# 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

Tavsanli 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 Tavsanli 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 Tavsanli 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 uygulanabileceği 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. Tavsanli 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 Tavsanli 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 Tavsanli.

# 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 bioavailibity 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. Virtification 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 verification 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.

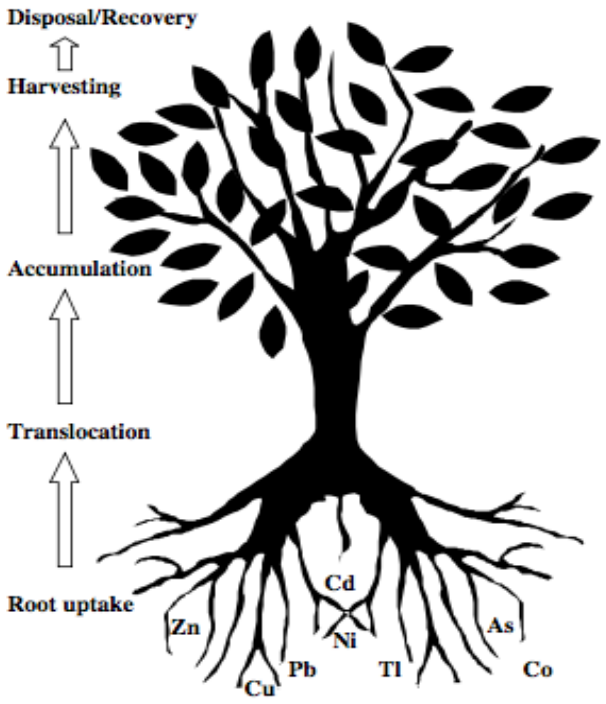


Figure 2.1. Schematic representation of phytoextraction of metals (Alkorta et al., 2004).

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 (Winquist 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).

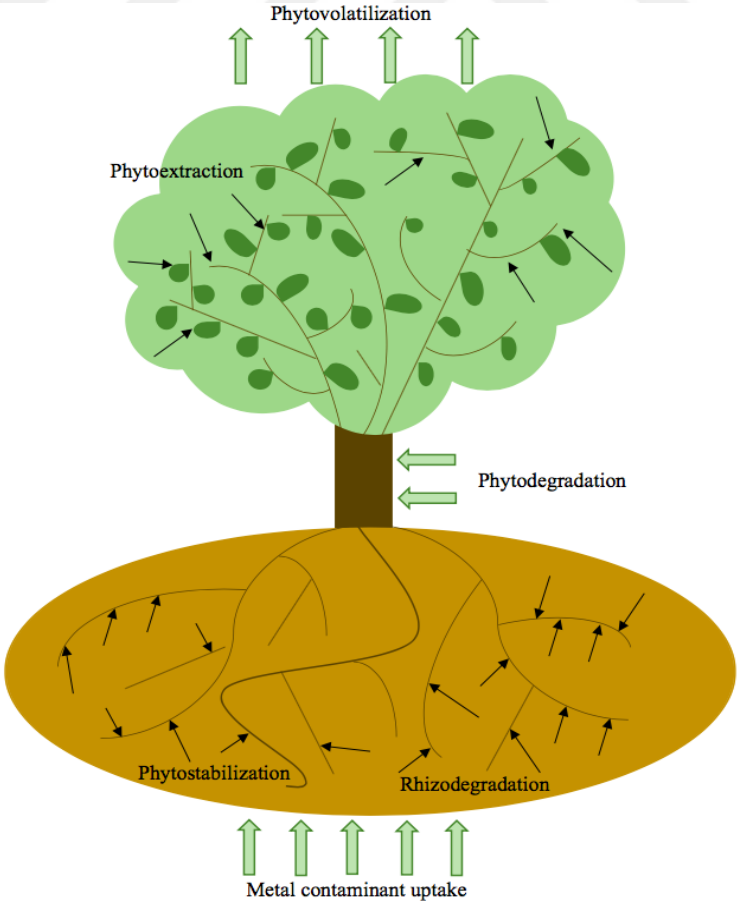


Figure 2.2. Representation of metal uptake by plant through phytoremediation processes.

## 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).

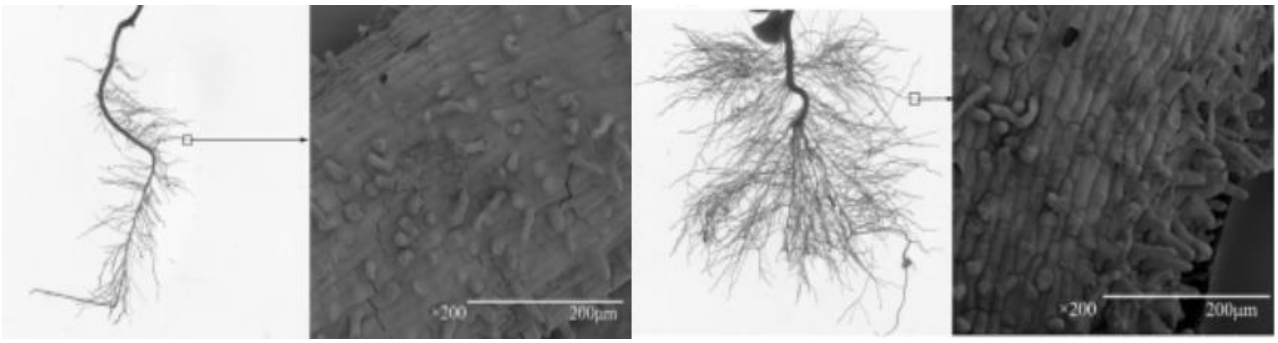


Figure 2.4. Representation of root morphology of cv. Fuding Dabaicha inoculated with mixed-AMF and non-inoculated (Shao et al., 2018).

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).

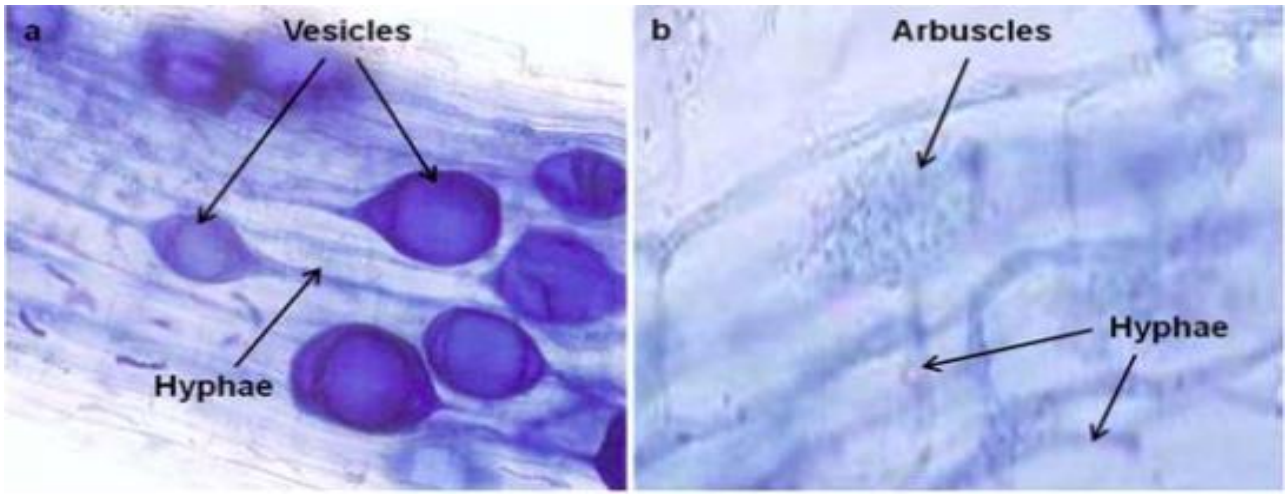


Figure 2.5. Microscopic observation of arbuscular mycorrhizal fungi (Akhtar et al., 2014).

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 (Gorzelak 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 Paraglomeracea 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 Glomeramycota 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 Tavsanli 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 Tavsanli, 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 the 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 25$^{th}$ of August in 2017 while the second trap culture system was planted on 13$^{th}$ of June in 2018. First of all, stream sand was sterilized in oven under 151℃ 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.

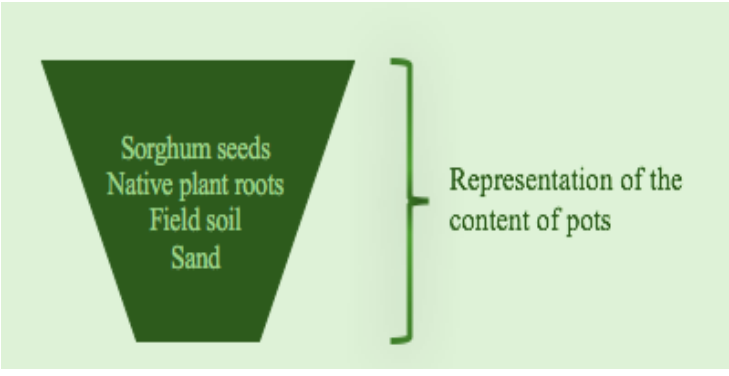


Figure 3.1. Schematic representation of the content of each pot in trap culture systems.

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

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 Column[3] 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 Column[3] 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 Column[3] and was centrifuged at 12000 RPM for 1 minute. Then, Zymo-Spin™ IIC Column[3] 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.

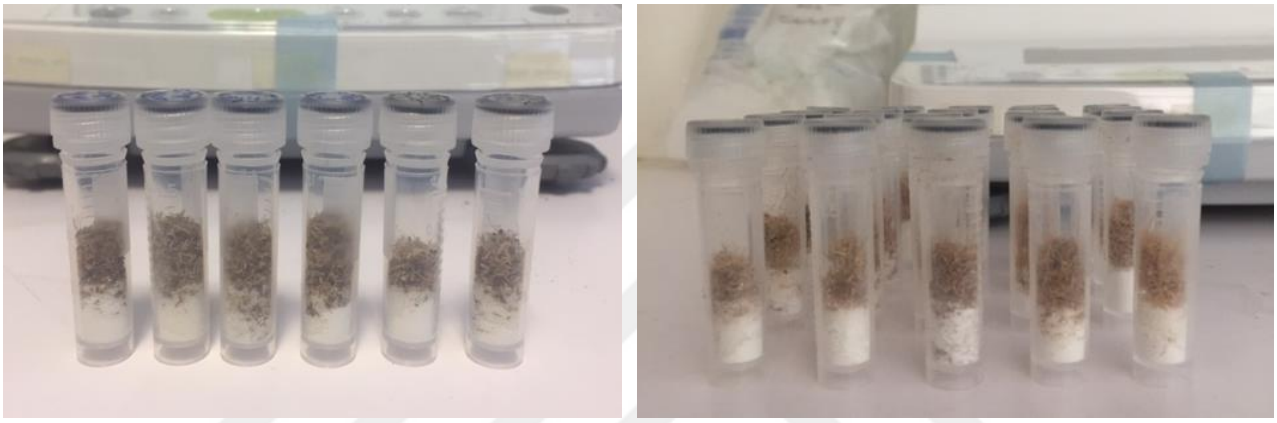

Figure 3.7. Sliced roots prior to DNA extraction from initial *(left)* and second *(right)* trap culture systems.

### 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.
95°C, time: 30 seconds
56°C, time: 30 seconds } 40 cycles
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.

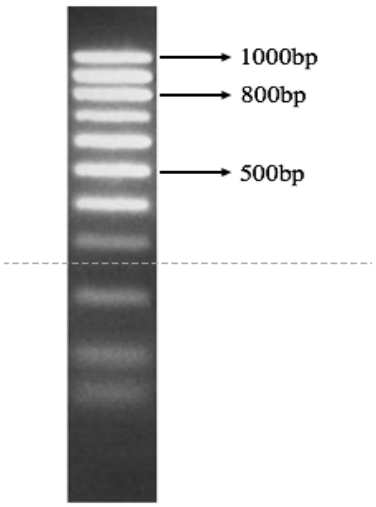


Figure 3.8. The demonstration of bands and their equivalent base pairs in the ladder used for the PCR products from the initial trap culture system.

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 0.5µg/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.

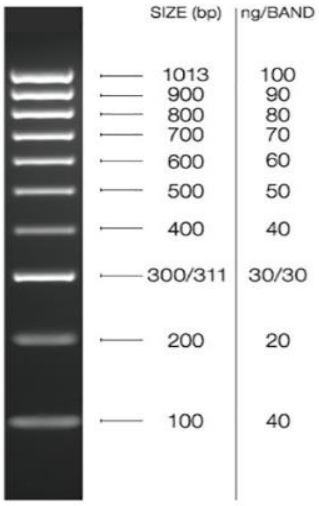


Figure 3.9. The demonstration of bands and their equivalent base pairs in the ladder used for the PCR products from the second trap culture system.

## 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 $OD_{260}/OD_{280}$ rate between 1.8-2.0, $OD_{260}/OD_{230}$ 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 lenght 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'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA ATCC-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 } 25 cycles
72°C, time: 5 minutes

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 } 8 cycles
72°C, time: 30 seconds
72°C, time: 5 minutes

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 hibridization buffer (HT1) and denaturised 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.

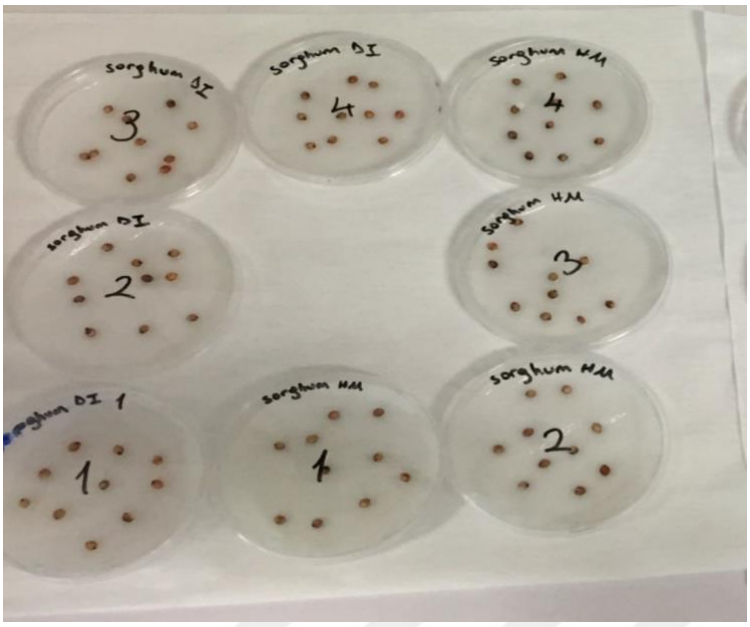


Figure 4.1. Petri dishes containing Sorghum bicolor L. seeds (Dishes labelled as DI only contained distilled water while dishes labelled as HM contained Fe and Al solutions).

### 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 12$^{th}$ week of plantation. The related graphs are given in Figure 4.2., Figure 4.3. and Figure 4.4.

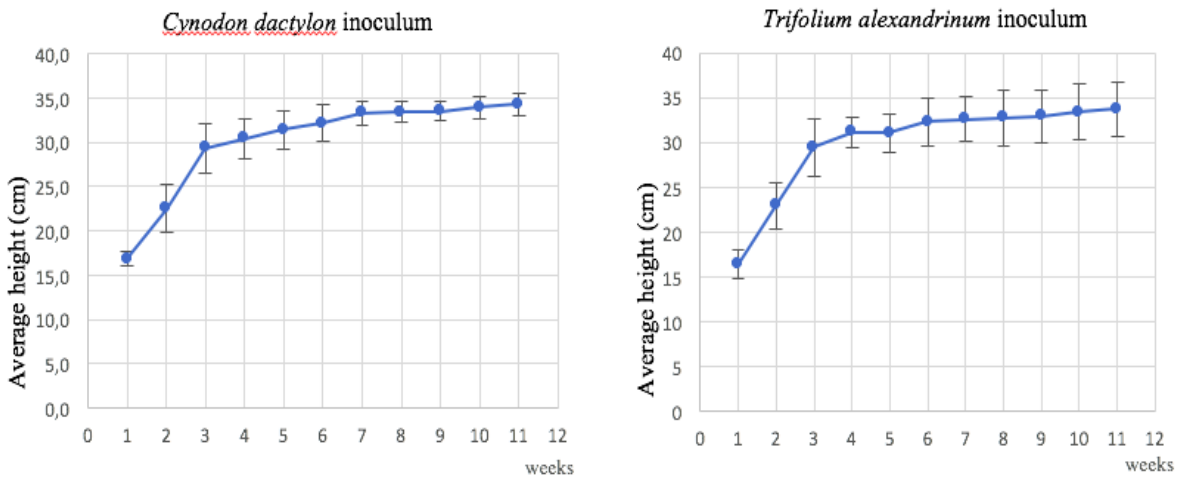


Figure 4.2. The average height of *Sorghum bicolor* L. plants in *Cynodon dactylon* inoculum *(left)* and *Trifolium alexandrinum* inoculum *(right)*.

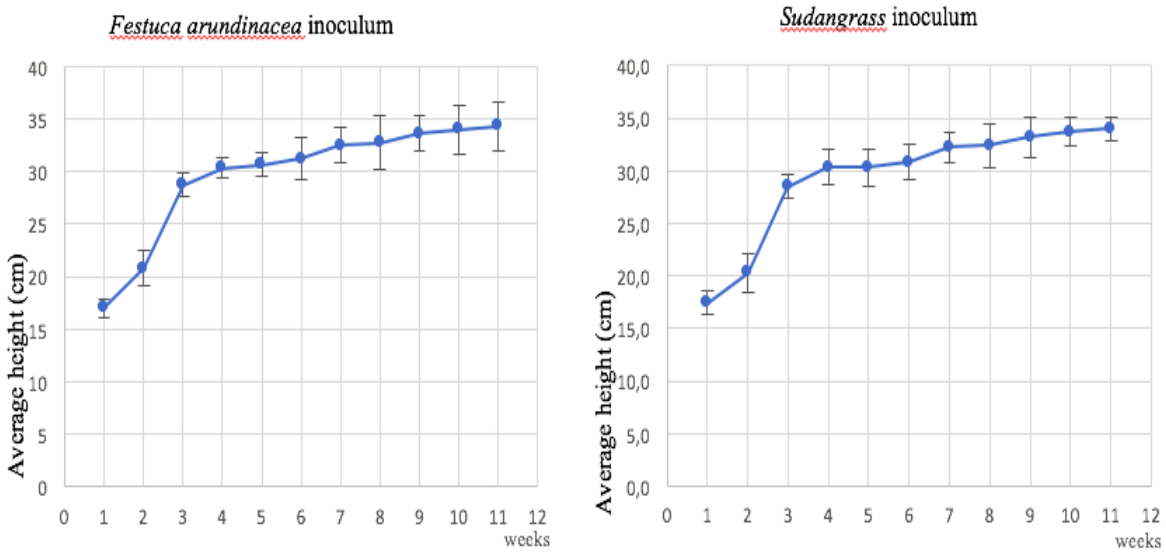


Figure 4.3. The average height of *Sorghum bicolor* L. plants in *Festuca arundinacea* inoculum *(left)* and *Sudangrass* inoculum *(right)*.

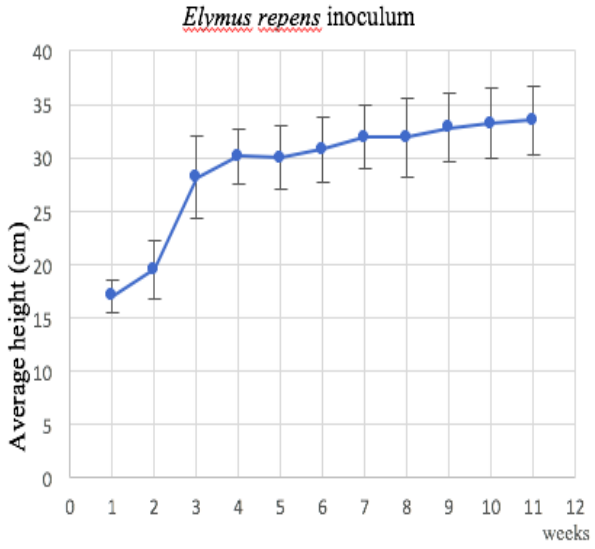


Figure 4.4. The average height of *Sorghum bicolor* L. plants in *Elymus repens* inoculum.

### 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.

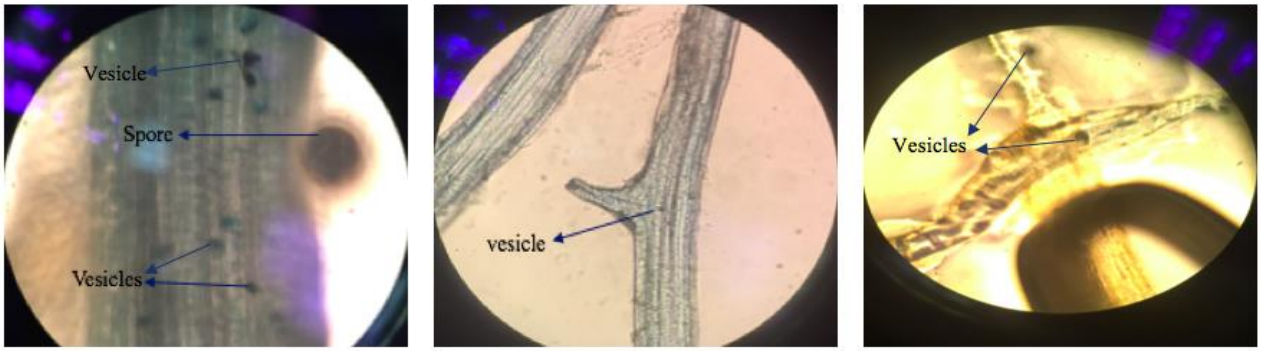


Figure 4.5. Spore and vesicular forms of AMF in roots of *Sorghum bicolor* L. in *Cynodon dactylon* inoculum from the initial *(left)* and second *(middle and right)* trap culture systems.

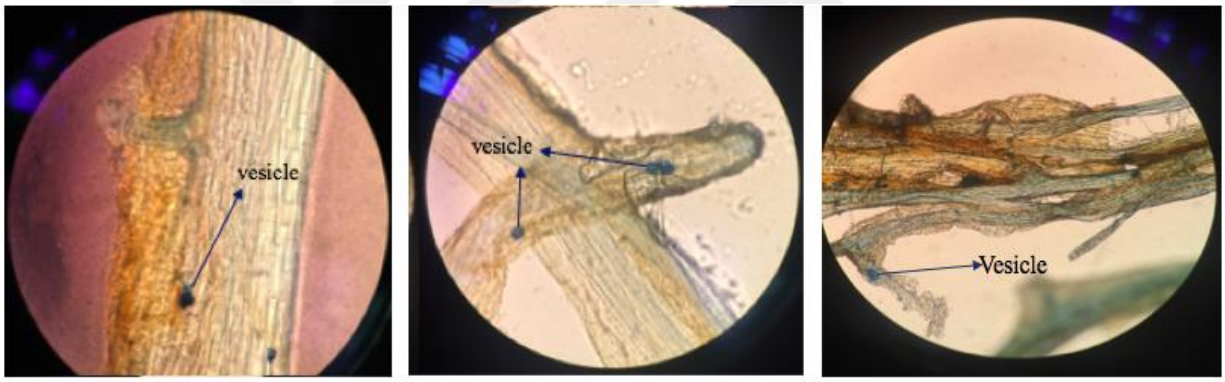


Figure 4.6. Vesicular forms of AMF in roots of *Sorghum bicolor* L. in *Sudangrass* inoculum from the initial *(left)* and second *(middle and right)* trap culture systems.

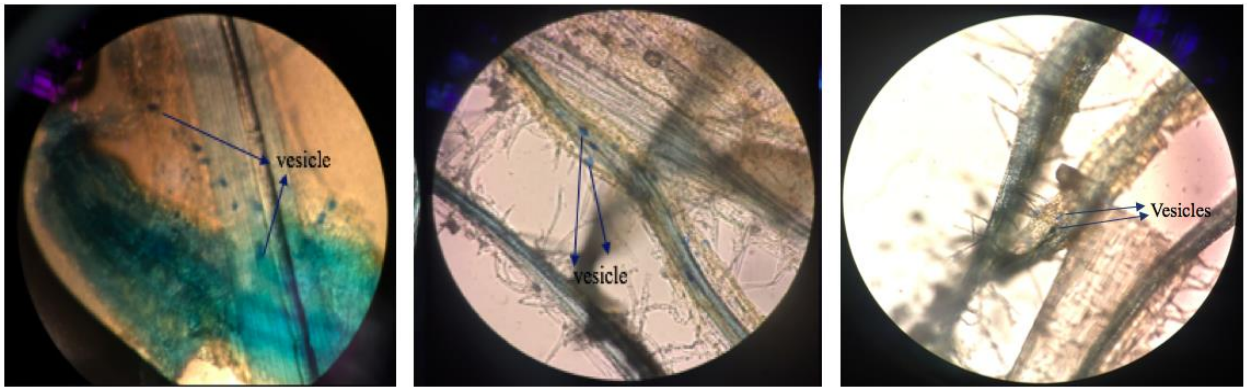


Figure 4.7. Vesicular forms of AMF in roots of *Sorghum bicolor* L. in *Elymus repens* inoculum from the initial *(left)* and second *(middle and right)* trap culture systems.

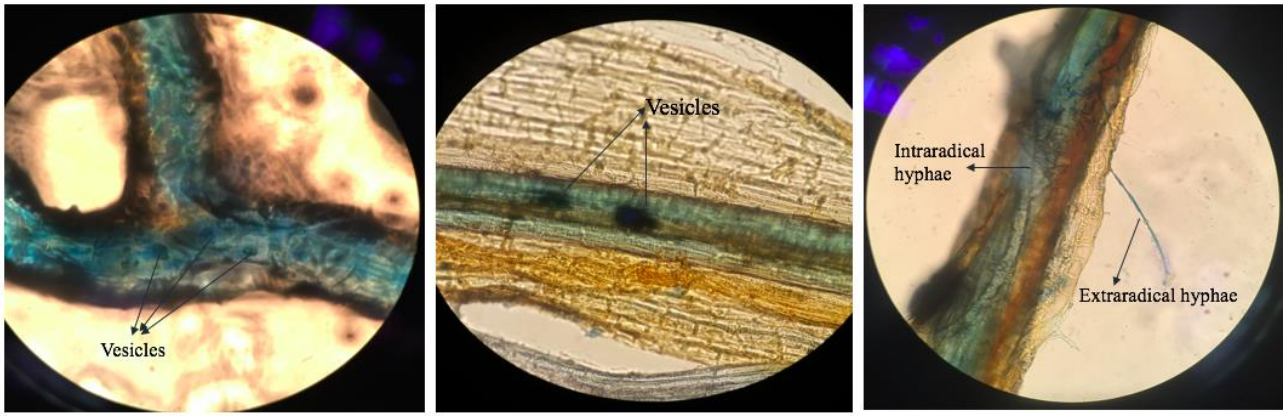


Figure 4.8. Vesicular forms, intraradical and extraradical hyphal structure of AMF in roots of *Sorghum bicolor* L. in *Trifolium alexandrinum* inoculum from the initial *(left)* and second *(middle and right)* trap culture systems.

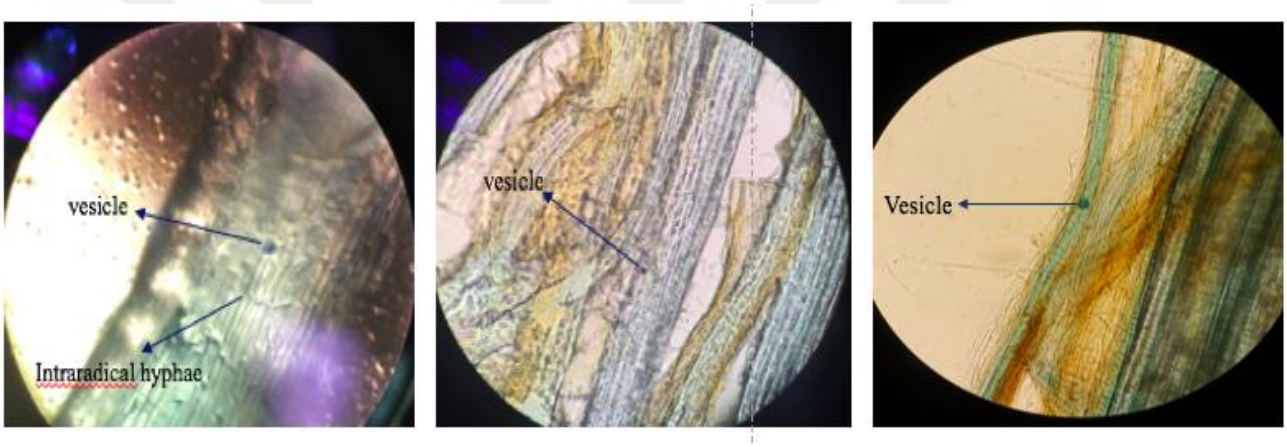


Figure 4.9. Vesicular forms and intraradical hyphal structure of AMF in roots of *Sorghum bicolor* L. in *Festuca arundinacea* inoculum from the initial *(left)* and second *(middle and right)* trap culture systems.

### 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.

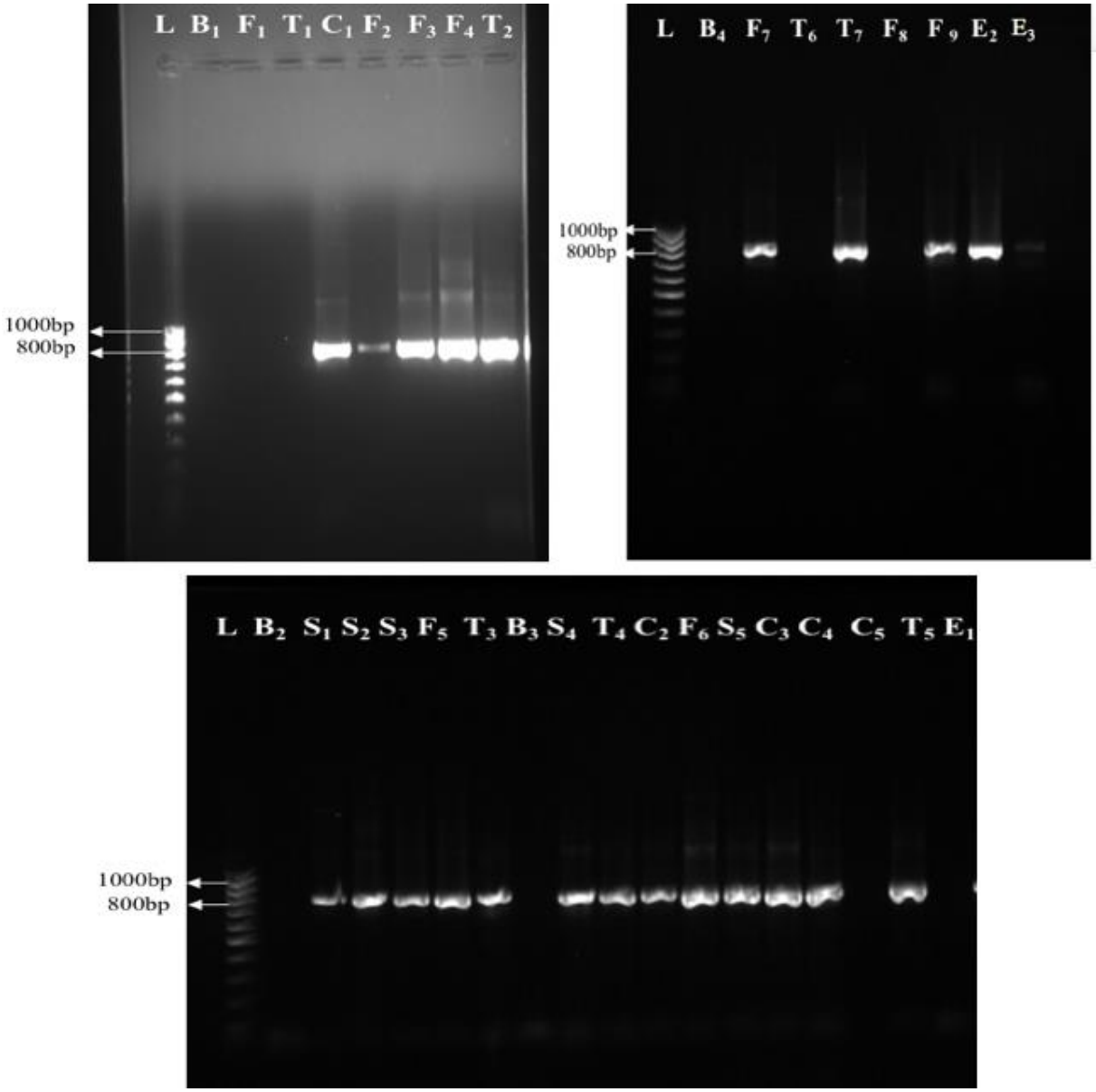


Figure 4.10. The gel images obtained from the PCR process of DNA isolates from initial trap culture system *(L: ladder, B:Blank, F: PCR product of Festuca arundinacea inoculum, T: PCR product of Trifolium alexandrinum inoculum, C: PCR product of Cynodon dactylon inoculum, E: PCR product of Elymus repens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

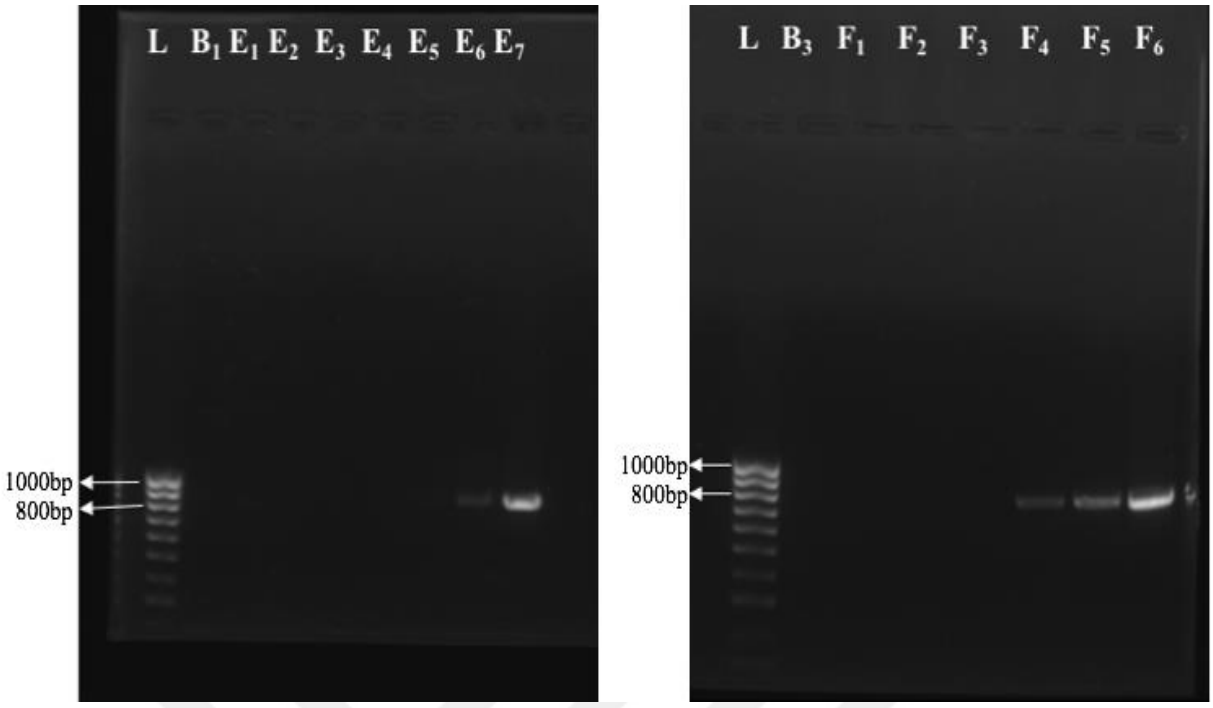


Figure 4.11. The gel images obtained from the PCR process of DNA isolates from second trap culture system *(L: Ladder, B: Blank, F: PCR product of Festuca arundinacea inoculum, T: PCR product of Trifolium alexandrinum inoculum, C: PCR product of Cynodon dactylon inoculum, E: PCR product of Elymus repens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

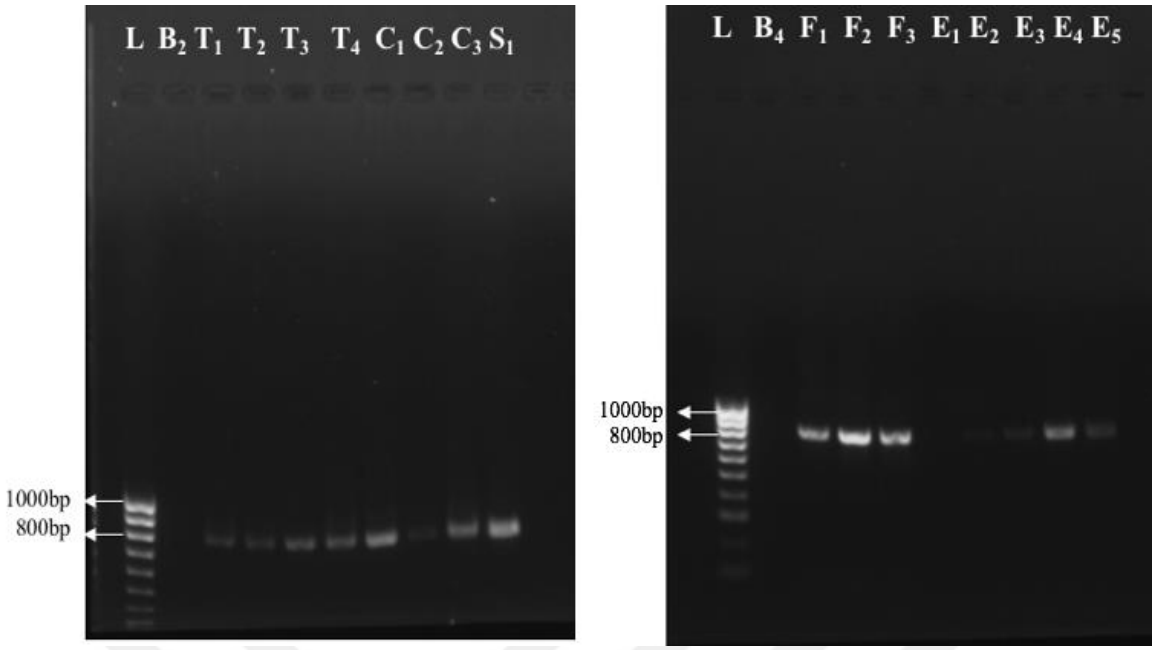


Figure 4.12. The gel images obtained from the PCR process of DNA isolates from second trap culture system *(L: ladder, B:Blank, F: PCR product of Festuca arundinacea inoculum, T: PCR product of Trifolium alexandrinum inoculum, C: PCR product of Cynodon dactylon inoculum, E: PCR product of Elymus repens 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 $E_1$, $E_2$, $E_3$, $E_4$, $E_5$ and $F_1$, $F_2$, $F_3$ samples. As demonstrated above, every PCR product in the gel except for $E_1$ 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 $C_1$, $S_1$, $F_2$, $T_3$ and $E_7$ from second trap culture system were send 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.

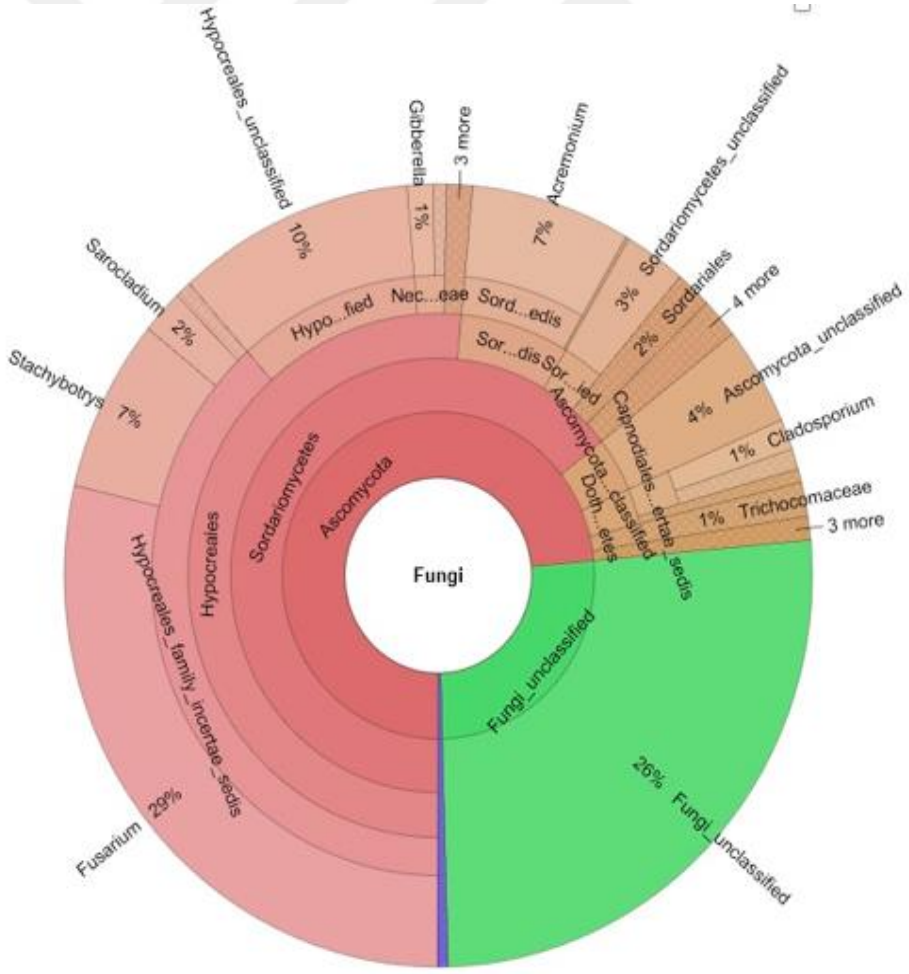


Figure 4.13. Fungal community profile of DNA extracted from *Sorghum bicolor* L. roots in *Cynodon dactylon* inoculum.

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.

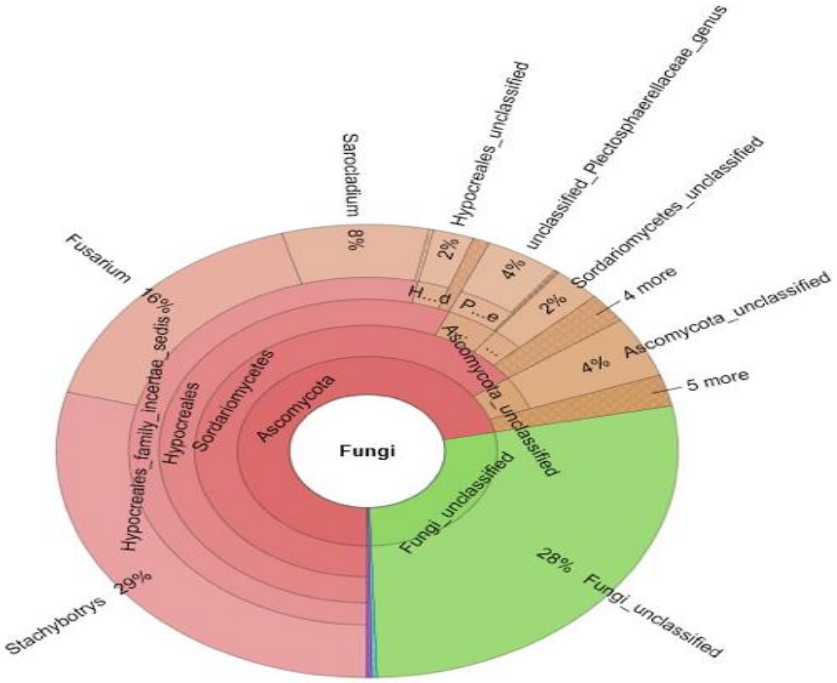


Figure 4.14. Fungal community profile of DNA extracted from *Sorghum bicolor* L. roots in *Trifolium alexandrinum* inoculum.

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.

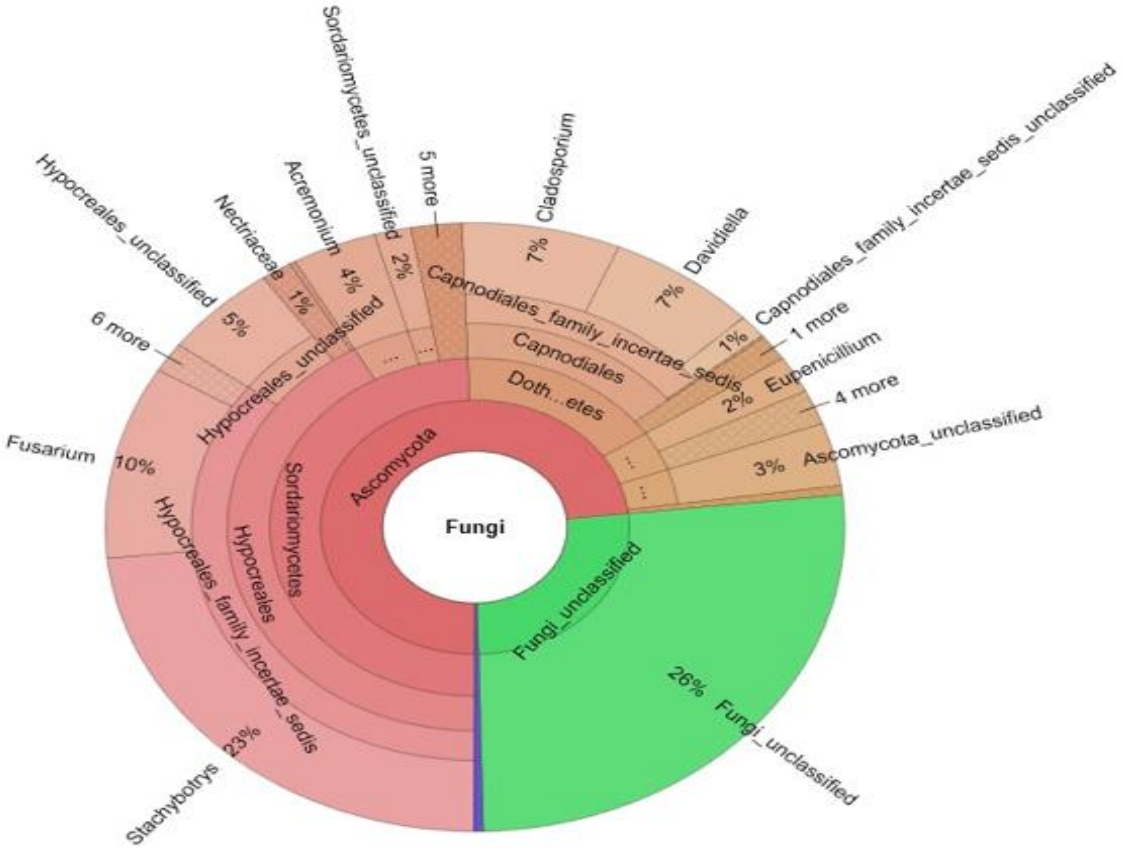


Figure 4.15. Fungal community profile of DNA extracted from *Sorghum bicolor* L. roots in *Festuca arundinacea* inoculum.

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.

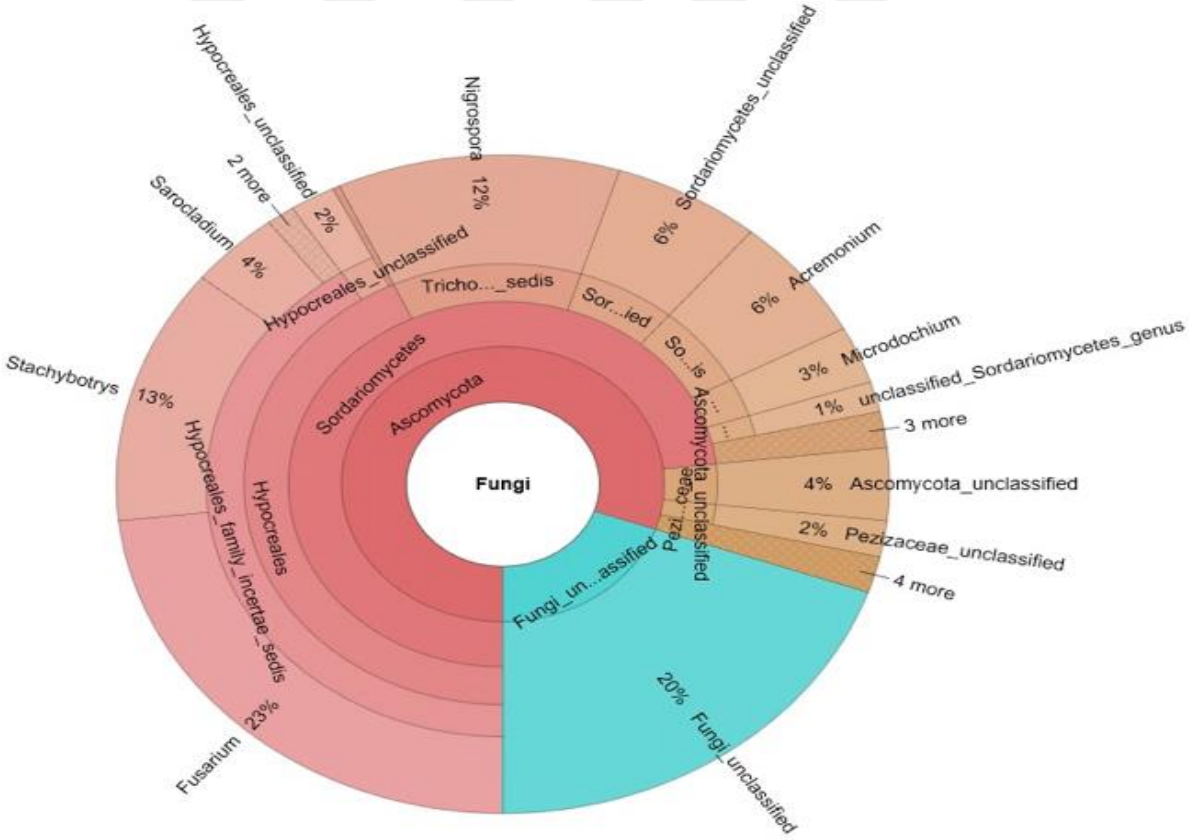


Figure 4.16. Fungal community profile of DNA extracted from *Sorghum bicolor* L. roots in *Sudangrass* inoculum.

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.

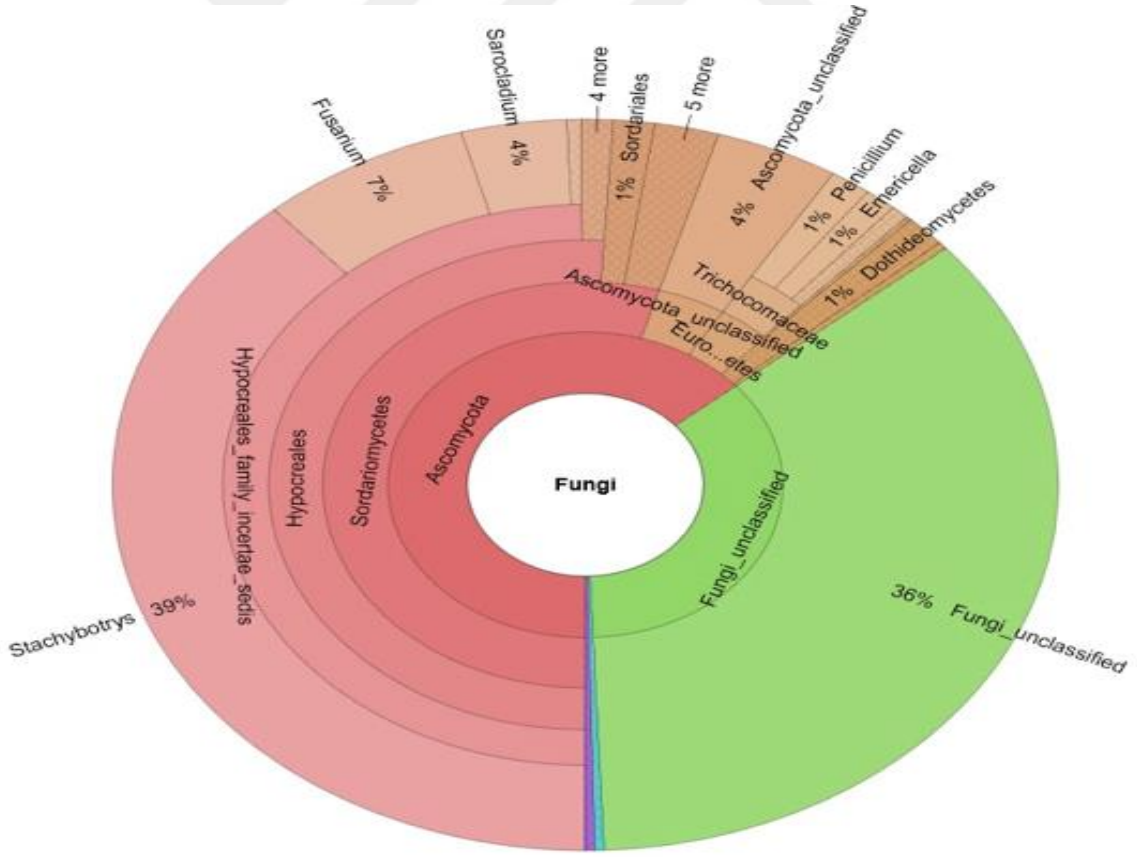


Figure 4.17. Fungal community profile of DNA extract from *Sorghum bicolor* L. rhizosphere in *Elymus repens* inoculum.

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 arundinacae*, *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 |
|---|---|---|---|
| *Elymus repens* | Mortierellales | Eurotiales | *Alternaria spp.*<br>*Chaetomium spp.*<br>*Fusarium spp.*<br>*Cladosporium spp.*<br>*Penicillium spp.* |
| *Sudangrass* | - | Pezizales | *Chaetomium spp.*<br>*Fusarium spp.*<br>*Cladosporium spp.*<br>*Penicillium spp.* |
| *Festuca arundinacea* | - | Pezizales<br>Eurotiales<br>Cantharellales<br>Ceratobasidiaceae | *Colletotrichum spp.*<br>*Alternaria spp.*<br>*Chaetomium spp.*<br>*Fusarium spp.*<br>*Cladosporium spp.*<br>*Penicillium spp.* |
| *Cynodon dactylon* | - | Pezizales<br>Eurotiales<br>Ceratobasidiaceae | *Colletotrichum spp.*<br>*Alternaria spp.*<br>*Chaetomium spp.*<br>*Fusarium spp.*<br>*Cladosporium spp.*<br>*Penicillium spp.* |
| *Trifolium alexandrinum* | Mortierellales | Eurotiales<br>Cantharellales<br>Ceratobasidiaceae | *Colletotrichum spp.*<br>*Alternaria spp.*<br>*Fusarium spp.*<br>*Cladosporium spp.*<br>*Penicillium spp.* |

# 6. CONCLUSION

In the present study, the presence of mycorrhizal fungi and fungal community profile of Tavsanli 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 Tavsanli 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 Tavsanli 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 |

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|---|---|---|---|---|---|---|
| **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 |

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|---|---|---|---|---|---|---|
| **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 | Microascaceae | 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 |

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|---|---|---|---|---|---|---|
| **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 |

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|---|---|---|---|---|---|---|
| **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 |

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|---|---|---|---|---|---|---|
| **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 | Orbiliales | 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 |

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| **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 |

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| **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 | Lasiosphaeriaceae | 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 |

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| **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 |