the technique. Moreover, soil properties are negatively affected by thermal desorption that may lead to the degradation of soil organic matter (Shukla et al. 2017).

Chemical remediation includes vitrification, soil leaching, chemical fixation and electrokinetic remediation. Vitrification is a technique that the soil is incinerated at high temperature and mineral matter is molten (Chibuike and Obiora, 2014). Temperature stands for the main factor regarding effectiveness and the cost of the process. The electrical conductivity of the soil may also decrease the efficiency of the process. Although vitrification can be applied in soils with small areas, it is not efficient in large-scale remediation of soils due to its high cost (Gong et al., 2018). Soil leaching or chemical leaching is a method in which polluted soil is washed with specific reagents followed by the removal of heavy metal complex. Following the separation, the recycling of heavy metals from extracting solution is applied. Heavy metals are then transferred from soil to liquid phase by the extractant and retrieved from the leachate (Ferraro et al., 2015). Although soil leaching is a rapid process that could fulfil the requirements for remediation for a short-term, it is not suitable for lasting a longer period. Besides, the wastewater generated from the process needs a further treatment which could increase the cost (Wuana and Okieimen, 2011). Chemical fixation is implemented by adding immobilizing agents into the polluted soil in an attempt to decrease mobility of heavy metals in soil (Zhou et al., 2004). Clay mineral is commonly used for this method due to its ability to fix almost all heavy metals (Ramesh and Reddy, 2011). Despite of being a simple and a rapid process, chemical fixation is not usually feasible due to its lack of removal of heavy metals from the soil (Gong et al., 2018). The principle of electrokinetic remediation is the utilization of certain particles and fluid interactions in an electrolytic tank in order to reduce the contamination of soil. The lack of by-products, easy applicability and protecting the original nature of soil are some of the advantages of the system, however, low effectiveness for heterogeneous soils, higher energy cost and probability to cause a fluctuation in the soil pH are the downsides of the technique (Zhao et al., 2016).

2.2.1. Bioremediation

Bioremediation is simply defined as the process which involves the use of organisms such as plants/microorganisms to biologically remove or degrade the pollutants into a non-toxic form under controlled conditions (Mohan et al., 2006). Due to its applicability via natural processes, the method

is commonly accepted in the remediation of soil. The fully degradation of heavy metals by bioremediation does not occur, however, the transformation of heavy metals into non-toxic or harmless compounds due to the change in their oxidation state take place in bioremediation (Garbisu and Alkorta, 2015). The method includes all techniques and processes to biologically transform a polluted soil to an unpolluted condition which is mainly obtained by the function of microbes either in situ or ex situ (Garbisu and Alkorta, 2003). In situ bioremediation is performed by the addition of oxygen and nutrients via circulating system into the polluted soils in order to induce microbes to degrade pollutants. The method in which microbial consortium is used for the degradation of contaminants is cost-effective and feasible for saturated soils. Ex situ bioremediation is the treatment of contaminates in soil or water once it has been excavated or pumped out of the location at which it was found. It refers to the physical removal of polluted soil or water (Shukla et al., 2017).

The main advantage of bioremediation compared to conventional physical and chemical remediation techniques is cost reduction. Moreover, physical treatment is not feasible for the polluted soil on a large scale while chemical remediation disrupt the structure of the soil in the long run (Wang et al., 2012). As a consequence, bioremediation is broadly accepted due to its effectiveness, cheapness and eco-friendly nature. Microorganisms, plants or the combination of two can be used in order to achieve bioremediation of heavy metals (Khan and Jones, 2009).

2.2.1.1. Microbial Remediation. Heavy metals cannot fully be degraded or eliminated by the microorganisms, however, their translocation and transformation can be affected by microbes (Yao et al., 2012). Microbial remediation refers to the uptake, accumulation and then the transformation of heavy metals into non-toxic, harmless or valuable compounds by microorganisms (Wang et al., 2017). Several mechanisms and techniques used in the process mainly depend on the combinations of van der Waal forces, redox interactions, electrostatic relations and extracellular covalent bonding which vary highly according to the types of microorganisms (Aly et al., 2018).

Heavy metals can be taken up from soil by microorganisms either actively or passively. Due to the fact that the presence of polysaccharides, lipids and proteins in the microbial cell walls; heavy metal ions can be bound to various functional groups including hydroxyl and carboxylate (Girma, 2015).

Certain microorganisms that naturally inhabit in soil have the ability to utilize various compounds which are toxic to the environment and living organisms. These microorganisms include

bacteria, algae, fungi and yeast (Vieira et al., 2017). Certain algae and bacteria release specific excretions that can allure and bind metal ions in the soil. Due to the ability of microorganisms to survive in extreme conditions such as drought, high or low temperatures, acidic and/or anaerobic conditions; they can be used in removing or remediating hazardous compounds (White et al., 1997).

The essential requirement for microorganisms to degrade heavy metals in soil is an energy and a carbon source (Vidali, 2001). Therefore, the microorganisms that use metal ions as final electron acceptor have the ability to contribute the removal of heavy metals from the environment (Aly et al., 2018). These microorganisms can be isolated from nearly every environmental condition and either indigenous or extraneous and could be used in bioremediation process in the removal of heavy metals. (Prescott, 2002).

2.2.1.1.1. Microbial Remediation by Fungi. Fungi stand for the essential component of soil food web due to their ability to provide nutrients for other living organisms in the soil (Rhodes, 2013). Myco-remediation refers to the use of fungi in bioremediation (Baldrian, 2003). Fungi form a structure called hyphae that can interact with soil particles, roots, rhizosphere and rocks which help them to survive under harsh conditions such as acidity, high heavy metal concentration, high/low temperature and pH (Anand et al., 2006). Mycorrhizal fungi also produce oxalate crystals that could immobilize and transform heavy metals into non-toxic forms (Gadd et al., 2014).

Various species of fungi have been shown the ability to remove heavy metals from contaminated soils. Among these, species of Ascomycetes and Basidiomycetes have been reported most frequently for heavy metal removal from polluted soils (Narendrula-Kotha and Nkongolo, 2017). Filamentous hyphae of mycorrhizal fungi play a vital role in chelation or adsorption of heavy metal due to its ability to penetrate into the deep soil. Apart from that, release of root exudates induces the formation of metal complexes along with providing nutrient and energy sources that used by microbial communities hence serve a function in the alteration of metal bioavailability (Aly et al., 2018). Fungi belonging to the genus *Aspergillus* such as *A. flavus* and *A. niger* have the ability to reduce and degrade heavy metals (Bennett et al., 2013). Moreover, fungi from genus *Aspergillus* and *Rhizopus* which extracted from heavy metal polluted soil; have been reported as silver lining for the biosorption of Cd^{++} and Cr^{++} (Zafar et al., 2007). It has been shown that marine fungi have also high tolerance to excessive concentrations of lead and copper (Gazem and Nazareth, 2013).

Fungal endophytes also have the ability to enhance the tolerance of host plant under abiotic and biotic stresses such as heavy metal contamination. Fungal endophytes are in symbiotic relationship with host plant and are able to form an association without harming the plant (Rahim et al., 2017). Fungal endophytes possess a promising future in phytoremediation due to their heavy metal tolerance which could increase the uptake and extraction of a pollutant (Firmin et al., 2015). They have been isolated from diversified species of plants including herbaceous crops and woody species representing their global existence in many higher plants (Chen et al., 2014, 2010). In a study conducted by Soleimani et al. (2010), *Festuca arundinacea* and *Festuca pratensis* inoculated with fungal endophytes (*Neotyphodium* spp.) in soil contaminated with Cd resulted in higher production yield and increased Cd accumulation compared to the plants not inoculated with endophytes. The toxic effect of heavy metals can be alleviated by heavy metal resistant endophytic fungi via biochemical processes which take place in the plant tissue through the generation of various hormones including phytohormones that aid the host plant to cope with heavy metal toxicity. Moreover; metal extraction, growth of host plants and biomass production can be enhanced by heavy metal resistant endophytic fungi (Rahim et al., 2017).

2.2.1.2. Phytoremediation. Phytoremediation, a specific form of bioremediation, involves plants to cleanse the nature due to their ability to absorb, detoxify and accumulate contaminants from soil, water and air via physical, chemical or biological processes (Buendía-González et al., 2010). Green plants such as herbs (e.g. *Thlaspi caerulescens*, *Brassica juncea*) and woody species (e.g. *Salix* spp., *Populus* spp.) are used in phytoremediation due to their ability to accumulate, uptake or transform environmental pollutants including heavy metals, organic and radioactive compounds (Tahir et al., 2016). Phytoremediation which includes natural mechanisms, is an eco-friendly and cost-effective process compared to physical and chemical remediation techniques. Moreover, phytoremediation lowers the risk of contaminants spread and is practicable for the revegetation of areas with diversified contaminants (Cristaldi et al., 2017).

The efficiency of phytoremediation process is affected by several soil and plant factors including the exudates of plants and microbes, the physical and chemical properties of the soil, metal bioavailability, the capability of plant species to uptake, accumulate, translocate, sequester and detoxify metals (Jabeen et al., 2009). The root system of plants is the main tool that prevents toxicity for the uptake of contaminants. Due to the deep root distribution in soil, high surface area is provided which can absorb and accumulate contaminants in addition to water and essential nutrients (Raskin et al. 2000). Plants with high efficiency of phytoremediation processes are named hyperaccumulators.

Such plants can accumulate and tolerate heavy metals in soil 100 times more than non-accumulating plants, however, the production of biomass is not high (Van Oosten and Maggio, 2015). Various species of plants have the ability to accumulate lead, cadmium, chromium and arsenic in their tissues (Tangahu et al., 2011). Biomass production affects the extraction efficiency of contaminants; if biomass increases the quantity of accumulated metals will also increase, however, more harvests will be required as a result (Sharma and Pandey, 2014).

The main drawback of phytoremediation is the requirement of long time to lower the metal concentration in soil to the levels that are non-toxic to the biota and the environment as a result of the restricted growth and low biomass of hyperaccumulator plants (McGrath and Zhao, 2003; Peuke and Rennenberg, 2005). However, the lack of interference to the ecosystem, requirement of low labor, inexpensiveness, the applicability of in situ and being well accepted by inhabitants are stated as advantages of the process (Cristaldi et al., 2017).

There are several mechanisms of phytoremediation depending on the type of pollutants and soil characteristics. Phytostabilization, rhizodegradation, phytofiltration, phytodegradation and phytovolatilization processes are used in the removal of organic contaminants whereas phytovolatilization, phytoextraction, phytofiltration and phytostabilization are used in the removal of inorganics (Tangahu et al., 2011).

Phytoextraction, i.e. phytoaccumulation is the process in which plant roots take up and translocate metals that are present in the soil into the above ground biomass (shoot, leaves etc.). The mechanism is based on the absorption, concentration and precipitation of toxic metals from polluted soils by hyperaccumulator plants in order to remove pollution from the area (Taylor et al. 2012). Due to the high levels of uptake and translocation by approximately 400 species of plants; nickel, zinc and copper are the most suitable metals for the removal by phytoextraction (Etim, 2012). Schematic representation of phytoextraction mechanism is demonstrated in Figure 2.1.

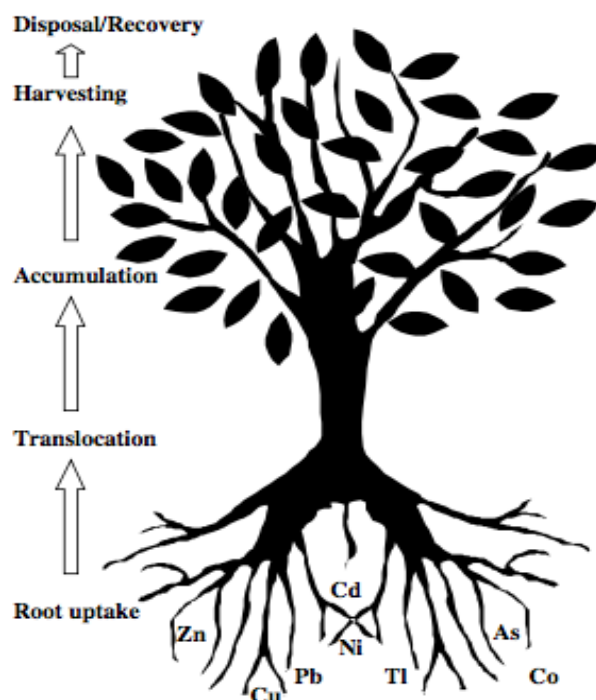


Figure 2.1. Schematic representation of phytoextraction of metals (Alkorta et al., 2004).

Phytostabilization refers to the reduction of the bioavailability of both organic and inorganic contaminants through stabilization, immobilization and prevention of their migration by root exudates (Tangahu et al., 2011; Taylor et al., 2012). The immobilization of contaminants in soil occur via absorption by plant roots or precipitation within the rhizosphere (Vara et al., 2003).

Phytovolatilization is the process in which the inorganic contaminant is absorbed by plant and later on volatilized so that the less toxic form could be released to the atmosphere (Tangahu et al., 2011; Taylor et al., 2012). Metals such as selenium, arsenic and mercury can be transformed into the volatile forms including dimethyl selenide and subsequently evaporated into the atmosphere (Muthusaravanan et al., 2018).

Phytofiltration is the use of plants to remove contaminants from surface and ground waters. The process includes rhizofiltration and blastofiltration; the former is the use of plant roots while the latter is the use of plant seedlings for the removal (Ali et al., 2013). The plant roots/seedlings are used to adsorb, absorb and/or precipitate metal contaminants or excess nutrients that are present in aqueous solution outlying root zone (Wuana and Okieimen, 2011). Studies have shown that rhizofiltration can be used for the removal of Pb, Cd, Cu, Ni, Zn and Cr (USEPA, 2000).

Rhizodegradation, i.e. phytostimulation refers to the breakdown of contaminants in soil through the activity of bacteria, fungi and yeasts present in the rhizosphere (Cristaldi et al., 2017). The number of microorganisms in the rhizosphere is significantly higher than that in the surface due to the presence of several nutrients including sugars and amino acids (Tangahu et al., 2011). Plants stimulate the growth of microorganisms in the rhizosphere by releasing root exudates which are carbon and nitrogen source for microorganisms. Also, plant roots provide a great surface area for both microbial growth and the transfer of oxygen. The process is suitable for the removal of organic contaminants including polycyclic aromatic hydrocarbons (PAHs), pesticides, benzene and toluene from the soil (Etim, 2012).

Phytodegradation refers to the degradation of organic contaminants through the action of plant metabolism or enzymes following the uptake of contaminants by plant roots (Tangahu et al., 2011). The related enzymes are dehalogenase, peroxidase, nitroreductase, nitrilase and phosphatase (Winqvist et al., 2014). The process is feasible for the degradation of organic contaminants including herbicides, insecticides and chlorinated solvents along with inorganic nutrients (Jabeen et al., 2009).

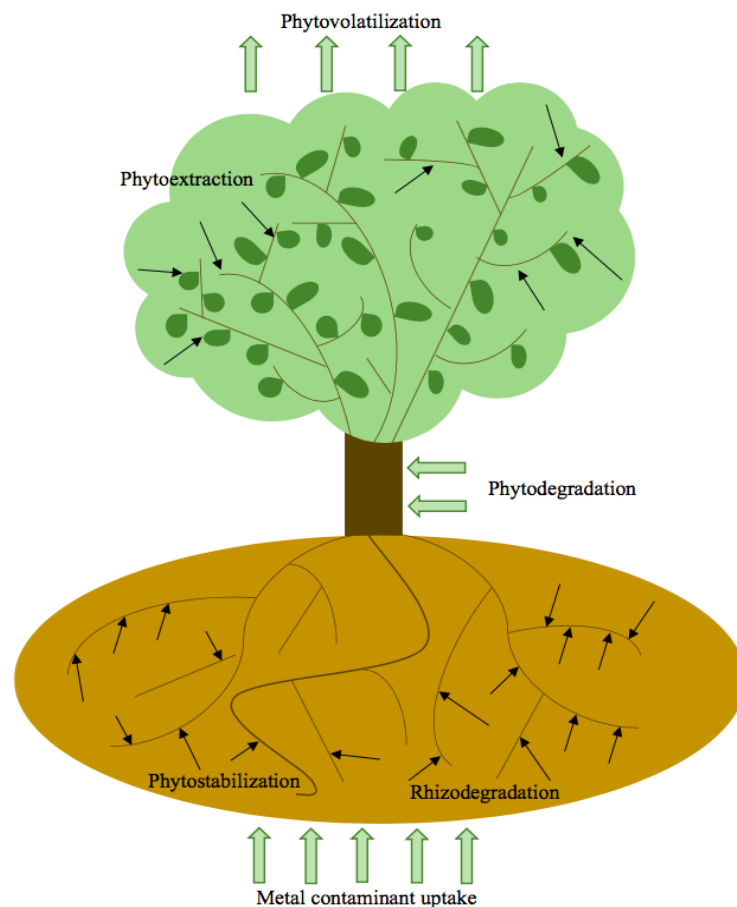


Figure 2.2. Representation of metal uptake by plant through phytoremediation processes.

2.3. Arbuscular Mycorrhizal Fungi (AMF)

The region of the soil in contact with the roots of a plant is called rhizosphere. It contains many microorganisms and its composition is affected by root activities. In another words, it is an environment under the influence of plant. The rhizosphere is the zone surrounding the roots of plants in which complex relations exist among the plant, the soil microorganisms and the soil itself (Symanczik et al., 2014). Among these microorganisms, mycorrhizal fungi which have emerged approximately 450 million years ago, have the ability to form a symbiotic relationship with host plants which facilitate the phytoremediation process (Mbodj et al., 2018). The relationship is based on fungi supporting plant growth by allowing increased uptake of essential elements such as nitrogen, phosphorus, potassium, calcium, copper, zinc while obtaining simple sugar and carbon compounds from the plant (Leung et al., 2013; Oyewole et al., 2017). The structures called hyphae which helps to increase the absorption of inorganic phosphorus and nitrogen is formed by mycorrhizal fungi in soil complex (Mbodj et al., 2018).

Mycorrhizas have been classified into two major groups depending on the level of the interaction with the root system; ectomycorrhizas and endomycorrhizas (Mbodj et al., 2018). Ectomycorrhizas involve the fungi from Ascomycota and Basidiomycota phylum in which fungal hyphae encircles the roots, however, do not enter the root tissue (Martino et al., 2000). On the other hand, endomycorrhizas consist of ericoid, orchid and arbuscular mycorrhizas which can penetrate and develop within root tissues. Among these, arbuscular mycorrhizas (AMs) which are included in Glomeromycota phylum are the most widespread mycorrhizas. Arbuscular mycorrhizal fungi have been divided into four orders; Diversisporales, Glomerales, Archaeosporales and Paraglomerales (Redecker et al., 2013).

Arbuscular mycorrhizal fungi associate with more than 80% of terrestrial plants by colonizing in the root cortex and developing an external mycelium in the root zone. Therefore, AMF enhance the growth of host plants, develop resistance to pathogens, and increase the levels of proteins, lipids and sugars. Geo et al. (2018) have shown that *Sorghum bicolor* L. plants inoculated with AMF (*Glomus intraradices*) in sand resulted in higher shoot and root height as well as the increase in the number of leaves. Pre and post treatment images of plants are demonstrated in Figure 2.3. (Geo et al., 2018). Shao et al. (2018) have reported that mixed- AMF inoculation (*Claroideoglomus etunicatum*, *Diversispora spurca*, *Diversispora versiformis*) to cv. Fuding Dabaicha seedlings significantly increased the total root length and volume compared to non-AMF treatment. Whole root morphology and root hairs are demonstrated in Figure 2.4. AMF have been shown to protect plants from heavy

metal toxicity by forming an interaction between metals and rhizosphere (Chen et al., 2007). It has been shown that plants living in symbiosis with AMF have better survival under conditions such as drought, toxicity etc. due to their higher access to nutrients and water (Christie et al., 2004). Moreover, AMF are responsible for the improvement of soil structure with the formation of external hyphae and its excretion (glomalin) and for the maintenance of plant biodiversity and ecosystem stability (Chen et al., 2007). Due to the fact that AMF are obligate symbionts, they require reciprocal interaction with plants in order to fulfil their life cycle (Bago and Bécard, 2002).



Figure 2.3. Positive growth effect of AMF in *Sorghum bicolor* L. plants (A: pre-treatment, B: post treatment) (Geo et al., 2018).

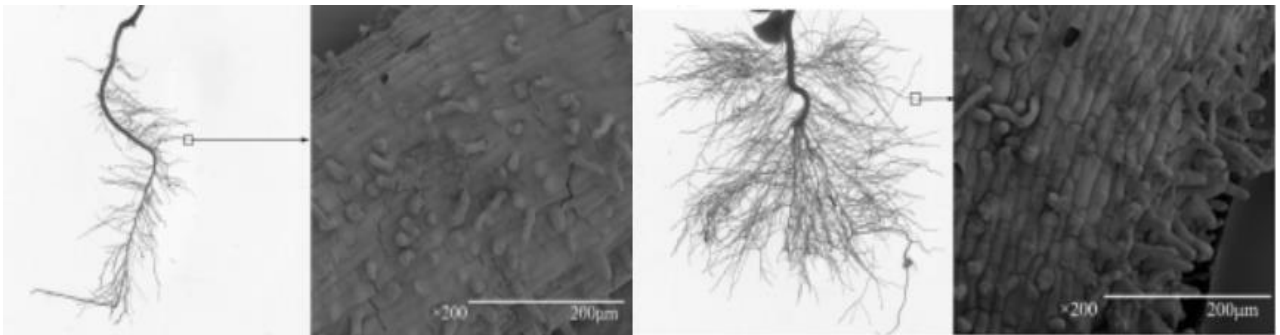


Figure 2.4. Representation of root morphology of cv. Fuding Dabaicha inoculated with mixed-AMF and non-inoculated (Shao et al., 2018).

AMF can endure various climates and tolerate extreme conditions including drought, acidity, aridity and soils contaminated with heavy metals (Miransari, 2011; Wu et al., 2010). The studies have shown that AMF are able to decrease the effects of metal toxicity to host plants through inducing interaction between root zone and heavy metals (Chen et al., 2004; Zhu et al., 2001). Heavy metals can be bound to the cell walls of AMF, compartmentalized in the vacuole or chelated in cytoplasm,

accordingly their mobilization into the plant is restricted (Leyval et al., 1997).

The fungal hyphae can utilize resources which are non-utilizable for plant roots by themselves. Therefore, AMF play a crucial role in the detoxification, accumulation and toleration of metals (Gaur and Adholeya, 2004). Consequently, AMF are significantly considerable solution for the revegetation of soils contaminated with metals (Khan, 2005). Wang et al. (2009) have shown that *Glomus versiforme* and *Glomus mosseae* promoted the white clover root growth and nitrogen absorption in conditions similar to coal mine tailings, hence white clover inoculated with AMF could be used in the restoration of coal mine.

The primary role of AMF in supporting plant growth in stressed conditions is linked with their ability to provide especially phosphate and trace elements to host plants (Jakobsen et al., 2002). Hence, AMF have a significant place in revegetation of heavy metal contaminated lands including mine tailing ponds other than the selection of proper plant species (Chen et al., 2007). The implementation of AMF alongside plants have numerous advantages compared to the use of hyperaccumulating plants alone. Accordingly, the application of both AMF and plants is the most up and coming green remediation method of our age (Wei et al., 2016).

AMF stands for the most important regulators of plant productivity in heavy metal polluted soils (Wei et al., 2015). Park et al. (2016) have reported that AMF spore communities in the mining area of Korea were remarkably influenced by the accumulation of heavy metals including As and Zn. Also, due to the fact that mycorrhizal symbiosis with host plants is affected by higher concentrations of heavy metals, the rates of mycorrhizal colonization in the mining area were found to be greater than that in the forest area. Solís-Domínguez et al. (2011) have reported that revegetation of mine tailings can be supported by the inoculation of AMF. Sánchez-Castro et al. (2017) have shown that AMF colonization occur in *F. arvernensis* and *K. vallesiana* species and concluded that these plants have an adaptation to high heavy metal concentrations in mine tailings. They have also reported that AMF diversity is inversely proportional to the heavy metal concentration. Zhao *et al.* (2015) have demonstrated that AMF colonization which obtained by the inoculation of *Rhizophagus intraradices* species enhanced the drought tolerance and development of maize (*Zea mays* L.) in coal mine tailings.

2.4. Identification of AMF

2.4.1. Morphological Identification

Arbuscular mycorrhizae are composed of two defined morphological types; *Arum*-type and *Paris*-type. *Arum*-type fungi form structures called intercellular hyphae and intracellular arbuscules which the former is in between cortical cells while the latter is inside them. *Paris*-type fungi create comprehensive intracellular hyphae and arbuscules (branched structures) within root cortex (Cavagnaro et al., 2010). The intercellular phase of colonization is not included in the *Paris*-type. Smith and Smith (1997) reported that *Arum*-type is formed by 30 families while *Paris*-type is formed by 41 families and 21 families include members of both types. All types of AMF are characterized by the formation of vesicle. The demonstrations of hyphae, vesicle and arbuscule is shown in Figure 2.5. (Akhtar et al., 2014).

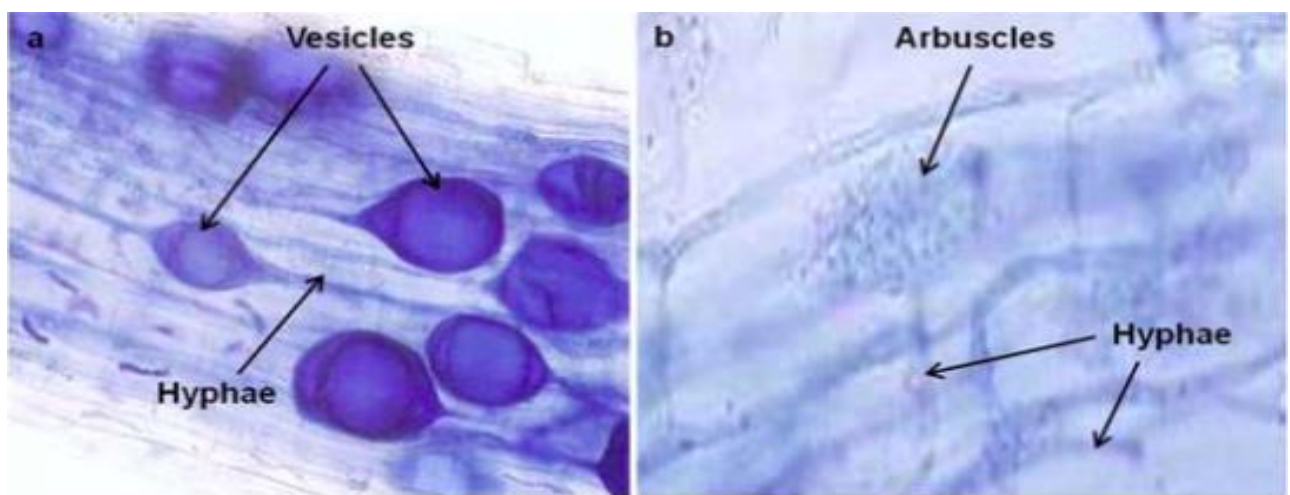


Figure 2.5. Microscopic observation of arbuscular mycorrhizal fungi (Akhtar et al., 2014).

Studies conducted on various plant and fungal symbiosis have shown that morphological type of AMF is mainly depends on plant species (Smith and Read, 2010). However, it has been demonstrated that the fungal genome also plays a role in the morphology of AMF. Several studies have reported that colonization with various fungal species have shown certain variance in the morphology of AMF in the same plant. Dickson et al. (1999) have shown that *Daucus carota* (carrot) inoculated with *G. mosseae* formed intercellular hyphae while *Daucus carota* inoculated with *Glomus coronatum* formed comprehensive intracellular coils and arbuscules.

2.4.2. Molecular Identification

There are more than 160 species of arbuscular mycorrhizal fungi (AMF) that has been classified recently. AMF belong to the Glomeromycota phylum currently which is composed of 4 orders and

10 families (Silva et al., 2013).

DNA-based techniques for the identification of AMF have started with the studies of White et al. (1990) and improved significantly since then (Buck et al., 2019). Several methods have been carried out to identify AMF diversity including molecular techniques, root colonization pattern, and morphological characteristics (Gardes and Bruns, 1993). However, molecular techniques have become the standard for the identification of AMF (Gorzalak et al., 2012). The identification and diversity analysis of AMF have been performed by using the morphological characters of AMF spore as the main tool in the traditional method (Del Val et al., 1999). Due to the fact that certain AMF species do not differ significantly in spore wall characters and the morphology of spore diversifies in particular phases of spore formation, molecular methods have arisen for the community structure studies of AMF which result in more precise data than traditional method (Krishnamoorthy et al., 2015).

High efficiency sequencing technologies including 454 amplicon pyrosequencing is frequently used for the identification of microbial communities due to its high performance (De Beenhouwer et al., 2014; Lekberg et al., 2012). The nuclear ribosomal RNA (rRNA) operon is also often used for molecular identification of AMF based upon its great resolution ability across extensive taxonomic groups (Schoch et al., 2012). However, closely related species could not be identified by this region (Stockinger et al., 2010). Accordingly, Stockinger et al. (2010) suggested a region of 1500 bp consisting of a segments of the small subunit (SSU) rRNA gene and large subunit (LSU) rRNA gene, and ITS region for DNA barcoding of AMF. Using the Internal Transcribed Spacer (ITS) region in AMF species is the general method for the molecular identification of AMF. Schoch et al. (2012) have suggested the internal transcribed spacer (ITS) region as the standard fungal barcode. Two variable non-coding regions are present in ITS and primers have been designed in the aim of binding these regions. The sequences on the 18S rRNA and the 28S rRNA are the binding regions of these primer sets and the ITS region in higher fungi is amplified by that (Gardes and Bruns, 1993). Various primers and techniques have been used in order to amplify the ITS region. Examination of the diversity of AMF is obtained by using the ITS primer set. AMF communities have been investigated by various primer pairs that used to amplify various fragments of SSU rRNA. NS31-AM1 primer pair stands for the first pair that has been used for the investigation of AMF by 454 pyrosequencing (Öpik et al., 2009). However, it has been shown that the species of AMF from Ambiosporaceae, Archaeosporaceae and Paraglomeraceae families could not be detected by this primer set (Daniell et al., 2001). It has been reported that AMV4.5NF and AMDGR primer set has amplified and detected

a wider region of Glomeromycota phylum in comparison to NS31-AM1 primer pair (Lumini et al., 2010). However, Lee et al. (2008) have reported that more reliable results are obtained by using AML1 and AML2 primer set when compared to other primer pairs. It is reported that the AML primers bind to the 18 rRNA gene in all identified AMF species.

3. MATERIALS AND METHODS

Five native plants were collected randomly during the target mine site survey in Tavsanlı mine tailings. The plants were randomly observed upon their healthy and well-grown. Those plants were identified as; *Cynodon dactylon*, *Elymus repens*, *Trifolium alexandrinum*, *Sudangrass* and *Festuca arundinacea*. Besides, several soil samplings were obtained immediately next to plants that were grown in mine tailing area (field soil). The soil samples were obtained and used in the experiment in order to determine and identify the AMF species they host by trap culture. Collected plants were used as a potential AMF inoculum and attached hyphae fragments to the rhizosphere. Trap culture method in order to obtain healthy spores of mycorrhizal fungi was applied in this study. Due to their ability to trap fungi spores from other plants' rhizosphere (Oehl et al., 2005), *Sorghum bicolor* L. seeds were employed as host plant. The growth in accordance with a living host plant inoculated with field soil in pots in a greenhouse is required for the propagation of cultures of arbuscular fungi (Velázquez and Cabello, 2011).

3.1. Soil and Native Plant Sampling

The soil and native plants that used for the experiments were obtained from a coal mine tailing area in Tavsanlı, Kütahya. The city is located in the north of Aegean region, Turkey.

3.2. Digestion Method

The mine-tailing soil was taken as ~0.25 gr triplicates and these triplicates were digested with 9 mL of 65% nitric acid, 1 mL of 35% hydrogen peroxide and 1 mL of distilled water under 800 psi pressure at 1600 watt and 180°C via MARS 6 Microwave Accelerated Reaction System Instrument (CEM), USA. Ramp time and hold time were 5.25 and 11 minutes, respectively. The method was adapted from USEPA 3052 (USEPA, 1996). Following the digestion process, the volume of samples was completed to 50 mL with distilled water.

3.3. Metals Analysis of Soil Samples

Analysis of the metals in the soil was carried out by using ICP-OES method after the digestion process in order to determine the concentrations of Cr, Mn, Fe, Ni, Cu, Zn, Al, Cd, Pb, Si, Co and

Mo in the soil.

3.4. Germination Assay

The germination test was carried out in order to determine the germination capacity of *Sorghum bicolor* L. seeds in heavy metal contamination. The seeds were obtained from Turkish Commercial Farming Business located in Izmir. The seeds were washed with 70% ethanol and tap water, respectively. The cycle was repeated 3 times and lastly the seeds were washed with distilled water for one minute. Whatman No.4 filter papers were cut in the shape of petri dishes and put inside them. The distilled water was added to petri dishes enough to cover 1/3 of *Sorghum bicolor* L. seeds. Fe and Al in concentrations of 700 ppm and 100 ppm, respectively were added to the 4 petri dishes among 8 in order to simulate the mine tailing conditions. The petri dishes were covered with aluminum foil and black nylon. Germination and growth of seeds were monitored for one week.

3.5. Cultivation of Plants and Mycorrhizal Fungi Community

Two trap culture systems were initiated in the greenhouse of Bogazici University in order to get more material for fungal DNA. The initial trap culture system was set up on 25th of August in 2017 while the second trap culture system was planted on 13th of June in 2018. First of all, stream sand was sterilized in oven under 151°C for 20 hours and left cooling for 2 days. The seeds of *Sorghum bicolor* L. were used as a host plant. The process started with washing seeds with 70% ethanol solution and distilled water, respectively. The cycle was repeated three times. Afterwards pots with 0.7L of volume were filled with approximately 500g of sand. Then, 50 mL of distilled water was added to each pot. 1-2 mm pieces of length of root samples from native plants and 10g of the field soil that each of the plants grow in were added to each pot. Afterwards, seeds of *Sorghum bicolor* L. were sown into each pot and covered with sand. The representation of each pot is shown in Figure 3.1.

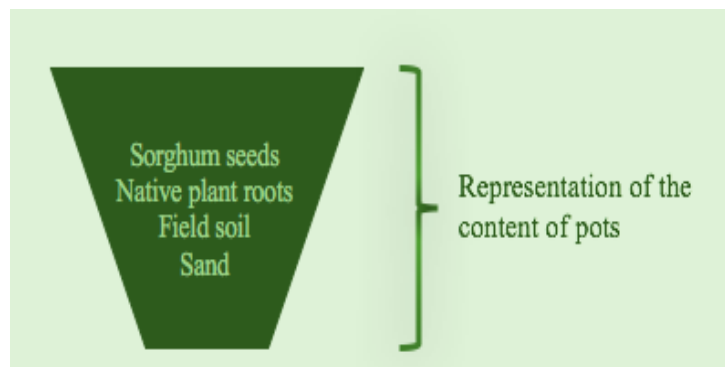


Figure 3.1. Schematic representation of the content of each pot in trap culture systems.

In order to complete the cultivation process, 50mL of Modified Strullu-Romand (MSR) medium was added to pots in order to provide nutrients to support plant growth. The content of the medium is demonstrated in Table 3.1.

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

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

The initial trap culture system included six parallels of each native plant as inoculum source therefore 30 pots were cultivated in total. The initial trap culture system were carried out for 12 weeks. *Sorghum bicolor* L. plants on the 1st and 12th week of plantation are shown in Figure 3.2. Distilled water was added to each pot three times a week throughout 12 weeks, however, nutrient solution was added to pots in every 4 weeks. Several morphological features such as plants height was measured every week and recorded in order to observe if *Sorghum bicolor* L. plants were sufficiently healthy to form a symbiotic relationship with AMF.



Figure 3.2. The pots on the 1st day (left) and 12th week (right) of plantation from initial trap culture system.

Second set of the trap culture system was cultivated on the 13th of June, 2018 in order to obtain possible AMF spores which might be present in the rhizosphere. This could provide us more precise results and obtain more material for fungal DNA. The steps from the initial trap culture system were followed in cultivation process, however, rather than native plant roots the roots of *Sorghum bicolor* L. plants from the initial trap culture system were used as inoculum. Trap culture system was carried out for 12 weeks with the same irrigation conditions as the initial trap culture system. *Sorghum bicolor* L. plants on the 1st and 12th week of plantation are shown in Figure 3.3.



Figure 3.3. *Sorghum bicolor* L. plants on the 1st week (left) and 12th week (right) of cultivation from second trap culture system.

3.6. Plant Harvesting and AMF Sampling

After 16 weeks of plantation, *Sorghum bicolor* L. plants were harvested and pots were covered with black nylon bags in both initial and second trap culture systems as demonstrated in the Figure 3.4. The pots were left in the dark for two months prior to DNA extraction in order to increase the propagation of AMF spores.



Figure 3.4. Pots covered with black nylon bags.

Root staining process was carried out for each pot of every native plant inoculum from the initial trap culture system in order to examine the presence of AMF morphologically. However, due to the higher root development in the second trap culture system, only the small part of roots was used for root staining instead of the whole pot. Initially, root samples were preserved in 70% ethanol solution at 4°C prior to root staining. The staining process was carried out in following order; each root sample carefully packed in tulle and kept in 10% KOH solution (w/v) for 4 hours in water bath at 60°C. This procedure was held to clean the roots by extracting tannins from root samples and dissolving them in KOH solution. The roots wrapped in tulle and placed in falcon tubes containing %10 KOH solution are shown in Figure 3.5.

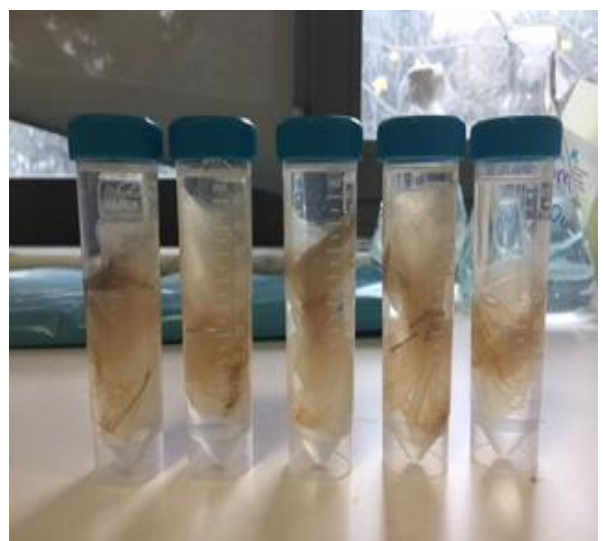


Figure 3.5. Representation of roots packed in tulle (*left*) and kept in KOH solution in falcon tubes for every native plant inoculum (*right*).

Afterwards, roots were contacted with 1% HCl solution for 3 minutes and rinsed carefully with distilled water. The roots then were kept in 0.05% (w/v) trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) for 4 days (Phillips & Hayman, 1970). This staining method eliminates the fungi other than Arbuscular mycorrhizal fungi. After staining process is completed all root samples handled carefully and aligned on glass slides horizontally for microscopic examination. Microscopic images of each slide from both initial and second trap culture systems are demonstrated in Figure 3.6.

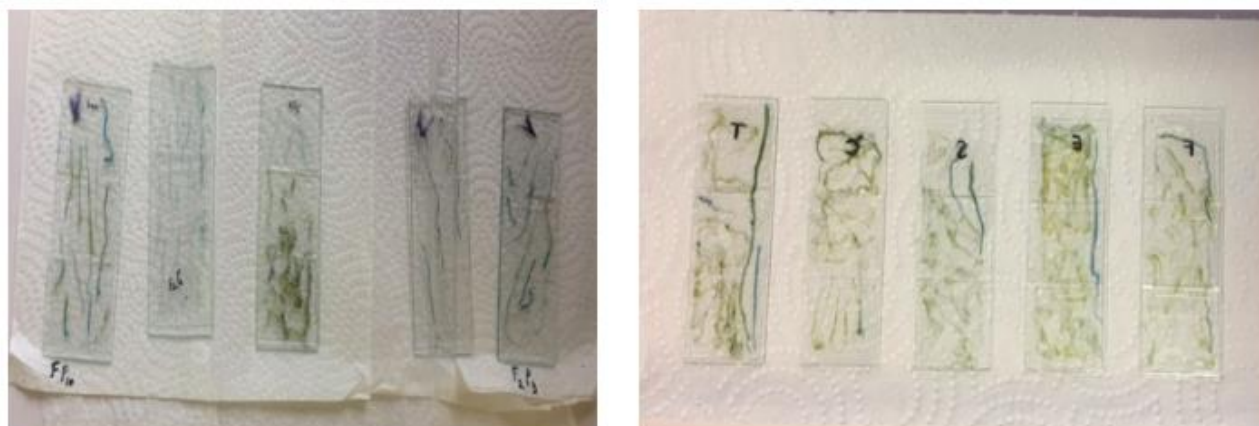


Figure 3.6. Prepared slides for the determination of mycorrhization in the initial trap culture system (*left*) and in the second trap culture system (*right*).

3.7. Fungal DNA Extraction from Host Plants Rhizosphere

Zymo Research Quick-DNA™ Fungal/Bacterial Miniprep Kit was used for both initial and second trap culture systems in order to extract fungal DNA from *Sorghum bicolor* L. roots. Primarily, the roots were cut into 1-2 mm pieces of length. 100mg of roots were put into ZR BashingBead™ lysis tubes and 750 µl Lysis Solution was added to tubes. The sliced roots from both trap culture systems prior to DNA extraction in lysis tubes are demonstrated in Figure 3.7. Subsequently, the tubes were run at 6000 RPM for 2 minutes by using MagNA Lyser Instrument from Roche in order to crash roots. Following the homogenization, the tubes were centrifuged at 12000 RPM for 1 minute via Hettich® Mikro 120 Centrifuge. 400 µl supernatant was transferred into Zymo-Spin™ IV Spin Filter in a Collection Tube and was centrifuged at 10000 RPM for 1 minute. 1200 µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube from the previous step. Later on, 800 µl of the mixture from the last step was transferred into Zymo-Spin™ IIC Column³ in a Collection Tube and was centrifuged at 12000 RPM for 1 minute. The flow was discarded from the Collection Tube and the previous step was repeated for remaining 800 µl mixture. Afterwards, 200 µl of DNA Pre-

Wash Buffer was added to the Zymo-Spin™ IIC Column³ in a new Collection Tube and was centrifuged at 12000 RPM for 1 minute. Following that, 500 µl g-DNA Wash Buffer was added to the Zymo-Spin™ IIC Column³ and was centrifuged at 12000 RPM for 1 minute. Then, Zymo-Spin™ IIC Column³ was transferred into a sterile 1.5 ml microcentrifuge tube and was centrifuged at 12000 RPM for 1 minute. Subsequently 50 µl of DNA Elution Buffer was added directly to the column matrix. After 2 minutes of waiting, the microcentrifuge tube was centrifuged at 12000 RPM for 1 minute. The procedure was optimized from the Manufacturer's instructions with a few differences. IMPLEN Nanophotometer® P360 was used to check the quality of isolated DNAs.

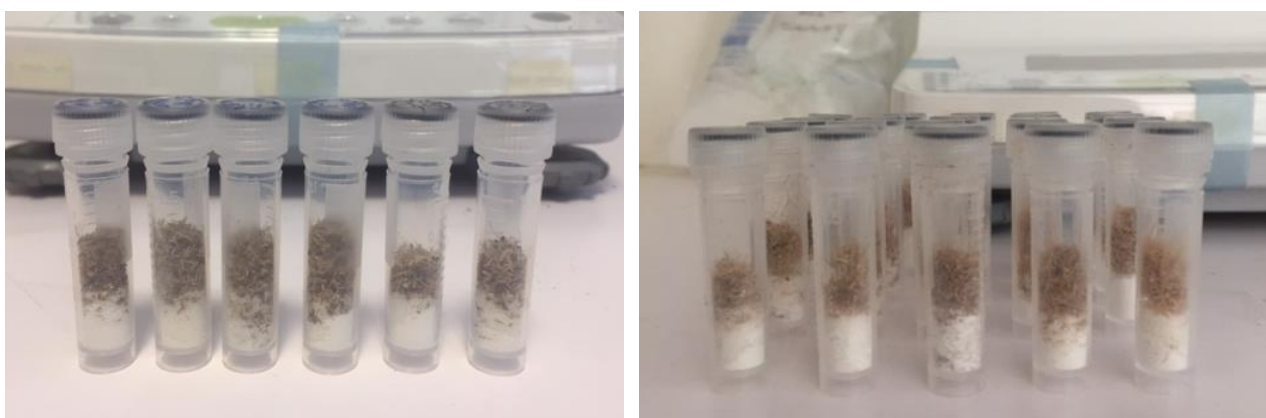


Figure 3.7. Sliced roots prior to DNA extraction from initial (*left*) and second (*right*) trap culture systems.

3.8. Polymerase Chain Reaction

Polymerase chain reaction (PCR) which is the technique to make many copies of specific DNA region (Focke et al., 2011) was applied to every sample following the DNA extraction. AML1 (forward) and AML2 (reverse) primers were used to identify arbuscular mycorrhizal fungi. The exact sequences of primers are shown in Table 3.2.

Table 3.2. The sequences of AML1 and AML2 primers.

AML_F	ATCAACTTTCGATGGTAGGATAGA
AML_R	GAACCCAAACACTTTGGTTTC

MyTaq HS DNA polymerase from Bioline was used in PCR procedure which was held for 40 cycles. Each PCR mix was prepared with 36.5 µl of nuclease free water, 10 µl of reaction buffer, 1 µl of each forward and reverse primers, 0.5 µl of sample DNA and 0.5 µl of Taq polymerase. Techne

TC-512 Gradient Thermal Cycler was used for all PCR experiments. The PCR cycle applied to DNAs from both initial and second trap culture system was as follows;

95°C, time: 5 minutes.	} 40 cycles
95°C, time: 30 seconds	
56°C, time: 30 seconds	
72°C, time: 20 seconds	
72°C, time: 5 minutes	

3.9. Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out following the PCR application in order to determine the length of found DNA fragments.

PCR products from the initial trap culture system was run on the gel prepared as 1% (w/v) with agarose and 1x TBE as buffer solution. Large and small gel tanks were used for all PCR products from the initial trap culture system. 210 mL of TBE solution was added to the 2.1 gr of agarose in order to prepare the agarose gel for large gel tank which is Sub-Cell® GT Cell from Bio-Rad. The solution was microwaved until the agarose is completely dissolved. Ethidium bromide (EtBr) was added to 0.5µg/mL final concentration when the agarose solution cooled down. EtBr is known to be its ability to visualize DNA in the agarose gel under ultraviolet (UV) light (Guo et al., 2017). Following it the solution was poured into the gel tray with the well comb inside. The comb was removed carefully after 20-30 minutes of waiting for agarose gel to solidify completely and the gel was placed into the tank. 5x DNA Loading Buffer Blue-Bioline was used to load DNA samples into the wells. 5 µl of PCR products were mixed with 1 µl of loading dye properly before loading samples to the agarose gel. GeneON 50 bp DNA ladder was used as a marker in the gel. The gel picture of the ladder is indicated in Figure 3.8. PCR products were run at 140V for 90 minutes. 50 mL of TBE solution was added to the 0.5 gr of agarose for the preparation of agarose gel used in small gel tank which is Thermo EC Minicell Primo EC-320. The same steps were followed as large tank, however, PCR products were run at 90V for 60 minutes in the small tank. Thermo EC 250-90 Electrophoresis Power supply was used as a source of power for electrophoresis. Molecular Imager® Gel Doc™ XR+ System from Bio-Rad was used for imaging both small and large gels.

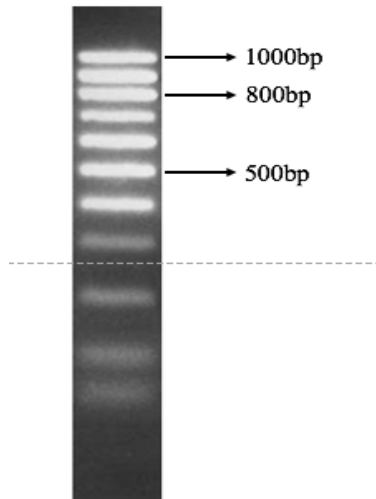


Figure 3.8. The demonstration of bands and their equivalent base pairs in the ladder used for the PCR products from the initial trap culture system.

PCR products from the second trap culture system were run on the gel prepared as 1% (w/v) with agarose and 1x TAE as buffer solution. EtBr was added to 0.5 μ g/mL final concentration in order to visualize DNA in the agarose gel. Only small gel tanks were used for every PCR product in the second trap culture system. HyperLadder™ 100bp 100 lanes from Bioline was used as a marker. The gel image of the ladder in gel is shown in Figure 3.9. All PCR products in the second trap culture system was run at 120V for 60 minutes. Thermo EC 135-90 Electrophoresis Power supply was used as a source of power for electrophoresis. Molecular Imager® Gel Doc™ XR+ System from Bio-Rad was used for imaging gels.

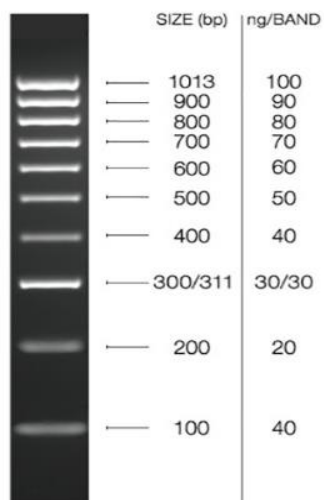


Figure 3.9. The demonstration of bands and their equivalent base pairs in the ladder used for the PCR products from the second trap culture system.

3.10. Metagenomic Analysis of Fungal Community

3.10.1. Purification of DNA

200 mg of sample was transferred into tubes containing glass beads with 0.1 mm of diameter and 300 µl of lysis buffer (200 mM Tris-HCl, pH 8.0; 20mM EDTA, 10% TritonX-100) and homogenized at 6000 RPM for 1 minute. The sample was transferred into a new tube in order to be separated from glass beads and incubated for 15 minutes while adding 10 µL of lysozyme solution (200 µg/ µL) to the new tube. During the incubation, 250 µL of digestion buffer (0,5 µg/µL Proteinase K, %5 Tween® 20, 3M Guanidine thiocyanate, 20 mM Tris-HCl, pH 8.0) was added to the sample and initially incubated at 70°C for 15 minutes later on at 95°C for 5 minutes. Over the course of incubation 250 µL of 2-propanol was added to the tube content and silica was loaded to the column. The sample was passed through the column by centrifuge at 13000 RPM for 1 minute so that present DNAs are hold in the silica column. Subsequently the sample was washed with washing solution (20 mM NaCl, 2 mM Tris-HCl, pH 8; 80% v/v Ethanol). The silica column was dried by centrifugation. DNAs that were hold in the silica column were taken from the column with 50 µL 100 mM Tris-HCl that prepared with nuclease-free, sterile deionized water (pH 7). The sample were stored at -20 °C prior to analysis. Following the purification process, the quality of DNA was assessed by spectrophotometric methods. DNAs with OD₂₆₀/OD₂₈₀ rate between 1.8-2.0, OD₂₆₀/OD₂₃₀ rate between 2.0-2.2 and the concentration of at least 10 ng/µL were chosen to be used for further analysis.

3.10.2. Next Generation Sequencing

In this study genome groups which have been determined by High Resolution Melting (HRM) were analysed by the combination of DNAs from each group to classify AMF in rhizosphere of each inoculum. The pair of primers to construct amplicon libraries targeted the region of 16S rRNA gene with the length of 460bp which embodies V3-V4 region (Klindworth et al. 2013). Connector DNA sequences were added to the 5' ends of target specific primer pairs for the compatibility of constructed library with Illumina adapter sequences. 16S rRNA and target specific primer-connector sequences were 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' for forward and reverse primers, respectively. The first PCR was applied with "Bio-Speedy® 2X qPCR HRM Fusion Mix" and 200 nm of each primer. The cycle was as following by using Biorad CFX Connect (Bio-Rad, USA);

95°C, time: 3 minutes
 95°C, time: 30 seconds
 55°C, time: 30 seconds
 72°C, time: 30 seconds
 72°C, time: 5 minutes

} 25 cycles

PCR product was run on agarose gel in order to verify the size (~550 bp) and purified with “Bio-Speedy® PCR Product Cleaning Kit” (Bioeksen, Turkey).

Illumina adapter sequences and binary index were inserted into the first purified PCR sample with second PCR procedure by using Nextera XT Index Kit (Illumina, USA). The cycle was as following;

95°C, time: 3 minutes
 95°C, time: 30 seconds
 55°C, time: 30 seconds
 72°C, time: 30 seconds
 72°C, time: 5 minutes

} 8 cycles

PCR product was purified with “Bio-Speedy® PCR Product Cleaning Kit” (Bioeksen, Turkey). “Bioanalyzer DNA 1000 chip” was used in order to verify the size (~630 bp). Last library was diluted to 4 nM by using 10 mM Tris pH 8.5 and aliquots of 5 µL were mixed to construct library pools. Library pools were denatured with NaOH, diluted with hybridization buffer (HT1) and denatured with temperature for the preparation of cluster construction and sequencing prior to MiSeq sequencing. “Illumina MiSeq v3” reaction kits were used in the run. Minimum %5 PhiX was added to each reaction as control.

Unprocessed sequence data were mined and analysed by using Mothur 1.36.1 version. Initially, the sequences of index and primers were cut and then novel sequences were identified. The novel sequences were aligned by RDP database sequences (<https://rdp.cme.msu.edu/>) and blast algorithm. RDP database sequences were cut in order to contain only V3-V4 region prior to the alignment. Unaligned sequences present in each end of sequences were removed by filtration method and error-checking was performed. The impurity was inhibited by pre-clustering. UCHIME (Edgar et al., 2011) code was used for chimera screening. The sequences were classified by Bayesian classifier. The

reference and taxonomy data were obtained from RDP database. After the selection of Operational Taxonomic Unit (OTU) and taxonomic identification according to RDP database, OTUs were clustered with regard to their phylotypes.

4. RESULTS

4.1. Metal Analysis of the Rhizosphere Soil Samples

Metals analysis of the rhizosphere soil samples was performed by using ICP-OES method after the digestion process. The concentrations of Cr, Mn, Fe, Ni, Cu, Zn, Al, Cd, Pb, Si, Co and Mo in the soil was determined. The results are given in Table 4.1. It has been noted that the soil was high in concentrations of Al and Fe compared to other metals.

Table 4.1. The results of soil metal analysis obtained by ICP-OES method.

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

4.2. Germination Assay

Sorghum bicolor L. seeds were put into eight petri dishes; four of them contained Fe and Al solutions stimulating mine tailings conditions and the next four dishes contained only distilled water as the control treatment. The growth of seeds was examined for one week and it was concluded that *Sorghum bicolor* L. seeds could germinate in high concentrations of Al and Fe hence in the soil used for experiments. Seeds on the first day of germination assay is shown in Figure 4.1.

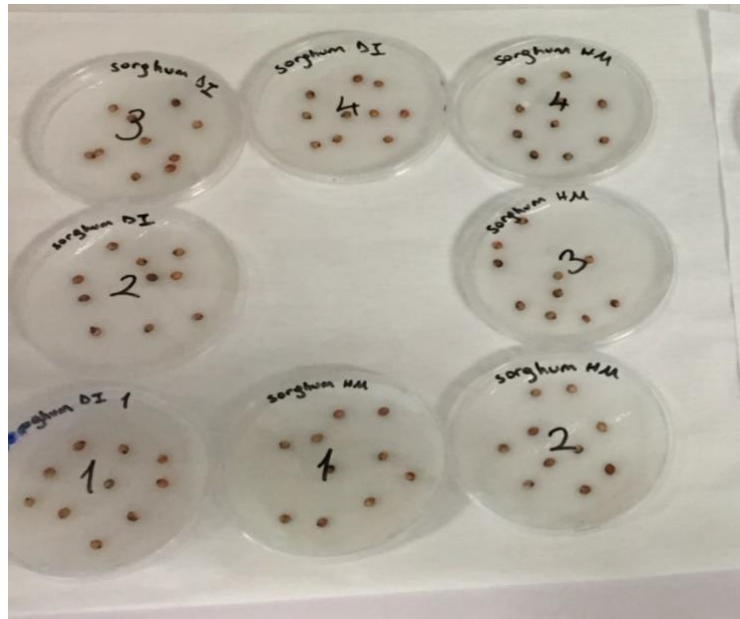


Figure 4.1. Petri dishes containing *Sorghum bicolor* L. seeds (Dishes labelled as DI only contained distilled water while dishes labelled as HM contained Fe and Al solutions).

4.3. Heights of *Sorghum bicolor* L. Plants

The heights of *Sorghum bicolor* L. plants in associated with rhizosphere of every native plant as inoculum source were measured weekly. The results indicated that plants were healthy enough to form symbiosis with AMF. The average height of *Sorghum bicolor* L. plants in every native plant inoculum was in between 30-35 centimeters on the 12th week of plantation. The related graphs are given in Figure 4.2., Figure 4.3. and Figure 4.4.

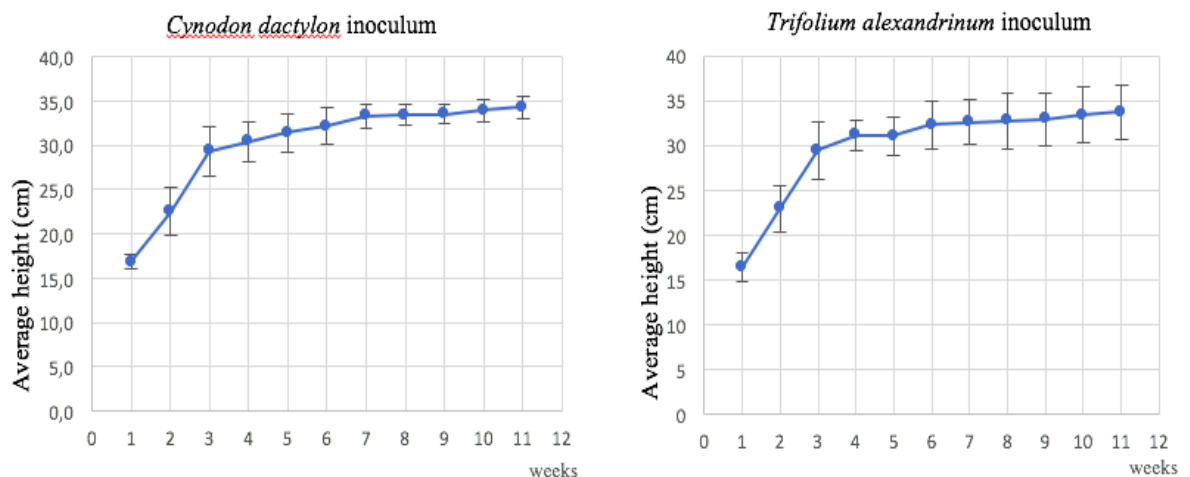


Figure 4.2. The average height of *Sorghum bicolor* L. plants in *Cynodon dactylon* inoculum (left) and *Trifolium alexandrinum* inoculum (right).

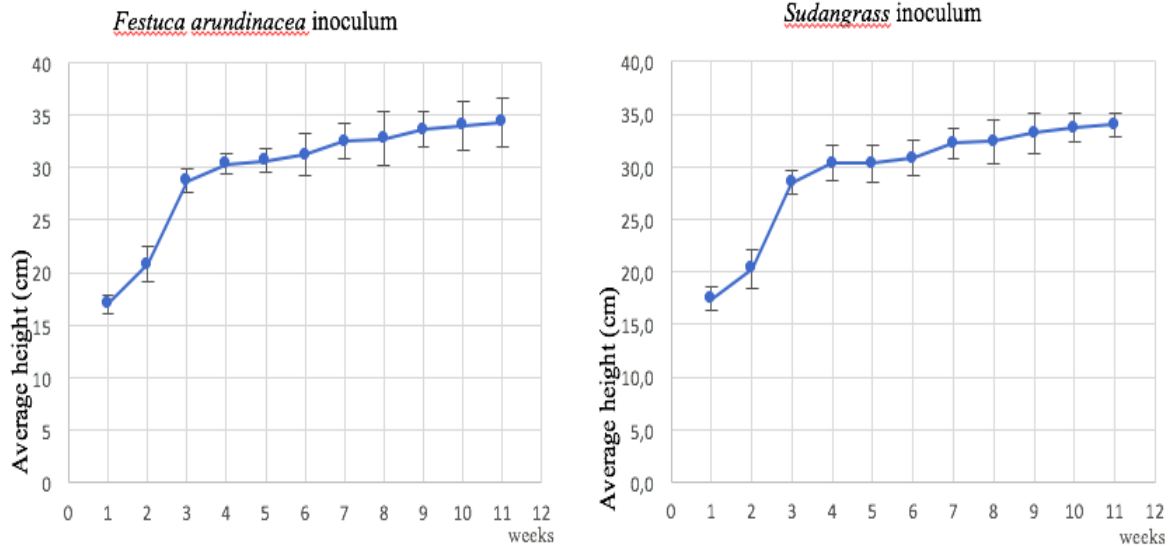


Figure 4.3. The average height of *Sorghum bicolor* L. plants in *Festuca arundinacea* inoculum (left) and *Sudangrass* inoculum (right).

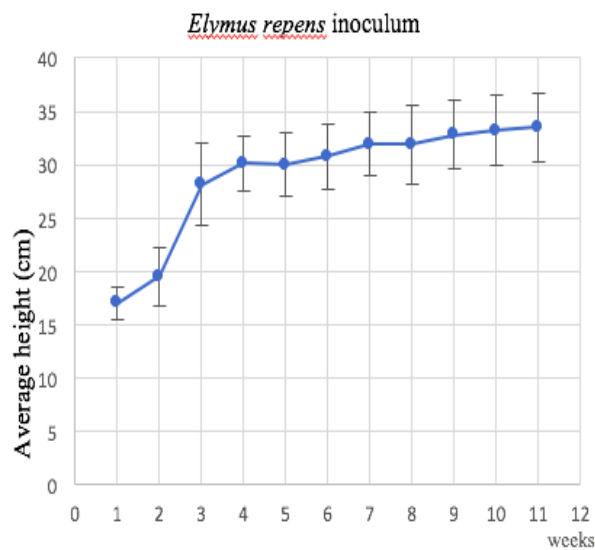


Figure 4.4. The average height of *Sorghum bicolor* L. plants in *Elymus repens* inoculum.

4.4. Root Staining

In order to examine the presence of AMF in the roots of *Sorghum bicolor* L. plant, roots were kept in 10% KOH solution prior to root staining. Roots then were contacted with distilled water for 3 minutes, 1% HCl for 3 minutes and distilled water for 3 minutes. Following the process roots were kept in 0.05% (w/v) trypan blue in lactoglycerol and aligned on glass slides for the microscopic examination. Various forms of AMF were observed in both trap culture systems in each native plant

inoculum as expected. The microscopic images of *Sorghum bicolor* L. roots in inoculums of *Cynodon dactylon*, *Sudangrass*, *Elymus repens*, *Trifolium alexandrinum* and *Festuca arundinacea* are shown in Figure 4.5., Figure 4.6., Figure 4.7., Figure 4.8. and Figure 4.9., respectively.



Figure 4.5. Spore and vesicular forms of AMF in roots of *Sorghum bicolor* L. in *Cynodon dactylon* inoculum from the initial (*left*) and second (*middle and right*) trap culture systems.

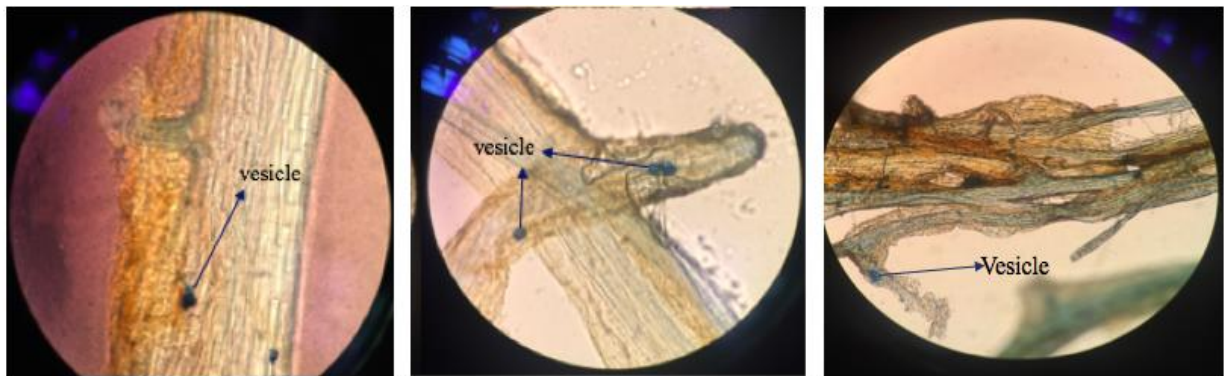


Figure 4.6. Vesicular forms of AMF in roots of *Sorghum bicolor* L. in *Sudangrass* inoculum from the initial (*left*) and second (*middle and right*) trap culture systems.

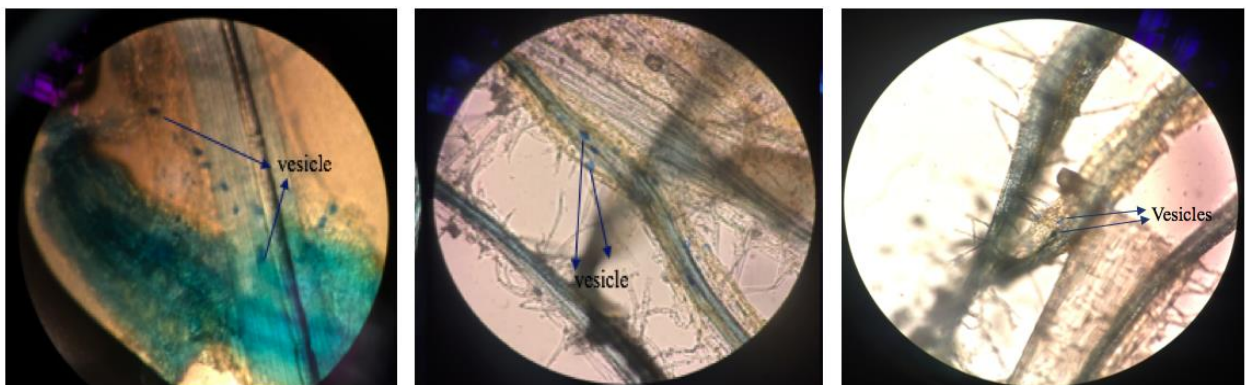


Figure 4.7. Vesicular forms of AMF in roots of *Sorghum bicolor* L. in *Elymus repens* inoculum from the initial (*left*) and second (*middle and right*) trap culture systems.

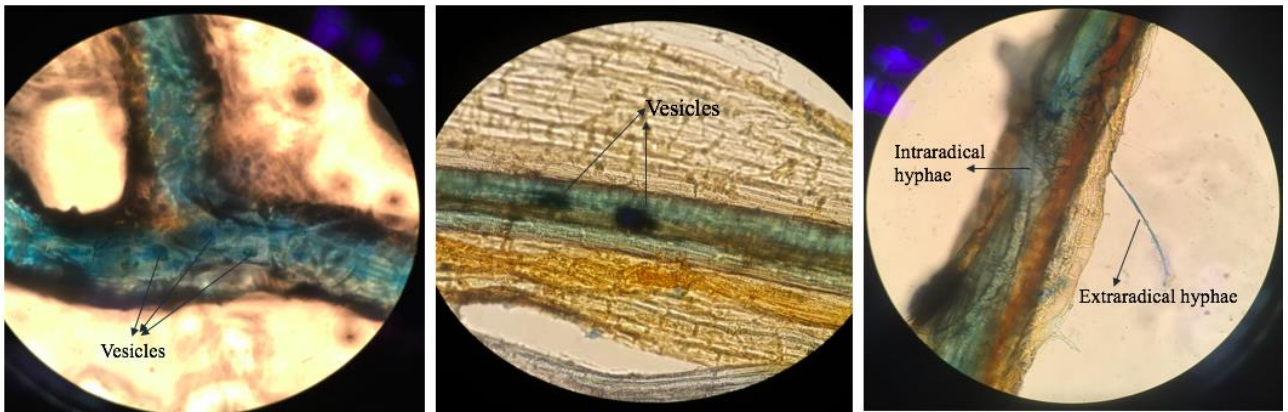


Figure 4.8. Vesicular forms, intraradical and extraradical hyphal structure of AMF in roots of *Sorghum bicolor* L. in *Trifolium alexandrinum* inoculum from the initial (*left*) and second (*middle and right*) trap culture systems.

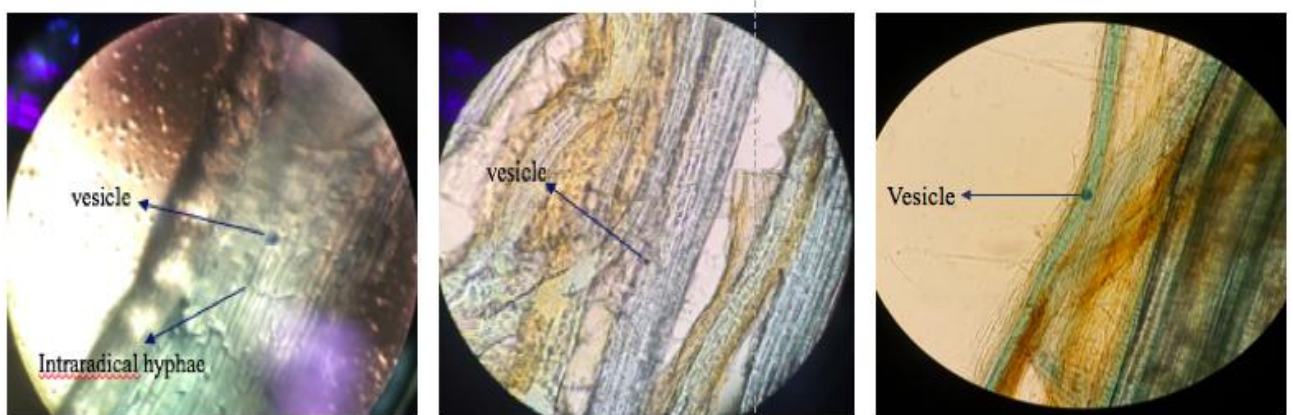


Figure 4.9. Vesicular forms and intraradical hyphal structure of AMF in roots of *Sorghum bicolor* L. in *Festuca arundinacea* inoculum from the initial (*left*) and second (*middle and right*) trap culture systems.

4.5. Agarose Gel Electrophoresis

Following the extraction of fungal DNA from roots of *Sorghum bicolor* L., DNA was copied by PCR process which is based on the use of ability to Taq DNA polymerase to synthesize new strands. Then PCR products were run on agarose gel in order to determine the length of amplified DNA fragments. The amplified sequence length targeted by AML1 and AML2 primers by PCR was 795bp, therefore it was expected to observe a band around 800bp on the agarose gel. The images of PCR products that run on agarose gel from initial trap culture system are demonstrated in Figure 4.10. while of that from second trap culture system is demonstrated in Figure 4.11. and Figure 4.12.

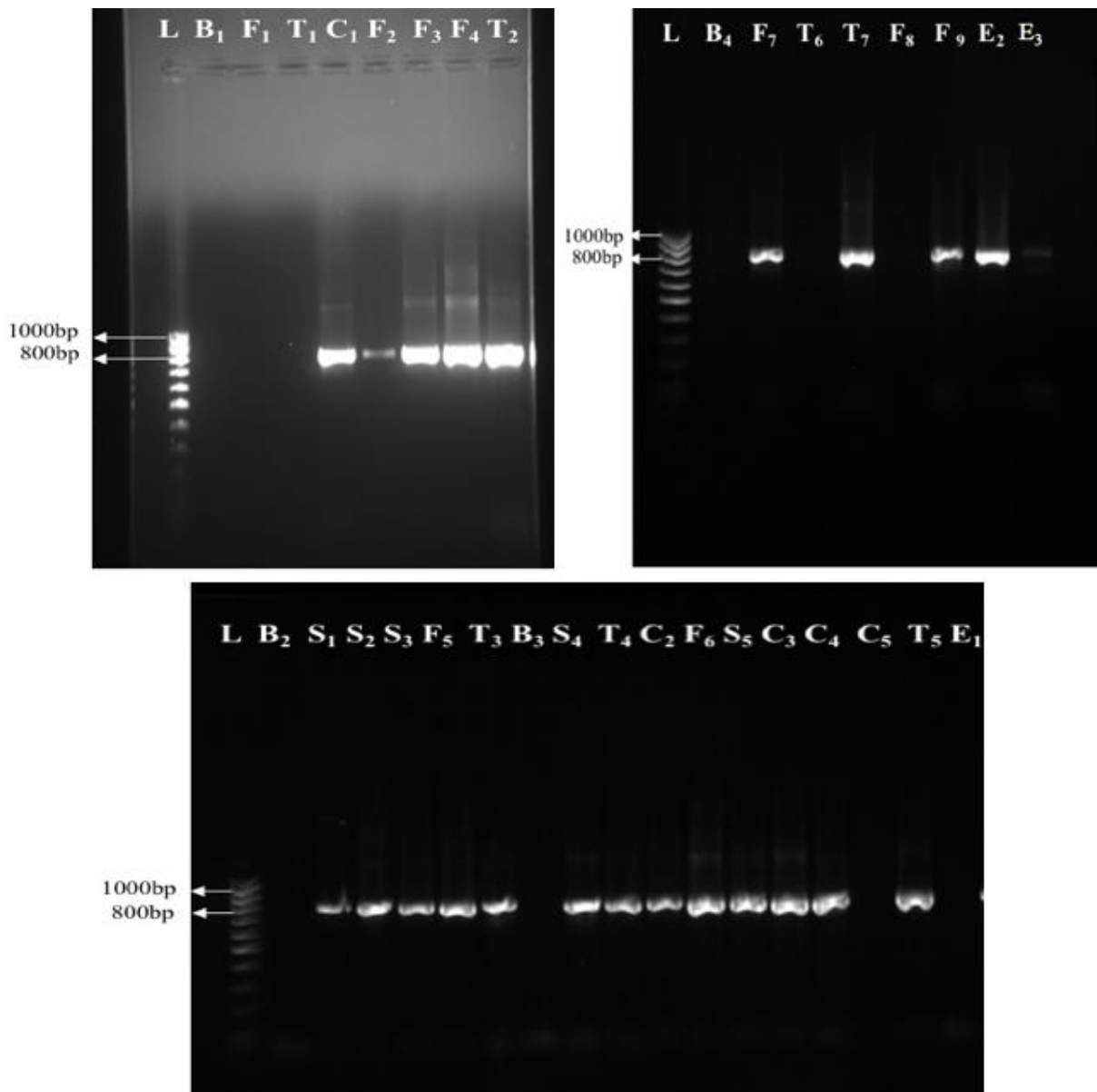


Figure 4.10. The gel images obtained from the PCR process of DNA isolates from initial trap culture system (*L*: ladder, *B*:Blank, *F*: PCR product of *Festuca arundinacea* inoculum, *T*: PCR product of *Trifolium alexandrinum* inoculum, *C*: PCR product of *Cynodon dactylon* inoculum, *E*: PCR product of *Elymus repens* inoculum and *S*: PCR product of *Sudangrass* inoculum).

Rendering to the gel images showing above, it has been indicated that every DNA that run on the agarose gel have given a band around 800bp as expected which meant that the samples did contain arbuscular mycorrhizal fungi DNA. 33 sample including 4 blanks were loaded in the gel and 23 of them created band at 800bp.

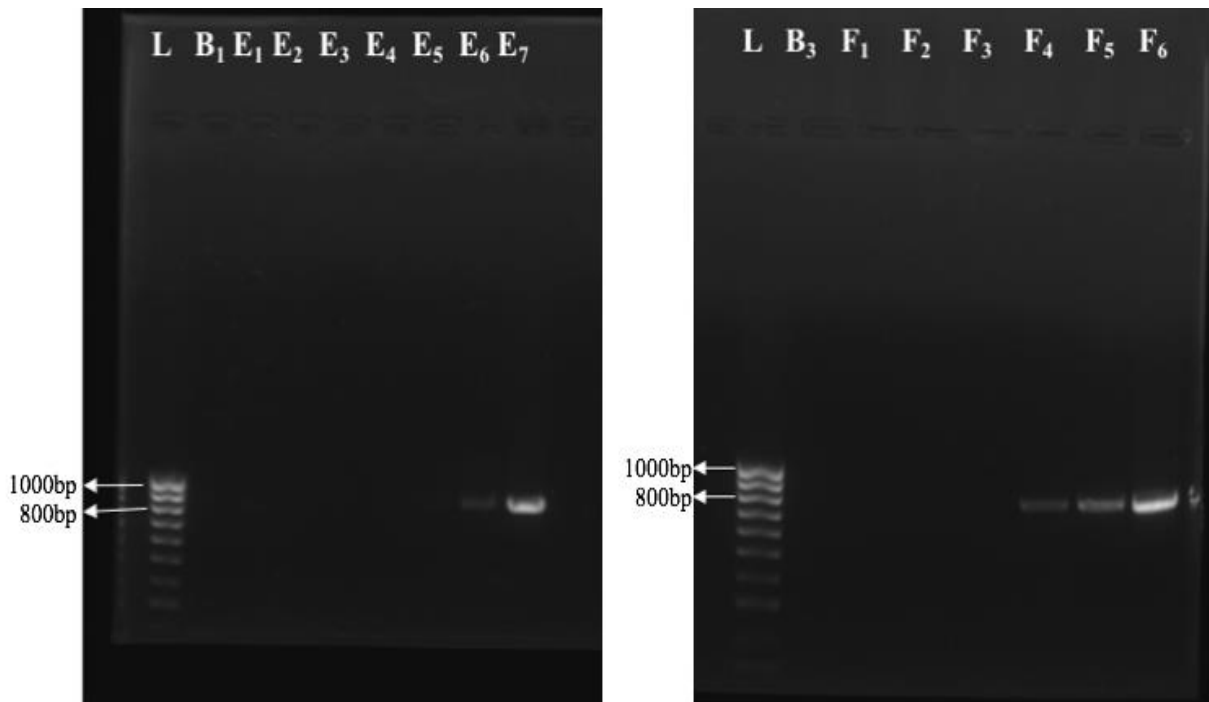


Figure 4.11. The gel images obtained from the PCR process of DNA isolates from second trap culture system (*L*: Ladder, *B*: Blank, *F*: PCR product of *Festuca arundinacea* inoculum, *T*: PCR product of *Trifolium alexandrinum* inoculum, *C*: PCR product of *Cynodon dactylon* inoculum, *E*: PCR product of *Elymus repens* inoculum and *S*: PCR product of Sudangrass inoculum).

As it observed from the gel image presented above on the left side, only the last two samples (*E*₆ and *E*₇) did create a band at 800bp however the first five samples (*E*₁, *E*₂, *E*₃, *E*₄ and *E*₅) did not. As can be seen from the gel image above on the right side, the last three samples (*F*₄, *F*₅, *F*₆) did run while the first three samples (*F*₁, *F*₂, *F*₃) did not.

As the results did not fulfill our satisfaction; it was thought that there was an experimental mistake due to possible technical error throughout the PCR process such as DNA was copied in the first place. PCR process was repeated and re-set up with same procedure with *E*₁, *E*₂, *E*₃, *E*₄, *E*₅ and *F*₁, *F*₂, *F*₃ samples more carefully. The PCR products then were run on 1% agarose gel. The image of the gel is indicated below on the right side in Figure 4.12.

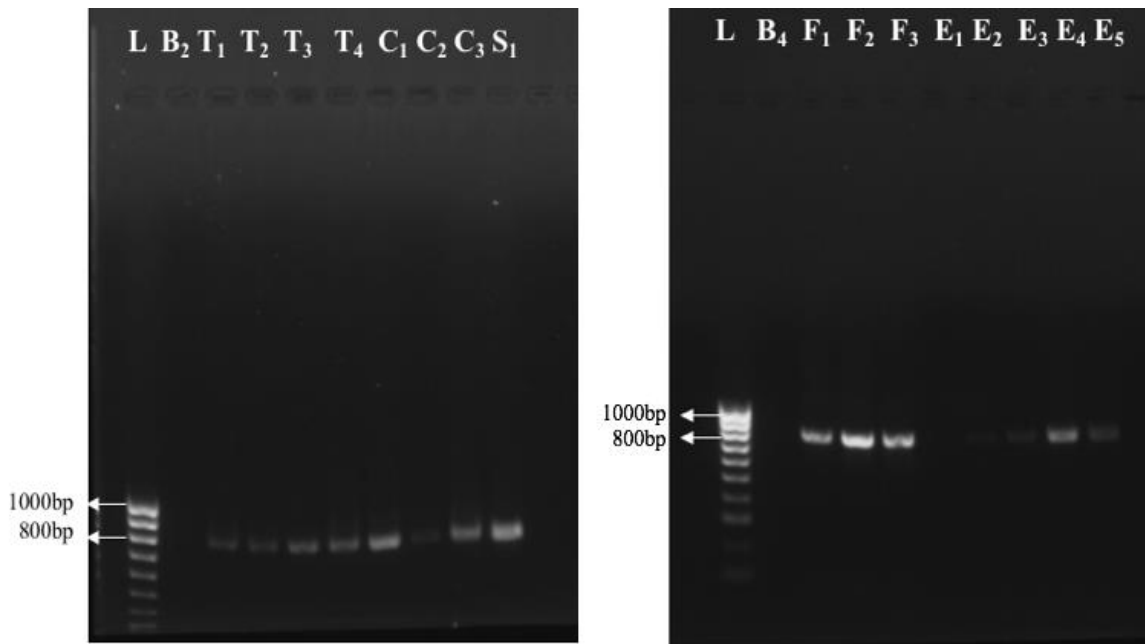


Figure 4.12. The gel images obtained from the PCR process of DNA isolates from second trap culture system (*L*: ladder, *B*:Blank, *F*: PCR product of *Festuca arundinacea* inoculum, *T*: PCR product of *Trifolium alexandrinum* inoculum, *C*: PCR product of *Cynodon dactylon* inoculum, *E*: PCR product of *Elymus repens* inoculum and *S*: PCR product of *Sudangrass* inoculum).

As indicated in the gel image placed above on the left side, every PCR product that loaded on the gel from different native plant inoculums created a band at 800bp. The gel image shown in the right side contains the second PCR products of E₁, E₂, E₃, E₄, E₅ and F₁, F₂, F₃ samples. As demonstrated above, every PCR product in the gel except for E₁ created a band around 800bp which meant that the samples did contain arbuscular mycorrhizal fungal DNA.

Following the morphological observation of AMF in the rhizosphere of host plant *Sorghum bicolor* L., molecular presence of AMF was supported by agarose gel electrophoresis. Afterwards, DNA isolates of C₁, S₁, F₂, T₃ and E₇ from second trap culture system were sent to Bioeksen R&D Technologies for metagenomics analysis of fungal community.

4.6. Metagenomic Analysis of Fungal Community Profile

Two procedures were involved in order to analyze the fungal community profile of DNA isolates obtained from roots of *Sorghum bicolor* L.; DNA purification and Next Generation sequencing which explained briefly in Materials & Methods.

Glomeromycota phylum which contains 160 genera of AMF did not identify in any of native plant inoculums even though the presence of AMF was observed both morphologically and molecularly. The situation is probably due to the amplification of NGS on DNA isolates rather than PCR products which obtained by AML1 and AML2 primers. Ribosomal DNA (rDNA) of AMF is highly polymorphic, however, for many other fungi several rDNA sequences are usually identical among a species such as Internal Transcribed Spacer (ITS). However, Fungi_unclassified is thought to be Glomeromycota phylum due to the observed presence of AMF.

4.6.1. Metagenomic Analysis of Fungal Community in *Cynodon dactylon* inoculum

The metagenomic analysis of fungal DNA obtained from *Cynodon dactylon* inoculum have been identified 3 phyla, 9 class, 19 orders, 31 families and 50 genera in total. The fungal community profile is shown in Figure 4.13.

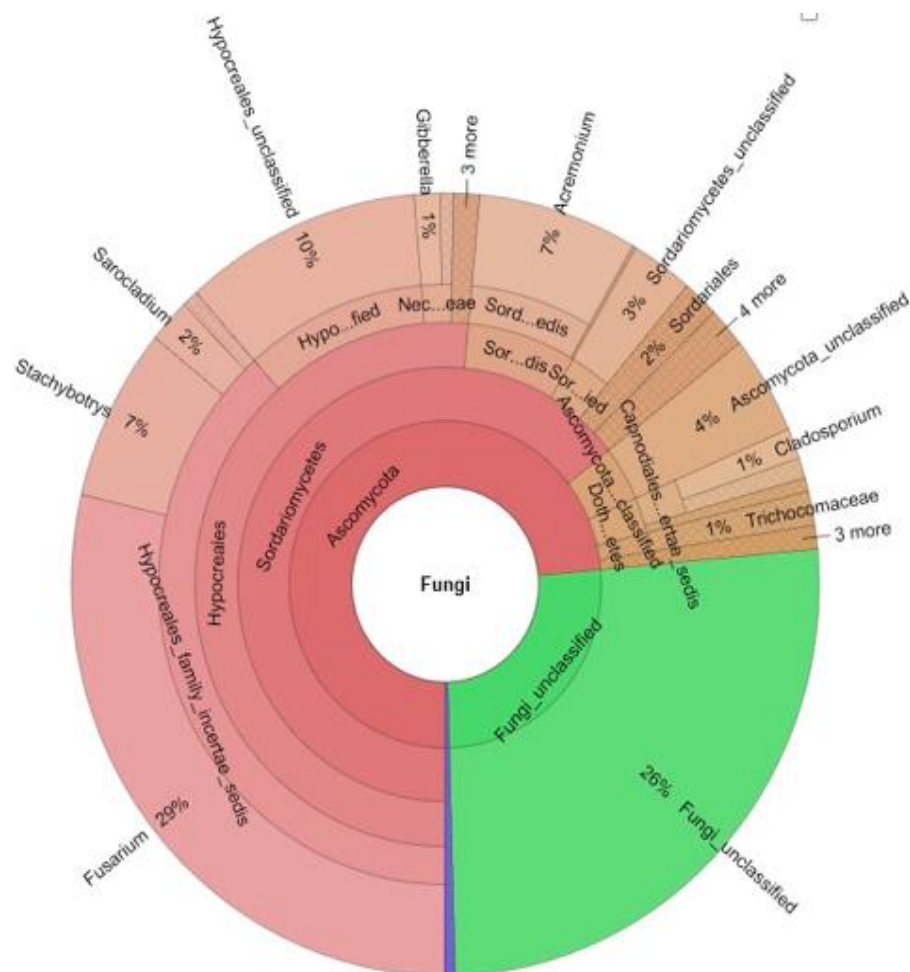


Figure 4.13. Fungal community profile of DNA extracted from *Sorghum bicolor* L. roots in *Cynodon dactylon* inoculum.

Fungi belonging to the phylum Ascomycota, Basidiomycota and Zygomycota are ectomycorrhizal fungi. As can be seen from the Figure 4.10., Ascomycota were discovered in the rhizosphere of *Sorghum bicolor* L. in *Cynodon dactylon* inoculum at high abundance along with Basidiomycota. According to comprehensive metagenomics analysis that cannot be seen on the scheme above due to relatively small abundance; ectomycorrhizal fungi orders Pezizales and Eurotiales were also discovered in the roots. Apart from that, Ceratobasidiaceae family which are ectomycorrhizal fungi were identified.

Other than ectomycorrhizal fungi, many genera of endophytes were also discovered in the roots of *Sorghum bicolor* L. These include, *Colletotrichum* spp., *Alternaria* spp., *Chaetomium* spp., *Fusarium* spp., *Cladosporium* spp. and *Penicillium* spp. As mentioned in Literature Review, fungal endophytes along with ectomycorrhizal fungi have the ability to support plant growth in stressed conditions by increasing nutrient uptake and enhancing tolerance to diseases caused by pathogens.

4.6.2. Metagenomic Analysis of Fungal Community in *Trifolium alexandrinum* inoculum

As a result of metagenomics analysis of fungal DNA obtained from *Trifolium alexandrinum* inoculum; 4 phyla, 10 classes, 18 orders, 24 families and 37 genera in total have been identified. The fungal community profile is demonstrated in Figure 4.14.

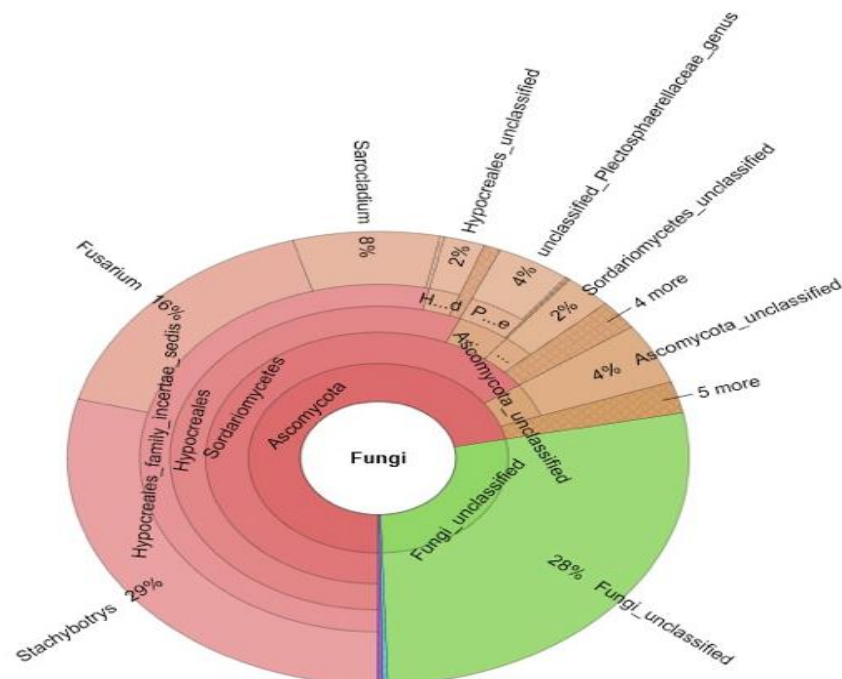


Figure 4.14. Fungal community profile of DNA extracted from *Sorghum bicolor* L. roots in *Trifolium alexandrinum* inoculum.

As given above, Ascomycota were the most abundant phylum discovered in *Trifolium alexandrinum* inoculum followed by Basidiomycota. Two different orders of ectomycorrhizal fungi were also discovered; Eurotiales and Cantharellales. Besides, Ceratobasidiaceae family (ectomycorrhizal fungi) were identified. Moreover, endomycorrhizal fungal order Mortierellales were discovered unlike *Cynodon dactylon* inoculum.

Several genera of fungal endophytes were also discovered from the rhizosphere of *Sorghum bicolor* L. in *Trifolium alexandrinum* inoculum. These include, *Colletotrichum spp.*, *Alternaria spp.*, *Fusarium spp.*, *Cladosporium spp.* and *Penicillium spp.*

4.6.3. Metagenomic Analysis of Fungal Community in *Festuca arundinacea* inoculum

3 phyla, 11 classes, 20 orders, 31 families and 49 genera in total have been identified as a result of metagenomic analysis applied to fungal DNA obtained from *Festuca arundinacea* inoculum. The fungal community profile is given in Figure 4.15.

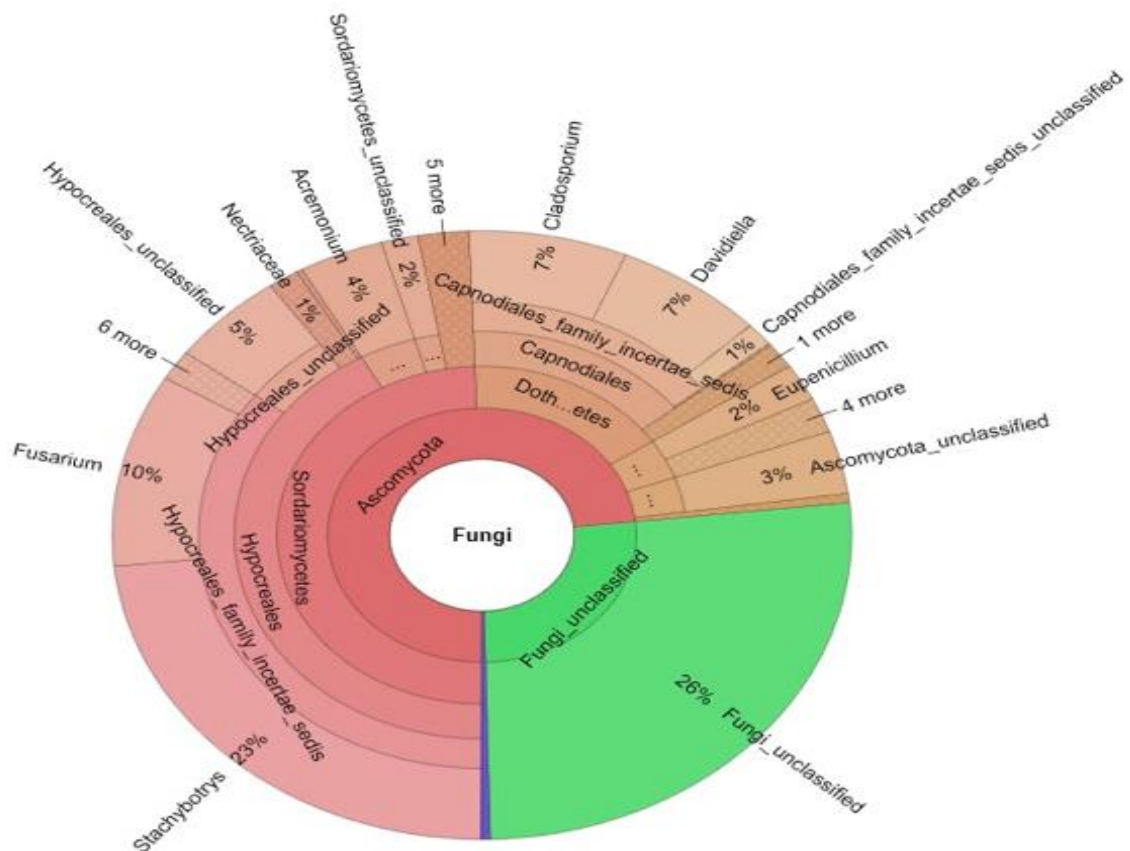


Figure 4.15. Fungal community profile of DNA extracted from *Sorghum bicolor* L. roots in *Festuca arundinacea* inoculum.

According to comprehensive metagenomics analysis; ectomycorrhizal Basidiomycota and Ascomycota phyla were discovered in the *Sorghum bicolor* L. roots in *Festuca arundinacea* inoculum with higher abundance of Ascomycota. Ectomycorrhizal fungi orders Eurotiales, Cantharellales and Pezizales were also identified. Moreover, Ceratobasidiaceae family were also discovered from the roots of *Sorghum bicolor* L. in *Festuca arundinacea* inoculum.

Six genera of fungal endophytes were also identified in *Festuca arundinacea* inoculum. These genera include; *Colletotrichum* spp., *Alternaria* spp., *Chaetomium* spp., *Fusarium* spp., *Cladosporium* spp. and *Penicillium* spp.

4.6.4. Metagenomic Analysis of Fungal Community in *Sudangrass* inoculum

As a result of metagenomic analysis of fungal DNA obtained from the roots of *Sorghum bicolor* L. in *Sudangrass* inoculum; 2 phyla, 8 classes, 16 orders, 19 families and 26 genera have been identified. The fungal community profile is shown in Figure 4.16.

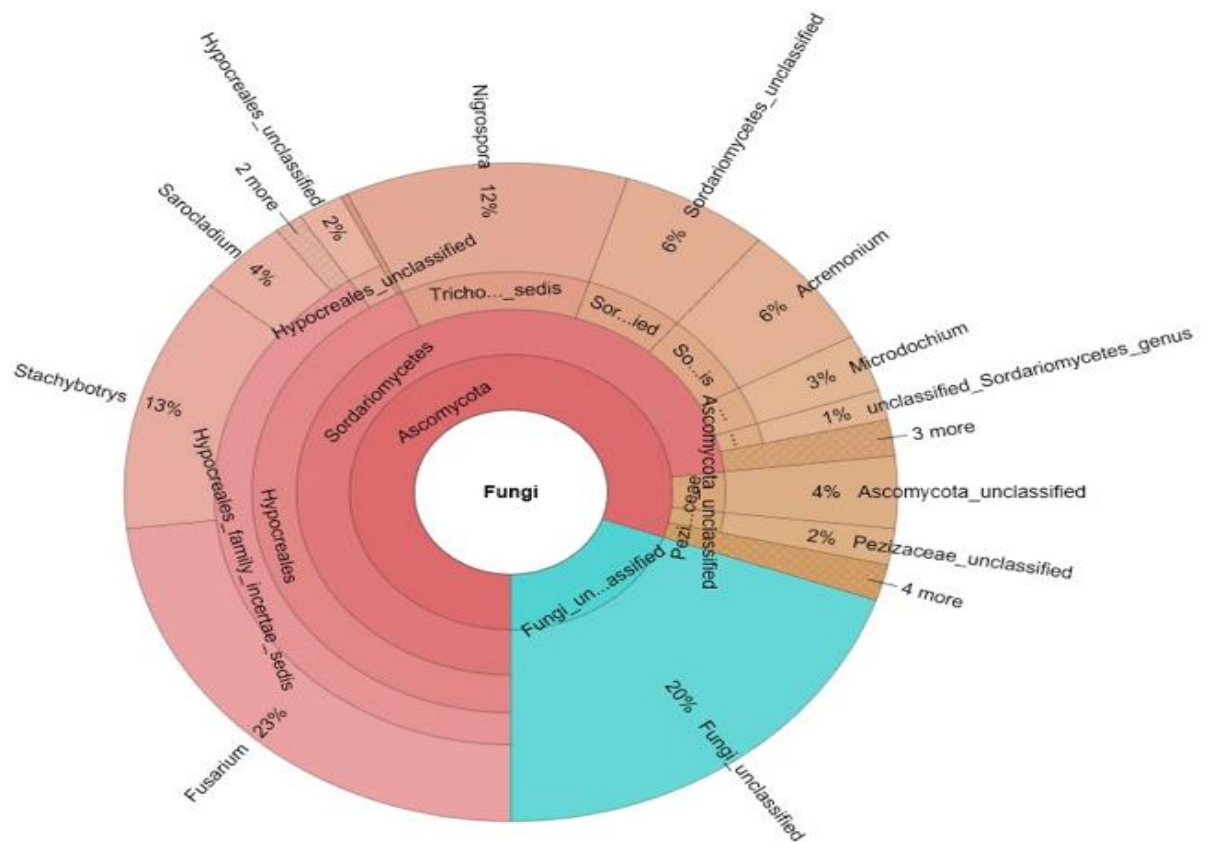


Figure 4.16. Fungal community profile of DNA extracted from *Sorghum bicolor* L. roots in *Sudangrass* inoculum.

Ascomycota stands for the most abundant phylum in *Sudangrass* inoculum as in *Cynodon dactylon*, *Trifolium alexandrinum* and *Festuca arundinacea*. However, Basidiomycota were not discovered unlike other native plants' inoculums. Only one ectomycorrhizal fungi order; Pezizales were identified in the rhizosphere of *Sorghum bicolor* L. in *Sudangrass* inoculum source.

Other than ectomycorrhizal fungi, four genera of fungal endophytes were also discovered in the roots of *Sorghum bicolor* L. These include, *Chaetomium* spp., *Fusarium* spp., *Cladosporium* spp. and *Penicillium* spp.

4.6.5. Metagenomic Analysis of Fungal Community in *Elymus repens* inoculum

The metagenomic analysis of fungal DNA obtained from *Elymus repens* inoculum have identified 4 phyla, 9 classes, 18 orders, 24 families and 37 genera. The fungal community profile is demonstrated in Figure 4.17.

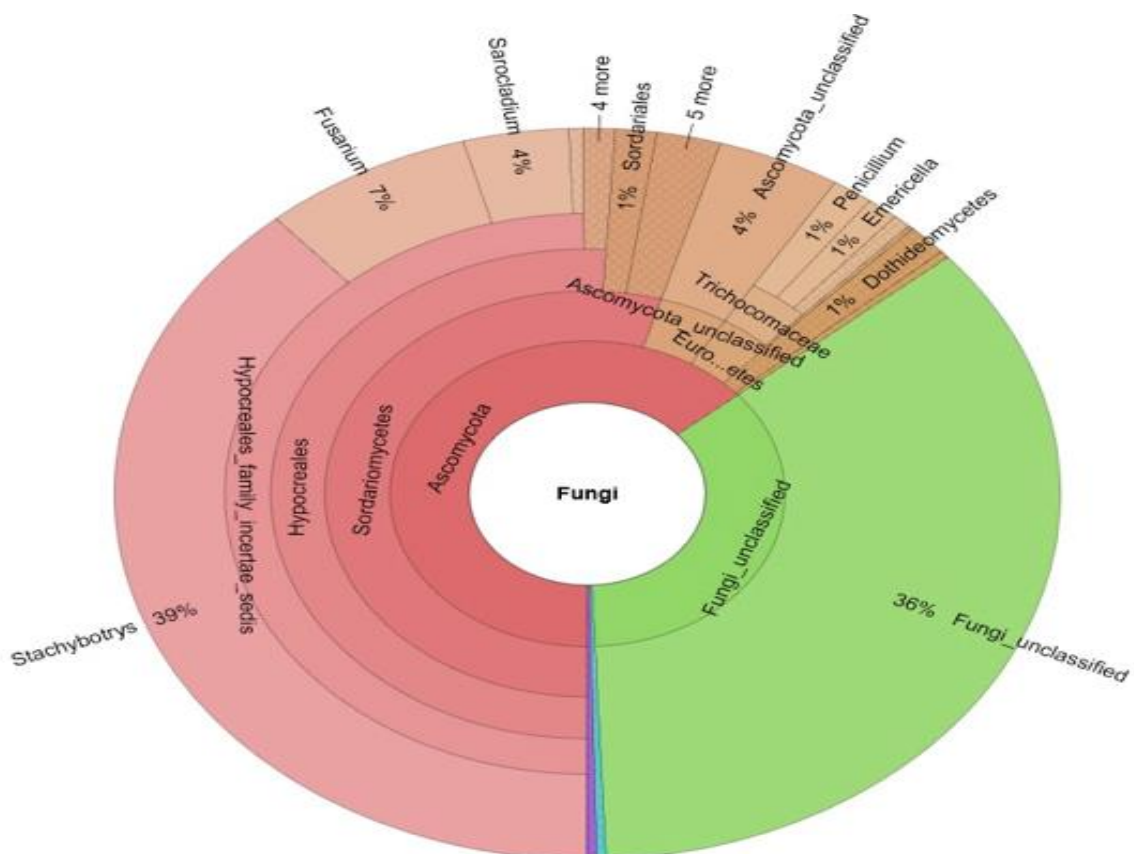


Figure 4.17. Fungal community profile of DNA extract from *Sorghum bicolor* L. rhizosphere in *Elymus repens* inoculum.

According to comprehensive metagenomics analysis ectomycorrhizal Basidiomycota and Ascomycota phyla were discovered in the *Sorghum bicolor* L. rhizosphere in *Elymus repens* inoculum with higher abundance of Ascomycota. Eurotiales, an ectomycorrhizal fungi order was also identified. Other than that, endomycorrhizal fungi order, Mortierellales were discovered.

Various genera of fungal endophyte were also identified in the roots of *Sorghum bicolor* L. in *Elymus repens* inoculum. These genera include; *Alternaria spp.*, *Chaetomium spp.*, *Fusarium spp.*, *Cladosporium spp.* and *Penicillium spp.*

5. DISCUSSION

Tavsanli is one of the most contaminated area by heavy metals in Turkey due to mining operations. Therefore, this site and its surroundings pose a threat to the environment and the biota. This study investigated soil-microbial relationships in five native plants from Tavsanli mine-tailings including *Sudangrass*, *Trifolium alexandrinum*, *Festuca arundinaceae*, *Elymus repens* and *Cynodon dactylon* based on soil and native plant sampling, cultivation of fungal community in trap culture system, harvesting of *Sorghum bicolor* L. plants, fungal DNA extraction, PCR, agarose gel electrophoresis and Next Generation Sequencing. Trap culture is the technique that used for isolating the fungal community members such as AMF which cannot be detected by spore extraction (Liu and Wang 2003).

Morphological and molecular techniques were used to identify arbuscular mycorrhizal fungi. AML1 and AML2 primers were used to determine if the DNA sample extracted from sorghum roots contained any AMF species. These primers are designed to bind all AMF species as reported by Lee et al. (2008). The amplified region by AML1 and AML2 primers was as long as 795bp and bands were observed around 800bp as expected. Afterwards, extracted DNAs were sequenced in order to analyze the fungal community profile.

In consequence of metagenomic analysis, different number of taxonomic categories were discovered from every native plant inoculum. The metagenomic analysis of fungal DNA obtained from *Cynodon dactylon* inoculum have identified 3 phyla, 9 class, 19 orders, 31 families and 50 genera. As a result of metagenomics analysis of fungal DNA obtained from *Trifolium alexandrinum* inoculum; 4 phyla, 10 classes, 18 orders, 24 families and 37 genera in total have been identified. 3 phyla, 11 classes, 20 orders, 31 families and 49 genera in total have been identified as a result of metagenomic analysis applied to fungal DNA obtained from *Festuca arundinacea* inoculum. As a result of metagenomic analysis of fungal DNA obtained from the roots of *Sorghum bicolor* L. in *Sudangraass* inoculum; 2 phyla, 8 classes, 16 orders, 19 families and 26 genera have been identified. The metagenomic analysis of fungal DNA obtained from *Elymus repens* inoculum have revealed 4 phyla, 9 classes, 18 orders, 24 families and 37 genera. All the fungi identified in this study belong to two distinct phyla which include Ascomycota and Basidiomycota. The phylum Ascomycota was discovered in every native plant inoculum, however, the phylum Basidiomycota was identified in each inoculum except for *Sudangrass*. The majority of fungi are the members of Ascomycota.

Bourceret et al. (2015) have reported that pyrosequencing of 16S rRNA gene revealed five distinct fungal phyla with higher abundance of Ascomycota in heavy metal contaminated soil. It has also been reported that in Cd-contaminated soil, next generation sequencing revealed two certain phyla in which Ascomycota being the most abundant followed by Basidiomycota (Yang et al., 2019). According to results, it can be said that the phylum Ascomycota has higher tolerance to the heavy metals compared to the phylum Basidiomycota as suggested by Likar (2009).

Metagenomic analysis also revealed that ectomycorrhizal fungal orders Pezizales, Eurotiales and Cantharellales were present in various native plant inoculums. All three orders have been discovered in *Festuca arundinacea* inoculum. Pezizales and Eurotiales have been identified in *Cynodon dactylon* inoculum while Eurotiales and Cantharellales have been discovered in *Trifolium alexandrinum* inoculum. However, *Sudangrass* inoculum only included Pezizales whereas *Elymus repens* inoculum only contained Eurotiales. According to results, it is concluded that different fungal orders are specific to different native plants. *Festuca arundinacea* stands for the most suitable native plant for ectomycorrhizal associations due to its ability to associate with high number of fungi. Moreover, Ceratobasidiaceae; an ectomycorrhizal fungi family was also discovered in *Festuca arundinacea*, *Trifolium alexandrinum* and *Cynodon dactylon* inoculum.

An endomycorrhizal fungal order, Mortierellales, was also identified in the inoculums of *Elymus repens* and *Trifolium alexandrinum*. The order was not identified in any other native plant inoculums. Narendula-Kotha et al. (2017) has reported that Mortierellales were found to be more abundant in soil exposed to heavy metals compared to non-contaminated soil.

Apart from mycorrhizal fungi, the results indicated that several genera of fungal endophytes were also discovered in every native plant inoculum. As explained previously, endophytic fungi are known for their high diversity and ability to allow host plants to cope with stressed conditions such as heavy metal contamination by altering the functions of the host plant (Khan et al., 2017). The discovered genera include *Colletotrichum spp.*, *Alternaria spp.*, *Chaetomium spp.*, *Fusarium spp.*, *Cladosporium spp.* and *Penicillium spp.* Each genus was discovered in the DNA obtained from the inoculums of *Cynodon dactylon* and *Festuca arundinacea* in which *Fusarium spp.* being in the highest abundance. Only *Chaetomium spp.* and *Colletotrichum spp.* among six genera were not identified in the *Trifolium alexandrinum* and *Elymus repens* inoculums, respectively. Every genus excluding *Colletotrichum spp.* and *Alternaria spp.* were discovered in the DNA obtained from the *Sudangrass* inoculum. Khan et al. (2016) have reported that fourteen distinct genera in which

Colletotrichum being the dominant genus were discovered as a consequence of ITS region analysis of endophytic fungi isolated from roots of Cd hyperaccumulator *Solanum nigrum* L. The detailed demonstration of identified fungi in each native plant inoculum is shown in Figure 5.1.

Table 5.1. The overall demonstration of identified fungal taxonomic groups from each native plant.

Inoculum	Endomycorrhiza	Ectomycorrhiza	Fungal Endophyte
<i>Elymus repens</i>	Mortierellales	Eurotiales	<i>Alternaria spp.</i> <i>Chaetomium spp.</i> <i>Fusarium spp.</i> <i>Cladosporium spp.</i> <i>Penicillium spp.</i>
<i>Sudangrass</i>	-	Pezizales	<i>Chaetomium spp.</i> <i>Fusarium spp.</i> <i>Cladosporium spp.</i> <i>Penicillium spp.</i>
<i>Festuca arundinacea</i>	-	Pezizales Eurotiales Cantharellales Ceratobasidiaceae	<i>Colletotrichum spp.</i> <i>Alternaria spp.</i> <i>Chaetomium spp.</i> <i>Fusarium spp.</i> <i>Cladosporium spp.</i> <i>Penicillium spp.</i>
<i>Cynodon dactylon</i>	-	Pezizales Eurotiales Ceratobasidiaceae	<i>Colletotrichum spp.</i> <i>Alternaria spp.</i> <i>Chaetomium spp.</i> <i>Fusarium spp.</i> <i>Cladosporium spp.</i> <i>Penicillium spp.</i>
<i>Trifolium alexandrinum</i>	Mortierellales	Eurotiales Cantharellales Ceratobasidiaceae	<i>Colletotrichum spp.</i> <i>Alternaria spp.</i> <i>Fusarium spp.</i> <i>Cladosporium spp.</i> <i>Penicillium spp.</i>

6. CONCLUSION

In the present study, the presence of mycorrhizal fungi and fungal community profile of Tavsanlı mine-tailings was investigated through the use of five native plants as an inoculum source and *Sorghum bicolor L.* as a host plant. It was hypothesized that native plants were likely to be associated with microorganisms such as mycorrhizal fungi in order to cope with harsh environmental conditions in the tailing such as high heavy metal concentrations (Fe and Al). The morphological observation indicated that AMF were present in the roots of every native plant hence in Tavsanlı area. Following the morphological observation, agarose gel electrophoresis supported the presence of AMF in molecular method.

According to molecular analysis of fungal DNA obtained from host plant's roots, Ascomycota was found to be the dominant fungal phylum in each of the native plant inoculums namely *Festuca arundinacea*, *Sudangrass*, *Cynodon dactylon*, *Elymus repens* and *Trifolium alexandrinum*.

Ectomycorrhizal fungi were also identified in every collected native plant inoculum. This can be supporting that native plants are in symbiotic relationship with fungi. Moreover, endomycorrhizal fungi was identified in the inoculums of *Elymus repens* and *Trifolium alexandrinum*. It was concluded that various mycorrhizal associations could be formed in the rhizosphere of each native plant in order to revegetate the Tavsanlı area. Although the presence of AMF was supported with morphological and molecular methods, the phylum Glomeromycota was not discovered as a result of metagenomic analysis. However, unclassified fungi represented in the fungal community profile, is thought to be AMF. The reason that AMF could not be classified could be using DNA extracts for sequencing rather than PCR products which were amplified by using AML1 and AML2 primers. Further study could be carried out in order to identify certain AMF species that exist in mine-tailing area.

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APPENDIX

Table A.1. Comprehensive metagenomic analysis results from DNA in *Elymus repens* inoculum.

Data Rank	Tax Level	Tax ID	Taxon	Taxon	rRNA Seq Qty	Relative Qty %
1	1	0.1	Super Kingdom	Fungi	638	100
2	2	0.1.1	Phylum	Ascomycota	407	63,793
3	3	0.1.1.1	Class	Ascomycota_class_incertae_sedis	1	0,157
4	4	0.1.1.1.1	Order	Ascomycota_order_incertae_sedis	1	0,157
5	5	0.1.1.1.1.1	Family	Ascomycota_family_incertae_sedis	1	0,157
6	6	0.1.1.1.1.1.1	Genus	Harzia	1	0,157
7	3	0.1.1.2	Class	Ascomycota_unclassified	27	4,232
8	4	0.1.1.2.1	Order	Ascomycota_unclassified	27	4,232
9	5	0.1.1.2.1.1	Family	Ascomycota_unclassified	27	4,232
10	6	0.1.1.2.1.1.1	Genus	Ascomycota_unclassified	27	4,232
11	3	0.1.1.3	Class	Dothideomycetes	9	1,411
12	4	0.1.1.3.1	Order	Capnodiales	7	1,097
13	5	0.1.1.3.1.1	Family	Capnodiales_family_incertae_sedis	7	1,097
14	6	0.1.1.3.1.1.1	Genus	Capnodiales_family_incertae_sedis_unclassified	1	0,157
15	6	0.1.1.3.1.1.2	Genus	Cladosporium	3	0,470
16	6	0.1.1.3.1.1.3	Genus	Davidiella	3	0,470
17	4	0.1.1.3.2	Order	Dothideales	1	0,157
18	5	0.1.1.3.2.1	Family	Dothioraceae	1	0,157
19	6	0.1.1.3.2.1.1	Genus	Aureobasidium	1	0,157
20	4	0.1.1.3.3	Order	Pleosporales	1	0,157
21	5	0.1.1.3.3.1	Family	Pleosporaceae	1	0,157
22	6	0.1.1.3.3.1.1	Genus	Alternaria	1	0,157
23	3	0.1.1.4	Class	Eurotiomycetes	21	3,292
24	4	0.1.1.4.1	Order	Chaetothyriales	1	0,157

25	5	0.1.1.4.1.1	Family	Herpotrichiellaceae	1	0,157
26	6	0.1.1.4.1.1.1	Genus	unclassified_Herpotrichiellaceae_genus	1	0,157
27	4	0.1.1.4.2	Order	Eurotiales	20	3,135
28	5	0.1.1.4.2.1	Family	Trichocomaceae	20	3,135
29	6	0.1.1.4.2.1.1	Genus	Emericella	7	1,097
30	6	0.1.1.4.2.1.2	Genus	Eupenicillium	3	0,470
31	6	0.1.1.4.2.1.3	Genus	Neosartorya	1	0,157
32	6	0.1.1.4.2.1.4	Genus	Penicillium	9	1,411
33	3	0.1.1.5	Class	Pezizomycetes	1	0,157
34	4	0.1.1.5.1	Order	Pezizales	1	0,157
35	5	0.1.1.5.1.1	Family	Ascobolaceae	1	0,157
36	6	0.1.1.5.1.1.1	Genus	unclassified_Ascobolaceae_genus	1	0,157
37	3	0.1.1.6	Class	Sordariomycetes	348	54,545
38	4	0.1.1.6.1	Order	Glomerellales	1	0,157
39	5	0.1.1.6.1.1	Family	Plectosphaerellaceae	1	0,157
40	6	0.1.1.6.1.1.1	Genus	Plectosphaerella	1	0,157
41	4	0.1.1.6.2	Order	Hypocreales	325	50,940
42	5	0.1.1.6.2.1	Family	Hypocreaceae	1	0,157
43	6	0.1.1.6.2.1.1	Genus	Hypocrea	1	0,157
44	5	0.1.1.6.2.2	Family	Hypocreales_family_incertae_sedis	318	49,843
45	6	0.1.1.6.2.2.1	Genus	Emericellopsis	1	0,157
46	6	0.1.1.6.2.2.2	Genus	Fusarium	46	7,210
47	6	0.1.1.6.2.2.3	Genus	Hypocreales_family_incertae_sedis_unclassified	2	0,313
48	6	0.1.1.6.2.2.4	Genus	Sarocladium	23	3,605
49	6	0.1.1.6.2.2.5	Genus	Stachybotrys	246	38,558
50	5	0.1.1.6.2.3	Family	Hypocreales_unclassified	4	0,627
51	6	0.1.1.6.2.3.1	Genus	Hypocreales_unclassified	4	0,627
52	5	0.1.1.6.2.4	Family	Nectriaceae	1	0,157
53	6	0.1.1.6.2.4.1	Genus	Nectriaceae_unclassified	1	0,157
54	5	0.1.1.6.2.5	Family	unclassified_Hypocreales_family	1	0,157

55	6	0.1.1.6.2.5.1	Genus	unclassified_Hypocreales_genus	1	0,157
56	4	0.1.1.6.3	Order	Microascales	1	0,157
57	5	0.1.1.6.3.1	Family	Microasceae	1	0,157
58	6	0.1.1.6.3.1.1	Genus	Petriella	1	0,157
59	4	0.1.1.6.4	Order	Sordariales	9	1,411
60	5	0.1.1.6.4.1	Family	Chaetomiaceae	5	0,784
61	6	0.1.1.6.4.1.1	Genus	Chaetomium	3	0,470
62	6	0.1.1.6.4.1.2	Genus	Corynascus	1	0,157
63	6	0.1.1.6.4.1.3	Genus	Thielavia	1	0,157
64	5	0.1.1.6.4.2	Family	Lasiosphaeriaceae	4	0,627
65	6	0.1.1.6.4.2.1	Genus	Lasiosphaeriaceae_unclassified	1	0,157
66	6	0.1.1.6.4.2.2	Genus	Podospora	2	0,313
67	6	0.1.1.6.4.2.3	Genus	unclassified_Lasiosphaeriaceae_genus	1	0,157
68	4	0.1.1.6.5	Order	Sordariomycetes_order_incertae_sedis	3	0,470
69	5	0.1.1.6.5.1	Family	Apiosporaceae	1	0,157
70	6	0.1.1.6.5.1.1	Genus	Arthrinium	1	0,157
71	5	0.1.1.6.5.2	Family	Sordariomycetes_family_incertae_sedis	2	0,313
72	6	0.1.1.6.5.2.1	Genus	Acremonium	2	0,313
73	4	0.1.1.6.6	Order	Sordariomycetes_unclassified	7	1,097
74	5	0.1.1.6.6.1	Family	Sordariomycetes_unclassified	7	1,097
75	6	0.1.1.6.6.1.1	Genus	Sordariomycetes_unclassified	7	1,097
76	4	0.1.1.6.7	Order	Trichosphaeriales	2	0,313
77	5	0.1.1.6.7.1	Family	Trichosphaeriales_family_incertae_sedis	2	0,313
78	6	0.1.1.6.7.1.1	Genus	Nigrospora	2	0,313
79	2	0.1.2	Phylum	Basidiomycota	2	0,313
80	3	0.1.2.1	Class	Basidiomycota_unclassified	2	0,313
81	4	0.1.2.1.1	Order	Basidiomycota_unclassified	2	0,313
82	5	0.1.2.1.1.1	Family	Basidiomycota_unclassified	2	0,313
83	6	0.1.2.1.1.1.1	Genus	Basidiomycota_unclassified	2	0,313
84	2	0.1.3	Phylum	Fungi_phylum_incertae_sedis	2	0,313

85	3	0.1.3.1	Class	Fungi_class_incertae_sedis	2	0,313
86	4	0.1.3.1.1	Order	Mortierellales	2	0,313
87	5	0.1.3.1.1.1	Family	Mortierellaceae	2	0,313
88	6	0.1.3.1.1.1.1	Genus	Mortierella	2	0,313
89	2	0.1.4	Phylum	Fungi_unclassified	227	35,580
90	3	0.1.4.1	Class	Fungi_unclassified	227	35,580
91	4	0.1.4.1.1	Order	Fungi_unclassified	227	35,580
92	5	0.1.4.1.1.1	Family	Fungi_unclassified	227	35,580
93	6	0.1.4.1.1.1.1	Genus	Fungi_unclassified	227	35,580

Table A.2. Comprehensive metagenomic analysis results from DNA in *Sudangrass* inoculum.

Data Rank	Tax Level	Tax ID	Taxon	Taxon	rRNA Seq Qty	Relative Qty %
1	1	0.1	Super Kingdom	Fungi	676	100
2	2	0.1.1	Phylum	Ascomycota	543	80,325
3	3	0.1.1.1	Class	Ascomycota_class_incertae_sedis	1	0,148
4	4	0.1.1.1.1	Order	Ascomycota_order_incertae_sedis	1	0,148
5	5	0.1.1.1.1.1	Family	Ascomycota_family_incertae_sedis	1	0,148
6	6	0.1.1.1.1.1.1	Genus	Engyodontium	1	0,148
7	3	0.1.1.2	Class	Ascomycota_unclassified	24	3,550
8	4	0.1.1.2.1	Order	Ascomycota_unclassified	24	3,550
9	5	0.1.1.2.1.1	Family	Ascomycota_unclassified	24	3,550
10	6	0.1.1.2.1.1.1	Genus	Ascomycota_unclassified	24	3,550
11	3	0.1.1.3	Class	Dothideomycetes	8	1,183
12	4	0.1.1.3.1	Order	Capnodiales	8	1,183
13	5	0.1.1.3.1.1	Family	Capnodiales_family_incertae_sedis	8	1,183
14	6	0.1.1.3.1.1.1	Genus	Cladosporium	4	0,592
15	6	0.1.1.3.1.1.2	Genus	Davidiella	4	0,592
16	3	0.1.1.4	Class	Eurotiomycetes	2	0,296
17	4	0.1.1.4.1	Order	Eurotiales	2	0,296
18	5	0.1.1.4.1.1	Family	Trichocomaceae	2	0,296
19	6	0.1.1.4.1.1.1	Genus	Aspergillus	1	0,148
20	6	0.1.1.4.1.1.2	Genus	Penicillium	1	0,148
21	3	0.1.1.5	Class	Orbiliomycetes	1	0,148
22	4	0.1.1.5.1	Order	Orbiliales	1	0,148
23	5	0.1.1.5.1.1	Family	Orbiliaceae	1	0,148
24	6	0.1.1.5.1.1.1	Genus	Orbilia	1	0,148
25	3	0.1.1.6	Class	Pezizomycetes	12	1,775
26	4	0.1.1.6.1	Order	Pezizales	12	1,775
27	5	0.1.1.6.1.1	Family	Pezizaceae	12	1,775

28	6	0.1.1.6.1.1.1	Genus	Pezizaceae_unclassified	12	1,775
29	3	0.1.1.7	Class	Sordariomycetes	495	73,225
30	4	0.1.1.7.1	Order	Glomerellales	1	0,148
31	5	0.1.1.7.1.1	Family	Plectosphaerellaceae	1	0,148
32	6	0.1.1.7.1.1.1	Genus	Plectosphaerella	1	0,148
33	4	0.1.1.7.2	Order	Hypocreales	291	43,047
34	5	0.1.1.7.2.1	Family	Bionectriaceae	1	0,148
35	6	0.1.1.7.2.1.1	Genus	Bionectria	1	0,148
36	5	0.1.1.7.2.2	Family	Hypocreales_family_incertae_sedis	276	40,828
37	6	0.1.1.7.2.2.1	Genus	Fusarium	157	23,225
38	6	0.1.1.7.2.2.2	Genus	Hypocreales_family_incertae_sedis_unclassified	3	0,444
39	6	0.1.1.7.2.2.3	Genus	Sarocladium	26	3,846
40	6	0.1.1.7.2.2.4	Genus	Stachybotrys	85	12,574
41	6	0.1.1.7.2.2.5	Genus	Trichothecium	5	0,740
42	5	0.1.1.7.2.3	Family	Hypocreales_unclassified	13	1,923
43	6	0.1.1.7.2.3.1	Genus	Hypocreales_unclassified	13	1,923
44	5	0.1.1.7.2.4	Family	Nectriaceae	1	0,148
45	6	0.1.1.7.2.4.1	Genus	Nectriaceae_unclassified	1	0,148
46	4	0.1.1.7.3	Order	Microascales	6	0,888
47	5	0.1.1.7.3.1	Family	Halosphaeriaceae	6	0,888
48	6	0.1.1.7.3.1.1	Genus	Periconia	6	0,888
49	4	0.1.1.7.4	Order	Sordariales	5	0,740
50	5	0.1.1.7.4.1	Family	Chaetomiaceae	5	0,740
51	6	0.1.1.7.4.1.1	Genus	Chaetomium	4	0,592
52	6	0.1.1.7.4.1.2	Genus	Corynascus	1	0,148
53	4	0.1.1.7.5	Order	Sordariomycetes_order_incertae_sedis	41	6,065
54	5	0.1.1.7.5.1	Family	Sordariomycetes_family_incertae_sedis	41	6,065
55	6	0.1.1.7.5.1.1	Genus	Acremonium	41	6,065
56	4	0.1.1.7.6	Order	Sordariomycetes_unclassified	42	6,213
57	5	0.1.1.7.6.1	Family	Sordariomycetes_unclassified	42	6,213

58	6	0.1.1.7.6.1.1	Genus	Sordariomycetes_unclassified	42	6,213
59	4	0.1.1.7.7	Order	Trichosphaeriales	80	11,834
60	5	0.1.1.7.7.1	Family	Trichosphaeriales_family_incertae_sedis	80	11,834
61	6	0.1.1.7.7.1.1	Genus	Nigrospora	80	11,834
62	4	0.1.1.7.8	Order	Xylariales	19	2,811
63	5	0.1.1.7.8.1	Family	Xylariales_family_incertae_sedis	19	2,811
64	6	0.1.1.7.8.1.1	Genus	Microdochium	19	2,811
65	4	0.1.1.7.9	Order	unclassified_Sordariomycetes_order	10	1,479
66	5	0.1.1.7.9.1	Family	unclassified_Sordariomycetes_family	10	1,479
67	6	0.1.1.7.9.1.1	Genus	unclassified_Sordariomycetes_genus	10	1,479
68	2	0.1.2	Phylum	Fungi_unclassified	133	19,675
69	3	0.1.2.1	Class	Fungi_unclassified	133	19,675
70	4	0.1.2.1.1	Order	Fungi_unclassified	133	19,675
71	5	0.1.2.1.1.1	Family	Fungi_unclassified	133	19,675
72	6	0.1.2.1.1.1.1	Genus	Fungi_unclassified	133	19,675

Table A.3. Comprehensive metagenomic analysis results from DNA in *Cynodon dactylon* inoculum.

Data Rank	Tax Level	Tax ID	Taxon	Taxon	rRNA Seq Qty	Relative Qty %
1	1	0.1	Super Kingdom	Fungi	711	100
2	2	0.1.1	Phylum	Ascomycota	523	73,558
3	3	0.1.1.1	Class	Ascomycota_unclassified	29	4,079
4	4	0.1.1.1.1	Order	Ascomycota_unclassified	29	4,079
5	5	0.1.1.1.1.1	Family	Ascomycota_unclassified	29	4,079
6	6	0.1.1.1.1.1.1	Genus	Ascomycota_unclassified	29	4,079
7	3	0.1.1.2	Class	Dothideomycetes	19	2,672
8	4	0.1.1.2.1	Order	Capnodiales	15	2,110
9	5	0.1.1.2.1.1	Family	Capnodiales_family_incertae_sedis	15	2,110
10	6	0.1.1.2.1.1.1	Genus	Cladosporium	9	1,266
11	6	0.1.1.2.1.1.2	Genus	Davidiella	6	0,844
12	4	0.1.1.2.2	Order	Dothideomycetes_order_incertae_sedis	1	0,141
13	5	0.1.1.2.2.1	Family	Eremomycetaceae	1	0,141
14	6	0.1.1.2.2.1.1	Genus	unclassified_Eremomycetaceae_genus	1	0,141
15	4	0.1.1.2.3	Order	Pleosporales	3	0,422
16	5	0.1.1.2.3.1	Family	Leptosphaeriaceae	1	0,141
17	6	0.1.1.2.3.1.1	Genus	Leptosphaeria	1	0,141
18	5	0.1.1.2.3.2	Family	Pleosporaceae	2	0,281
19	6	0.1.1.2.3.2.1	Genus	Alternaria	1	0,141
20	6	0.1.1.2.3.2.2	Genus	Cochliobolus	1	0,141
21	3	0.1.1.3	Class	Eurotiomycetes	10	1,406
22	4	0.1.1.3.1	Order	Eurotiales	10	1,406
23	5	0.1.1.3.1.1	Family	Trichocomaceae	10	1,406
24	6	0.1.1.3.1.1.1	Genus	Emericella	1	0,141
25	6	0.1.1.3.1.1.2	Genus	Eupenicillium	2	0,281
26	6	0.1.1.3.1.1.3	Genus	Neosartorya	1	0,141
27	6	0.1.1.3.1.1.4	Genus	Penicillium	4	0,563

28	6	0.1.1.3.1.1.5	Genus	Trichocomaceae_unclassified	2	0,281
29	3	0.1.1.4	Class	Leotiomycetes	1	0,141
30	4	0.1.1.4.1	Order	Helotiales	1	0,141
31	5	0.1.1.4.1.1	Family	Sclerotiniaceae	1	0,141
32	6	0.1.1.4.1.1.1	Genus	Sclerotiniaceae_unclassified	1	0,141
33	3	0.1.1.5	Class	Orbiliomycetes	5	0,703
34	4	0.1.1.5.1	Order	Orbiliiales	5	0,703
35	5	0.1.1.5.1.1	Family	Orbiliaceae	5	0,703
36	6	0.1.1.5.1.1.1	Genus	Arthrobotrys	5	0,703
37	3	0.1.1.6	Class	Pezizomycetes	1	0,141
38	4	0.1.1.6.1	Order	Pezizales	1	0,141
39	5	0.1.1.6.1.1	Family	Ascobolaceae	1	0,141
40	6	0.1.1.6.1.1.1	Genus	unclassified_Ascobolaceae_genus	1	0,141
41	3	0.1.1.7	Class	Sordariomycetes	458	64,416
42	4	0.1.1.7.1	Order	Glomerellales	3	0,422
43	5	0.1.1.7.1.1	Family	Glomerellales_family_incertae_sedis	1	0,141
44	6	0.1.1.7.1.1.1	Genus	Colletotrichum	1	0,141
45	5	0.1.1.7.1.2	Family	Plectosphaerellaceae	2	0,281
46	6	0.1.1.7.1.2.1	Genus	Plectosphaerella	1	0,141
47	6	0.1.1.7.1.2.2	Genus	unclassified_Plectosphaerellaceae_genus	1	0,141
48	4	0.1.1.7.2	Order	Hypocreales	366	51,477
49	5	0.1.1.7.2.1	Family	Bionectriaceae	4	0,563
50	6	0.1.1.7.2.1.1	Genus	Bionectria	4	0,563
51	5	0.1.1.7.2.2	Family	Clavicipitaceae	1	0,141
52	6	0.1.1.7.2.2.1	Genus	Metarhizium	1	0,141
53	5	0.1.1.7.2.3	Family	Hypocreales_family_incertae_sedis	273	38,397
54	6	0.1.1.7.2.3.1	Genus	Emericellopsis	1	0,141
55	6	0.1.1.7.2.3.2	Genus	Fusarium	204	28,692
56	6	0.1.1.7.2.3.3	Genus	Hypocreales_family_incertae_sedis_unclassified	1	0,141

57	6	0.1.1.7.2.3.4	Genus	Myrothecium	2	0,281
58	6	0.1.1.7.2.3.5	Genus	Purpureocillium	1	0,141
59	6	0.1.1.7.2.3.6	Genus	Sarocladium	13	1,828
60	6	0.1.1.7.2.3.7	Genus	Stachybotrys	51	7,173
61	5	0.1.1.7.2.4	Family	Hypocreales_unclassified	73	10,267
62	6	0.1.1.7.2.4.1	Genus	Hypocreales_unclassified	73	10,267
63	5	0.1.1.7.2.5	Family	Nectriaceae	12	1,688
64	6	0.1.1.7.2.5.1	Genus	Gibberella	8	1,125
65	6	0.1.1.7.2.5.2	Genus	Nectriaceae_unclassified	4	0,563
66	5	0.1.1.7.2.6	Family	unclassified_Hypocreales_family	3	0,422
67	6	0.1.1.7.2.6.1	Genus	unclassified_Hypocreales_genus	3	0,422
68	4	0.1.1.7.3	Order	Microascales	2	0,281
69	5	0.1.1.7.3.1	Family	Halosphaeriaceae	1	0,141
70	6	0.1.1.7.3.1.1	Genus	Periconia	1	0,141
71	5	0.1.1.7.3.2	Family	Microascaceae	1	0,141
72	6	0.1.1.7.3.2.1	Genus	Pseudallescheria	1	0,141
73	4	0.1.1.7.4	Order	Sordariales	11	1,547
74	5	0.1.1.7.4.1	Family	Chaetomiaceae	6	0,844
75	6	0.1.1.7.4.1.1	Genus	Chaetomiaceae_unclassified	1	0,141
76	6	0.1.1.7.4.1.2	Genus	Chaetomium	2	0,281
77	6	0.1.1.7.4.1.3	Genus	Thielavia	2	0,281
78	6	0.1.1.7.4.1.4	Genus	Zopfiella	1	0,141
79	5	0.1.1.7.4.2	Family	Lasiosphaeriaceae	4	0,563
80	6	0.1.1.7.4.2.1	Genus	Cladorrhinum	2	0,281
81	6	0.1.1.7.4.2.2	Genus	Lasiosphaeriaceae_unclassified	1	0,141
82	6	0.1.1.7.4.2.3	Genus	unclassified_Lasiosphaeriaceae_genus	1	0,141
83	5	0.1.1.7.4.3	Family	Sordariales_unclassified	1	0,141
84	6	0.1.1.7.4.3.1	Genus	Sordariales_unclassified	1	0,141
85	4	0.1.1.7.5	Order	Sordariomycetes_order_incertae_sedis	50	7,032
86	5	0.1.1.7.5.1	Family	Magnaporthaceae	1	0,141

87	6	0.1.1.7.5.1.1	Genus	Magnaporthaceae_unclassified	1	0,141
88	5	0.1.1.7.5.2	Family	Sordariomycetes_family_incertae_sedis	49	6,892
89	6	0.1.1.7.5.2.1	Genus	Acremonium	49	6,892
90	4	0.1.1.7.6	Order	Sordariomycetes_unclassified	19	2,672
91	5	0.1.1.7.6.1	Family	Sordariomycetes_unclassified	19	2,672
92	6	0.1.1.7.6.1.1	Genus	Sordariomycetes_unclassified	19	2,672
93	4	0.1.1.7.7	Order	Trichosphaeriales	5	0,703
94	5	0.1.1.7.7.1	Family	Trichosphaeriales_family_incertae_sedis	5	0,703
95	6	0.1.1.7.7.1.1	Genus	Nigrospora	5	0,703
96	4	0.1.1.7.8	Order	Xylariales	2	0,281
97	5	0.1.1.7.8.1	Family	Amphisphaeriaceae	1	0,141
98	6	0.1.1.7.8.1.1	Genus	Pestalotiopsis	1	0,141
99	5	0.1.1.7.8.2	Family	Xylariales_family_incertae_sedis	1	0,141
100	6	0.1.1.7.8.2.1	Genus	Microdochium	1	0,141
101	2	0.1.2	Phylum	Basidiomycota	3	0,422
102	3	0.1.2.1	Class	Agaricomycetes	3	0,422
103	4	0.1.2.1.1	Order	Agaricomycetes_unclassified	1	0,141
104	5	0.1.2.1.1.1	Family	Agaricomycetes_unclassified	1	0,141
105	6	0.1.2.1.1.1.1	Genus	Agaricomycetes_unclassified	1	0,141
106	4	0.1.2.1.2	Order	Cantharellales	2	0,281
107	5	0.1.2.1.2.1	Family	Ceratobasidiaceae	2	0,281
108	6	0.1.2.1.2.1.1	Genus	Ceratobasidium	2	0,281
109	2	0.1.3	Phylum	Fungi_unclassified	185	26,020
110	3	0.1.3.1	Class	Fungi_unclassified	185	26,020
111	4	0.1.3.1.1	Order	Fungi_unclassified	185	26,020
112	5	0.1.3.1.1.1	Family	Fungi_unclassified	185	26,020

Table A.4. Comprehensive metagenomic analysis results from DNA in *Trifolium alexandrinum* inoculum.

Data Rank	Tax Level	Tax ID	Taxon	Taxon	rRNA Seq Qty	Relative Qty %
1	1	0.1	Super Kingdom	Fungi	801	100
2	2	0.1.1	Phylum	Ascomycota	575	71,785
3	3	0.1.1.1	Class	Ascomycota_class_incertae_sedis	1	0,125
4	4	0.1.1.1.1	Order	Ascomycota_order_incertae_sedis	1	0,125
5	5	0.1.1.1.1.1	Family	Ascomycota_family_incertae_sedis	1	0,125
6	6	0.1.1.1.1.1.1	Genus	Ascomycota_family_incertae_sedis_unclassified	1	0,125
7	3	0.1.1.2	Class	Ascomycota_unclassified	35	4,370
8	4	0.1.1.2.1	Order	Ascomycota_unclassified	35	4,370
9	5	0.1.1.2.1.1	Family	Ascomycota_unclassified	35	4,370
10	6	0.1.1.2.1.1.1	Genus	Ascomycota_unclassified	35	4,370
11	3	0.1.1.3	Class	Dothideomycetes	5	0,624
12	4	0.1.1.3.1	Order	Capnodiales	4	0,499
13	5	0.1.1.3.1.1	Family	Capnodiales_family_incertae_sedis	4	0,499
14	6	0.1.1.3.1.1.1	Genus	Cladosporium	3	0,375
15	6	0.1.1.3.1.1.2	Genus	Davidiella	1	0,125
16	4	0.1.1.3.2	Order	Pleosporales	1	0,125
17	5	0.1.1.3.2.1	Family	Pleosporaceae	1	0,125
18	6	0.1.1.3.2.1.1	Genus	Alternaria	1	0,125
19	3	0.1.1.4	Class	Eurotiomycetes	6	0,749
20	4	0.1.1.4.1	Order	Eurotiales	6	0,749
21	5	0.1.1.4.1.1	Family	Trichocomaceae	6	0,749
22	6	0.1.1.4.1.1.1	Genus	Emericella	1	0,125
23	6	0.1.1.4.1.1.2	Genus	Eupenicillium	2	0,250
24	6	0.1.1.4.1.1.3	Genus	Penicillium	3	0,375
25	3	0.1.1.5	Class	Leotiomycetes	1	0,125

26	4	0.1.1.5.1	Order	Helotiales	1	0,125
27	5	0.1.1.5.1.1	Family	Sclerotiniaceae	1	0,125
28	6	0.1.1.5.1.1.1	Genus	Sclerotiniaceae_unclassified	1	0,125
29	3	0.1.1.6	Class	Orbiliomycetes	5	0,624
30	4	0.1.1.6.1	Order	Orbiliales	5	0,624
31	5	0.1.1.6.1.1	Family	Orbiliaceae	5	0,624
32	6	0.1.1.6.1.1.1	Genus	Orbilia	5	0,624
33	3	0.1.1.7	Class	Sordariomycetes	522	65,169
34	4	0.1.1.7.1	Order	Glomerellales	33	4,120
35	5	0.1.1.7.1.1	Family	Glomerellales_family_incertae_sedis	1	0,125
36	6	0.1.1.7.1.1.1	Genus	Colletotrichum	1	0,125
37	5	0.1.1.7.1.2	Family	Plectosphaerellaceae	32	3,995
38	6	0.1.1.7.1.2.1	Genus	Plectosphaerella	1	0,125
39	6	0.1.1.7.1.2.2	Genus	unclassified_Plectosphaerellaceae_genus	31	3,870
40	4	0.1.1.7.2	Order	Hypocreales	452	56,429
41	5	0.1.1.7.2.1	Family	Bionectriaceae	2	0,250
42	6	0.1.1.7.2.1.1	Genus	Bionectria	2	0,250
43	5	0.1.1.7.2.2	Family	Hypocreales_family_incertae_sedis	428	53,433
44	6	0.1.1.7.2.2.1	Genus	Emericellopsis	1	0,125
45	6	0.1.1.7.2.2.2	Genus	Fusarium	131	16,355
46	6	0.1.1.7.2.2.3	Genus	Myrothecium	1	0,125
47	6	0.1.1.7.2.2.4	Genus	Sarocladium	61	7,615
48	6	0.1.1.7.2.2.5	Genus	Stachybotrys	234	29,213
49	5	0.1.1.7.2.3	Family	Hypocreales_unclassified	17	2,122
50	6	0.1.1.7.2.3.1	Genus	Hypocreales_unclassified	17	2,122
51	5	0.1.1.7.2.4	Family	Nectriaceae	5	0,624
52	6	0.1.1.7.2.4.1	Genus	Cosmospora	2	0,250
53	6	0.1.1.7.2.4.2	Genus	Nectria	1	0,125
54	6	0.1.1.7.2.4.3	Genus	Neonectria	1	0,125
55	6	0.1.1.7.2.4.4	Genus	Pseudonectria	1	0,125

56	4	0.1.1.7.3	Order	Sordariales	11	1,373
57	5	0.1.1.7.3.1	Family	Chaetomiaceae	2	0,250
58	6	0.1.1.7.3.1.1	Genus	Chaetomiaceae_unclassified	1	0,125
59	6	0.1.1.7.3.1.2	Genus	Zopfiella	1	0,125
60	5	0.1.1.7.3.2	Family	Lasiosphaeriaceae	9	1,124
61	6	0.1.1.7.3.2.1	Genus	Lasiosphaeriaceae_unclassified	4	0,499
62	6	0.1.1.7.3.2.2	Genus	Podospora	5	0,624
63	4	0.1.1.7.4	Order	Sordariomycetes_order_incertae_sedis	3	0,375
64	5	0.1.1.7.4.1	Family	Sordariomycetes_family_incertae_sedis	3	0,375
65	6	0.1.1.7.4.1.1	Genus	Acremonium	3	0,375
66	4	0.1.1.7.5	Order	Sordariomycetes_unclassified	20	2,497
67	5	0.1.1.7.5.1	Family	Sordariomycetes_unclassified	20	2,497
68	6	0.1.1.7.5.1.1	Genus	Sordariomycetes_unclassified	20	2,497
69	4	0.1.1.7.6	Order	Trichosphaeriales	1	0,125
70	5	0.1.1.7.6.1	Family	Trichosphaeriales_family_incertae_sedis	1	0,125
71	6	0.1.1.7.6.1.1	Genus	Nigrospora	1	0,125
72	4	0.1.1.7.7	Order	Xylariales	2	0,250
73	5	0.1.1.7.7.1	Family	Amphisphaeriaceae	1	0,125
74	6	0.1.1.7.7.1.1	Genus	Bartalinia	1	0,125
75	5	0.1.1.7.7.2	Family	Xylariales_family_incertae_sedis	1	0,125
76	6	0.1.1.7.7.2.1	Genus	Microdochium	1	0,125
77	2	0.1.2	Phylum	Basidiomycota	2	0,250
78	3	0.1.2.1	Class	Agaricomycetes	2	0,250
79	4	0.1.2.1.1	Order	Cantharellales	2	0,250
80	5	0.1.2.1.1.1	Family	Ceratobasidiaceae	2	0,250
81	6	0.1.2.1.1.1.1	Genus	Ceratobasidium	2	0,250
82	2	0.1.3	Phylum	Fungi_phylum_incertae_sedis	2	0,250
83	3	0.1.3.1	Class	Fungi_class_incertae_sedis	2	0,250
84	4	0.1.3.1.1	Order	Fungi_order_incertae_sedis	1	0,125
85	5	0.1.3.1.1.1	Family	Olpidiaceae	1	0,125

86	6	0.1.3.1.1.1.1	Genus	Olpidium	1	0,125
87	4	0.1.3.1.2	Order	Mortierellales	1	0,125
88	5	0.1.3.1.2.1	Family	Mortierellaceae	1	0,125
89	6	0.1.3.1.2.1.1	Genus	Mortierella	1	0,125
90	2	0.1.4	Phylum	Fungi_unclassified	222	27,715
91	3	0.1.4.1	Class	Fungi_unclassified	222	27,715
92	4	0.1.4.1.1	Order	Fungi_unclassified	222	27,715
93	5	0.1.4.1.1.1	Family	Fungi_unclassified	222	27,715
94	6	0.1.4.1.1.1.1	Genus	Fungi_unclassified	222	27,715

Table A.5. Comprehensive metagenomic results from *DNA in Festuca arundinacae* inoculum.

Data Rank	Tax Level	Tax ID	Taxon	Taxon	rRNA Seq Qty	Relative Qty %
1	1	0.1	Super Kingdom	Fungi	809	100
2	2	0.1.1	Phylum	Ascomycota	593	73,300
3	3	0.1.1.1	Class	Ascomycota_class_incertae_sedis	1	0,124
4	4	0.1.1.1.1	Order	Ascomycota_order_incertae_sedis	1	0,124
5	5	0.1.1.1.1.1	Family	Ascomycota_family_incertae_sedis	1	0,124
6	6	0.1.1.1.1.1.1	Genus	Humicola	1	0,124
7	3	0.1.1.2	Class	Ascomycota_unclassified	27	3,337
8	4	0.1.1.2.1	Order	Ascomycota_unclassified	27	3,337
9	5	0.1.1.2.1.1	Family	Ascomycota_unclassified	27	3,337
10	6	0.1.1.2.1.1.1	Genus	Ascomycota_unclassified	27	3,337
11	3	0.1.1.3	Class	Dothideomycetes	131	16,193
12	4	0.1.1.3.1	Order	Capnodiales	120	14,833
13	5	0.1.1.3.1.1	Family	Capnodiales_family_incertae_sedis	119	14,710
14	6	0.1.1.3.1.1.1	Genus	Capnodiales_family_incertae_sedis_unclassified	10	1,236
15	6	0.1.1.3.1.1.2	Genus	Cladosporium	56	6,922
16	6	0.1.1.3.1.1.3	Genus	Davidiella	53	6,551
17	5	0.1.1.3.1.2	Family	unclassified_Capnodiales_family	1	0,124
18	6	0.1.1.3.1.2.1	Genus	unclassified_Capnodiales_genus	1	0,124
19	4	0.1.1.3.2	Order	Pleosporales	11	1,360
20	5	0.1.1.3.2.1	Family	Pleosporaceae	10	1,236
21	6	0.1.1.3.2.1.1	Genus	Alternaria	1	0,124
22	6	0.1.1.3.2.1.2	Genus	Lewia	9	1,112
23	5	0.1.1.3.2.2	Family	Pleosporales_family_incertae_sedis	1	0,124
24	6	0.1.1.3.2.2.1	Genus	Phoma	1	0,124
25	3	0.1.1.4	Class	Eurotiomycetes	31	3,832
26	4	0.1.1.4.1	Order	Eurotiales	31	3,832
27	5	0.1.1.4.1.1	Family	Trichocomaceae	31	3,832

28	6	0.1.1.4.1.1.1	Genus	Emericella	1	0,124
29	6	0.1.1.4.1.1.2	Genus	Eupenicillium	16	1,978
30	6	0.1.1.4.1.1.3	Genus	Neosartorya	1	0,124
31	6	0.1.1.4.1.1.4	Genus	Penicillium	5	0,618
32	6	0.1.1.4.1.1.5	Genus	Trichocomaceae_unclassified	8	0,989
33	3	0.1.1.5	Class	Leotiomycetes	1	0,124
34	4	0.1.1.5.1	Order	Helotiales	1	0,124
35	5	0.1.1.5.1.1	Family	Sclerotiniaceae	1	0,124
36	6	0.1.1.5.1.1.1	Genus	unclassified_Sclerotiniaceae_genus	1	0,124
37	3	0.1.1.6	Class	Orbiliomycetes	1	0,124
38	4	0.1.1.6.1	Order	Orbiliales	1	0,124
39	5	0.1.1.6.1.1	Family	Orbiliaceae	1	0,124
40	6	0.1.1.6.1.1.1	Genus	Orbilia	1	0,124
41	3	0.1.1.7	Class	Pezizomycetes	1	0,124
42	4	0.1.1.7.1	Order	Pezizales	1	0,124
43	5	0.1.1.7.1.1	Family	Ascobolaceae	1	0,124
44	6	0.1.1.7.1.1.1	Genus	unclassified_Ascobolaceae_genus	1	0,124
45	3	0.1.1.8	Class	Sordariomycetes	400	49,444
46	4	0.1.1.8.1	Order	Glomerellales	3	0,371
47	5	0.1.1.8.1.1	Family	Glomerellales_family_incertae_sedis	1	0,124
48	6	0.1.1.8.1.1.1	Genus	Colletotrichum	1	0,124
49	5	0.1.1.8.1.2	Family	Plectosphaerellaceae	2	0,247
50	6	0.1.1.8.1.2.1	Genus	Plectosphaerella	1	0,124
51	6	0.1.1.8.1.2.2	Genus	unclassified_Plectosphaerellaceae_genus	1	0,124
52	4	0.1.1.8.2	Order	Hypocreales	339	41,904
53	5	0.1.1.8.2.1	Family	Bionectriaceae	1	0,124
54	6	0.1.1.8.2.1.1	Genus	Bionectria	1	0,124
55	5	0.1.1.8.2.2	Family	Clavicipitaceae	1	0,124
56	6	0.1.1.8.2.2.1	Genus	Metarhizium	1	0,124
57	5	0.1.1.8.2.3	Family	Hypocreales_family_incertae_sedis	285	35,229

58	6	0.1.1.8.2.3.1	Genus	Cordyceps	1	0,124
59	6	0.1.1.8.2.3.2	Genus	Emericellopsis	1	0,124
60	6	0.1.1.8.2.3.3	Genus	Fusarium	83	10,260
61	6	0.1.1.8.2.3.4	Genus	Hypocreales_family_incertae_sedis_unclassified	1	0,124
62	6	0.1.1.8.2.3.5	Genus	Myrothecium	2	0,247
63	6	0.1.1.8.2.3.6	Genus	Sarocladium	5	0,618
64	6	0.1.1.8.2.3.7	Genus	Stachybotrys	189	23,362
65	6	0.1.1.8.2.3.8	Genus	Trichothecium	3	0,371
66	5	0.1.1.8.2.4	Family	Hypocreales_unclassified	41	5,068
67	6	0.1.1.8.2.4.1	Genus	Hypocreales_unclassified	41	5,068
68	5	0.1.1.8.2.5	Family	Nectriaceae	11	1,360
69	6	0.1.1.8.2.5.1	Genus	Gibberella	7	0,865
70	6	0.1.1.8.2.5.2	Genus	Nectria	1	0,124
71	6	0.1.1.8.2.5.3	Genus	Nectriaceae_unclassified	3	0,371
72	4	0.1.1.8.3	Order	Microascales	1	0,124
73	5	0.1.1.8.3.1	Family	Halosphaeriaceae	1	0,124
74	6	0.1.1.8.3.1.1	Genus	Periconia	1	0,124
75	4	0.1.1.8.4	Order	Sordariales	3	0,371
76	5	0.1.1.8.4.1	Family	Chaetomiaceae	1	0,124
77	6	0.1.1.8.4.1.1	Genus	Chaetomium	1	0,124
78	5	0.1.1.8.4.2	Family	Lasiochaetaceae	2	0,247
79	6	0.1.1.8.4.2.1	Genus	Cladorrhinum	1	0,124
80	6	0.1.1.8.4.2.2	Genus	Podospora	1	0,124
81	4	0.1.1.8.5	Order	Sordariomycetes_order_incertae_sedis	30	3,708
82	5	0.1.1.8.5.1	Family	Sordariomycetes_family_incertae_sedis	30	3,708
83	6	0.1.1.8.5.1.1	Genus	Acremonium	30	3,708
84	4	0.1.1.8.6	Order	Sordariomycetes_unclassified	13	1,607
85	5	0.1.1.8.6.1	Family	Sordariomycetes_unclassified	13	1,607
86	6	0.1.1.8.6.1.1	Genus	Sordariomycetes_unclassified	13	1,607
87	4	0.1.1.8.7	Order	Trichosphaeriales	2	0,247

88	5	0.1.1.8.7.1	Family	Trichosphaeriales_family_incertae_sedis	2	0,247
89	6	0.1.1.8.7.1.1	Genus	Nigrospora	2	0,247
90	4	0.1.1.8.8	Order	Xylariales	9	1,112
91	5	0.1.1.8.8.1	Family	Amphisphaeriaceae	1	0,124
92	6	0.1.1.8.8.1.1	Genus	Pestalotiopsis	1	0,124
93	5	0.1.1.8.8.2	Family	Diatrypaceae	1	0,124
94	6	0.1.1.8.8.2.1	Genus	Cryptovalsa	1	0,124
95	5	0.1.1.8.8.3	Family	Xylariales_family_incertae_sedis	1	0,124
96	6	0.1.1.8.8.3.1	Genus	Microdochium	1	0,124
97	5	0.1.1.8.8.4	Family	unclassified_Xylariales_family	6	0,742
98	6	0.1.1.8.8.4.1	Genus	unclassified_Xylariales_genus	6	0,742
99	2	0.1.2	Phylum	Basidiomycota	3	0,371
100	3	0.1.2.1	Class	Agaricomycetes	2	0,247
101	4	0.1.2.1.1	Order	Agaricomycetes_order_incertae_sedis	1	0,124
102	5	0.1.2.1.1.1	Family	Peniophoraceae	1	0,124
103	6	0.1.2.1.1.1.1	Genus	Peniophora	1	0,124
104	4	0.1.2.1.2	Order	Cantharellales	1	0,124
105	5	0.1.2.1.2.1	Family	Ceratobasidiaceae	1	0,124
106	6	0.1.2.1.2.1.1	Genus	Ceratobasidium	1	0,124
107	3	0.1.2.2	Class	Tremellomycetes	1	0,124
108	4	0.1.2.2.1	Order	Tremellales	1	0,124
109	5	0.1.2.2.1.1	Family	Tremellales_family_incertae_sedis	1	0,124
110	6	0.1.2.2.1.1.1	Genus	Cryptococcus	1	0,124
111	2	0.1.3	Phylum	Fungi_unclassified	213	26,329
112	3	0.1.3.1	Class	Fungi_unclassified	213	26,329
113	4	0.1.3.1.1	Order	Fungi_unclassified	213	26,329
114	5	0.1.3.1.1.1	Family	Fungi_unclassified	213	26,329